METHOD FOR IDENTIFICATION, SELECTION AND ANALYSIS OF TUMOUR CELLS

Field of the art

The present invention relates, in general, to diagnostic methods based upon identification, isolation, and subsequent analysis of circulating or disseminated rare cells. In particular, the invention relates to diagnostic methods that find application in the oncology sector, and that hence involve identification and isolation of circulating tumour cells (CTCs) or disseminated tumour cells (DTCs).

State of the art

The diagnosis of numerous pathological conditions, in particular that of tumours, is typically performed according to methods characterized, on the one hand, by different degrees of invasiveness and, on the other, by different levels of performance in terms of specificity, sensitivity, and reliability.

Some methods of screening are based upon analyses aimed at detecting the presence of biomarkers in the peripheral bloodflow (for example, CA-125, CEA, PSA) or in other biological fluids (for example, test of occult blood in the faeces or test on faecal DNA). Said methods, however, are based upon an indirect evaluation that is a function of the concentration of a biomarker and not on the direct analysis, for example, of tumoural tissue. Consequently, their sensitivity and specificity are not particularly high.

In the case, considered by way of example, of prostate cancer, one of the most widespread forms of cancer in the male population (approximately one quarter of all the cases of cancer, 300,000 new cases a year in Europe), screening on the basis of serological parameters (dosages of PSA) is widespread, but has a poor sensitivity and specificity.

More invasive procedures, such as digital rectal examination can at times complete the clinical picture, but more frequently it is necessary to carry out a prostatic biopsy to have a more accurate diagnosis, in an escalation of invasiveness that results in a growing discomfort for the patient and in non-negligible costs for the health system.

Similar situations arise in other contexts, such as cancer of the colon-rectum, where recourse is frequently had to the evaluation of occult blood in the faeces, or else an invasive analysis is performed via colonscopy and, possibly, a biopsy with sampling of tissues taken directly from the colon-rectum.

Since, in this context, early diagnosis enables on average a more effective treatment, it is obviously of interest to evaluate the onset of cancer at an early stage and with a high degree of sensitivity and specificity.

In this sense, recent studies (for example, Zieglschmid *et al.*, *Crit. Rev. Clin. Lab. Sci.* **42**, 155-196 (2005)) on circulating tumour cells (CTCs) have demonstrated that, in the peripheral bloodflow, cells deriving from the primary tumour are found. Even though they are extremely rare, the possibility of identifying them and isolating them constitutes a potentially very interesting alternative to the invasive methods described previously for obtaining specimens of tumoural tissue to be characterized, subsequently, for diagnostic purposes. Regulatory bodies, such as the FDA, have already approved some systems for clinical use based upon CTCs for evaluation of patients presenting metastases.

For the majority of patients affected by a cancer, in fact, it is not the main tumour that causes death in so far as, if identified at a sufficiently early stage, it can be eliminated surgically, by means of radiotherapy, chemotherapy, or by a

combination of said methods. Instead, the most frequent cause of death are metastases, i.e., tumoural colonies originated by malignant cells that detach from the main tumour and migrate towards other districts of the body, even at a considerable distance from the main tumoural site. Since they are difficult to identify and eliminate, it is frequently impossible to treat all the metastases successfully. Consequently, from the clinical standpoint their formation can be considered the conclusive event in the natural progress of a cancer.

Consequently, since there has been demonstrated, in the case of breast cancer, cancer of the colon, and cancer of the prostate, a correlation between the number of CTCs and the increased risk of unfavourable outcome of the illness, it appears evident that the possibility of isolating them and, by means of molecular analysis, obtaining a complete informative picture therefrom having clinical importance, would have very significant diagnostic repercussions.

It has moreover been proven that the analysis of disseminated tumour cells (DTCs), i.e., of tumour cells that can be found in the bone marrow or in the lymph nodes, is, in certain contexts, even more significant than the analysis of the primary tumour. Some research groups, for example, have noted (Klein *et al.*, *Tumour cell.* **13(5)**, 441-53 (2008)) that the over-expression of HER2, which can be detected in a single DTC but not in the primary tumour, proves predictive of a very high risk of death.

Furthermore, it is known, for example from the patent No. EP1109938, that the analysis of single multiple cells can be more informative as compared to the analysis of a set of cells. In particular, it has been found (Klein *et. al.*, *Proc. Natnl. Acad. Sci. USA* **96**, 4494-4499 (1999)) that different DTCs of one and the same patient have different chromosome variations (losses and amplifications in different sites).

At the current state of the art, for isolation of circulating and disseminated tumour cells there are, however, available approaches of a chiefly analytical nature that are very complex and laborious, which, however, have limited yields and generate specimens distinguished by a low degree of purity. Consequently, said specimens of CTCs or DTCs are not compatible with some of the most refined and reliable diagnostic procedures, such as sequencing. The limited purity, in fact, determines a high risk of false positives and false negatives caused by wrong calls in the bases of the sequence.

Attempts have been made (*Nagrath et al., Nature*, **450**, 1235-1241 (2007)) to isolate CTCs by means of microfluidic devices with an inner surface coated with anti-EpCAM antibodies (expressed in epithelial cells but not in leukocytes) that are able to process volumes of blood of the order of millilitres, without having to resort to preliminary treatments that entail operations with a high risk of loss of part of the cells of interest (for example, centrifugation, flushing and incubation). However, said methods have led to levels of purity on average of 50%-60%, which do not prove sufficient for carrying out, downstream, analyses for which the purity of the specimen is crucial, such as, for example, the evaluation of copy-number variations, microsatellite instability, loss of heterozygosity (LoH), gene expression in which a better signal-to-noise (S/N) ratio is fundamental for diagnostic applications, or identification of new drugs, or again in the sector of research into cancer-initiating cells.

Similar considerations can be extended to other methods the enormous diagnostic potential of which has been highlighted, which, however, cannot be concretely implemented since the known procedures of enrichment of biological specimens and isolation of a population of rare cells therein do not enable sufficient levels of purity to be obtained, if not following

upon a heavy, complex and costly analytical workload that cannot, consequently, find application in medical practice. For instance (Bonci *et al.* "The miR-15a-miR-16-1 cluster controls prostate cancer by targeting multiple oncogenic activities" October 19, 2008; doi:10.1038/nm.1880),

it has recently been demonstrated that some microRNAs (miRNAs), i.e., short single-strand non-encoding fragments of RNA, containing approximately 22 nucleotides, are directly involved in the development and progress of cancer. From a study of the expression of miR-15a and miR-16 in cells of a primary tumour (prostate cancer) by means of quantitative PCR, there has been noted a considerable under-regulation of both of the miRNAs as compared to the corresponding healthy tissue. Said datum has been confirmed by means of *in-situ* hybridation (ISH). It has moreover been demonstrated that the deletion of the miR-15a-miR-16 cluster is generally associated to advanced stages of the illness, even though in some cases the under-regulation of these miRNAs has been recorded already during the initial stages of the development of cancer. Consequently, from the diagnostic standpoint, the possibility of isolating tumour cells and of evaluating whether they present an under-regulation of these miRNAs would provide a precise indication on the state of advance of the illness, which is useful to the physician also for the choice of the most appropriate form of therapy.

To proceed with further considerations of a therapeutic nature, it is worthwhile recalling that some therapeutic approaches in the oncology sector are based upon the use of monoclonal antibodies that are able to have a direct influence upon the survival of tumour cells, depriving them of essential signals of extracellular proliferation, such as those mediated by growth factors through their cell receptors. For example, one of the targets of interest, in this context, is the epidermal-growth-factor receptor (EGFr). Binding between the

epidermal growth factor (EGF) and the corresponding receptor EGFr triggers a cascade of cellular biochemical events, which include autophosphorylation and internalization of EGFr, which culminates in cell proliferation.

It has been shown that both EGF and the transforming growth factor-α (TGF-α) bind to EGFr and lead to cell proliferation and growth of the tumour. In many cases, the increased expression of EGFr has been accompanied by the production of TGF-α or EGF by the tumour cells, thus suggesting involvement of an autocrine growth control in the progress of cancer. Consequently, there has been proposed the use of antibodies against EGF, TGF-α and EGFr in the treatment of tumours in which they are expressed or over-expressed. It has recently been demonstrated, as described in the international patent application published under No. WO28112274, the presence of a mutation of which in the K-RAS gene or in the B-RAF gene in tumour cells constitutes an indication on the fact that the tumour will not respond to the treatment with an agent designed to bind to a polypeptide of EGFr.

It will be understood that the possibility of detecting, starting from a specimen of tumour cells taken from a patient by means of a non-invasive procedure, the presence of such a mutation would provide the physician with a tool of enormous importance for evaluating whether to proceed with a therapy based upon the use of an anti-EGFr monoclonal antibody or else reject it, knowing beforehand that it would not be effective. Also in this perspective, however, the methodologies of enrichment, identification and isolation of rare cells in the blood, or in an some biological fluid, do not enable a sufficient purity to be obtained to guarantee that the subsequent analysis will be reliable (exclusion of false positives, false negatives, etc.) and, in some cases, that they can even materially be conducted (for example, for the cases in which, since the purity is low, the signal-to-noise

ratio is too low to obtain a valid reading).

Summary of the invention

Consequently, an aim of the present invention is to provide a method for identification, isolation, and subsequent analysis of circulating or disseminated rare cells obtained by means of a sampling of a non-invasive nature that will overcome the drawbacks of the known art described previously.

Consequently, provided according to the present invention are procedures for diagnostic purposes to be carried out on a specimen of cells of interest obtained starting from an organic fluid taken from a patient, which are identified and subsequently isolated until a purity of at least 90% is obtained, operating according to the method specified in Claim 1.

Preferably, the step of isolating at least one cell from amongst said at least one type of circulating tumour cells or disseminated tumour cells or portions thereof is performed so as to obtain a specimen of tumour cells or portions thereof having a purity of at least 95%. Even more preferably, the step of isolating at least one cell from amongst said at least one type of circulating tumour cells or disseminated tumour cells or portions thereof is performed so as to obtain a specimen of tumour cells or portions thereof having a purity of 100%.

In particular, according to a preferential embodiment of the present invention the step of isolation of the rare cells is performed according to a technology developed by the present applicant and based upon the use of a silicon microchip that integrates several hundreds of thousands of electrodes of micrometric dimensions by manipulating individual cells so as to isolate the cells of interest with a unitary purity rendering them available for molecular analysis. In this way,

the method according to the invention provides cells with a high purity, is highly automated for the most delicate part of the process (isolation of individual cells), and enables implementation of analytical techniques of high diagnostic and predictive reliability. In the light of what has been described previously, there will emerge clearly the innovative scope, at a diagnostic and therapeutic level, of the present invention, which enables improvement of the oncological treatment in the different stages of the course of the illness, rendering possible both a non-invasive early diagnosis having a level of reliability comparable with a biopsy, and a sensitive and accurate follow-up in the course of the treatment with specific cancer drugs and/or in the post-operative stage in order to follow the evolution of the illness and the quality of the response to the clinical treatments, both in the metastatic stage and in the adjuvant stage.

According to the present invention, an organic fluid taken in a non-invasive manner from a patient is, in a first instance, processed by making one or more passages of enrichment of the cells of interest according to one or more methodologies known in the art, such as Ficoll, selective lysis of the erythrocytes, filtration with filters based upon dimension of the cells - obtained by means of photolithographic micromachining or other technique, such as track-etched membranes, depletion or magnetic enrichment. The enriched cells are then labelled with specific antibodies for identifying the cells of interest and/or the contaminating cells, etc.

In this context, by the expression "organic fluid" or "corporeal fluid" reference is made to a fluid obtained starting from a corporeal specimen in which there is a high probability of finding cells of interest. The organic fluid can be obtained from the corporeal specimen, either directly -

such as for example, peripheral bloodflow, bone marrow, urine - or indirectly, such as for example, by means of trypsinization of a tissue from a lymph node.

In this context, by the expression "cells of interest" reference is made to cells, the characteristics of which, detectable by means of appropriate techniques of analysis, can provide indications of a diagnostic or therapeutic nature on a pathological condition of the patient.

For instance, the corporeal fluid is peripheral blood, which can be drawn in a substantially non-invasive way from the patient, according to the methodologies commonly in use, and the cells of interest are circulating tumour cells (CTCs).

As further example, the organic fluid is blood from bone marrow, and the cells of interest are disseminated tumour cells (DTC).

The method of the invention is then characterized by the use of a microfluidic system that is able to select individually single cells from the enriched specimen and isolate them in an automatic or semi-automatic non-manual way.

Used hereinafter for reasons of simplicity is the term "single cells" or "individual cells", but this term must be understood as including a single cell or a single aggregate of cells in so far as, in general, it may occur that the cells of interest present as not isolated but bound to one or more other cells, whether tumour cells or non-tumour cells. Furthermore, there must be understood also the possibility of analysing portions of cell, such as isolated nuclei.

Via said isolation of the cells in the microfluidic system, a set is obtained containing only cells of interest, i.e., a specimen of a purity equal to 100%, which thus proves suitable

for being subjected to a plurality of procedures of molecular analysis.

Advantageously, in the aforesaid microfluidic system for the individual selection of single cells, the selection is made on the basis of images of the cells themselves.

Advantageously, said images of the cells are acquired in the absence of flow in the medium of suspension of the cells themselves.

Advantageously, said images comprise images acquired in fluorescence.

By "microfluidic device" is understood, herein, a device designed to manage volumes of liquid in a laminar-flow regime, in which the space for containing the liquid during analysis has at least one dimension smaller than 1 mm.

By "device capable of selecting individually single cells" is here understood a device that is able to carry out selection of one or more single cells, one at a time or simultaneously, on the basis of parameters evaluated individually on each cell.

By "non-manual isolation" is understood a movement of the cells in which manuality of the operator is not required, such as for example, in the case of movement by means of mobile dielectrophoretic cages.

By "automatic isolation" is understood a movement of the cells in which intervention of the operator is not required, such as for example, in the case of movement by means of dielectrophoretic cages managed by a program executed in a substantially non-interactive way by a microprocessor.

By "semi-automatic isolation" is understood a movement of the cells in which the interaction with the operator enables exerting an *indirect* control on the manipulation, such as for example, in the case of movement, for example by means of dielectrophoretic cages, managed by an interactive program executed by a microprocessor.

Advantageously, the aforesaid selection is made in an automatic or semi-automatic way.

By "automatic selection" is understood herein a selection of the cells in which intervention of judgement of the operator is not required, such as for example, in the case where there is determined automatically with an automatic classifier which cells correspond effectively to tumour cells of interest. For example, this could be performed with a classifier of images that processes the acquired images of the cells, extracts the discriminating features therefrom and classifies them on the basis of an algorithm.

By "semi-automatic selection" is understood herein a selection of the cells in which intervention of judgement of the operator is required. For instance, this could be performed with a system that acquires the images of the cells, extracts the discriminating features therefrom, and proposes them to the operator for the final decision as to whether they should be selected.

The molecular analysis on the cells recovered can then be executed via different techniques - thanks to the high purity of the specimen recovered -, amongst which, by way of non-limiting example:

- sequencing (for example, for identification of mutations);
- microsatellite analysis (for example, with Quantitative Fluorescent PCR, i.e., QF-PCR);

- comparative genomic hybridization (CGH) ;
- array CGH;
- end-point PCR;
- real-time PCR;
- methylation analysis:
  - quantitative methylation analysis with pyrosequencing;
  - bisulphite-genomic-sequencing PCR (BSP);
  - methylation-specific PCR (MSP);
  - Combined Bisulphite Restriction Analysis of DNA (COBRA)
  - methylation-sensitive single-nucleotide primer extension (MS-SNuPE)
- analysis of gene expression;
  - RT-PCR;
  - single-cell gene expression;
  - digital PCR.

Further characteristics and advantages of the invention will emerge clearly from the ensuing description of some non-limiting examples of embodiment thereof, provided with reference to the figures of the annexed drawings.

Brief description of the figures

Figure 1 shows a flowchart summarizing an embodiment of the non-invasive method of diagnosis according to the present invention;

Figure 2 is a schematic illustration of an example of device for implementing the method (or the substantial and characterizing part thereof) according to the invention;

Figures 3A, 3B and 3D show images acquired in the course of scanning of circulating tumour cells according to the method of the invention;

Figures 4A, 4B, 4C and 4D show a series of electropherograms obtained in the course of the genetic analysis of circulating tumour cells identified and isolated according to the method

of the invention;

Figure 5 shows images acquired in the course of scanning of circulating tumour cells selected and isolated according to the method of the invention from peripheral bloodflow of patients affected by mCRC;

Figures 6 and 7 show, respectively, spectra regarding the detection of a mutation Gly2Val on K-RAS of CTCs isolated from the peripheral bloodflow of a patient affected by mCRC according to the method of the invention;

Figures 8, 9 and 10 each show five images acquired in the course of scanning of an enriched specimen, said images regarding the contents of five dielectrophoretic cages on a DEPArray™ chip. The cells of each figure were recovered together with one another and separately from the cells of the other figures. Figure 10 shows circulating tumour cells according to the method of the invention and Figures 8 and 9 the corresponding negative controls (normal leukocytes).

Detailed description

The subject of the present invention is a method of identification, isolation, and subsequent analysis of rare cells, in particular circulating tumour cells or disseminated tumour cells, obtained preferably by means of a sampling of a non-invasive nature.

Sampling

The specimens can be taken from the peripheral circulation of the patient, or else from bone marrow through various techniques known in the sector.

Pre-enrichment

The proportion of tumour cells in the specimen taken can be enriched using various methods, such as for example: centrifugation on density gradient, constituted by solutions such as Ficoll or Percoll; mechanical enrichment, such as filters of various types; enrichment by means of separation by

dielectrophoresis via a purposely provided device - dielectrophoretic-activated cell sorter (DACS); selective lysis, such as for example, selective lysis of erythrocytes not of interest; immunomagnetic separation, via immunomagnetic beads with positive selection (using beads bound to specific antibodies for the population to be recovered) or with negative selection (depletion of cell populations that are not of interest), and in which the two types of selection can be coupled in order to increase the specificity of the procedure; FACS, on cells labelled with a specific fluorescent antibody; solid-phase immunoseparation, advantageously by means of microfluidic systems presenting surfaces coated with specific antibodies for epithelial receptors (such as EpCAMs).

For the majority, these procedures can be automated and all the sorting procedures can be preceded by separation by centrifugation on density gradient or alternatively they can be applied on whole blood.

In general, the process starts with a dilution, but this is not strictly necessary for all techniques.

Other enrichment techniques

A further technique well known to persons skilled in the branch is the one referred to as MACS developed by Miltenyi Biotech, or else Easy-sep, developed by Stem-cell technologies.

Alternatively, it is possible to use paramegnetic beads of larger dimensions that do not require the use of a special column, but can also be used when working with wells or test tubes (such as, for example, anti-EpCAM Dynabeads).

To sum up, the step of enriching the specimen in the population of cells comprising at least one type of tumour cells can be performed via a method consisting of successive

steps by means of a selection of cells made on the basis of at least one parameter chosen from the group consisting of:

a. mass density;
b. morphology;
c. electrical properties;
d. chemical properties;
e. mechanical properties;
f. expression of surface antigens;
g. expression of intracytosolic antigens;
h. dielectric properties;
i. magnetic properties;
l. geometrical properties (size, etc.); and
m. optical properties.

or combinations thereof.

Advantageously, the enrichment of the tumour cells is then further obtained by means of a second step, in which the positive or negative selection of cells is made from the mononucleated cells recovered in the first step. Obviously, the second enrichment step can comprise a selection made on the basis of the capacity to express or not a specific antigen, evaluated with one of the following techniques:

a. MACS, i.e., Magnetic-Activated Cell Sorter;
b. DACS, i.e., Dielectrophoretic-Activated Cell Sorter;
c. FACS, i.e., Fluorescence-Activated Cell Sorter.

Isolation of single tumour cells

Next, the specimen containing the tumour cells is inserted in a microfluidic device that is able to select individually single cells and isolate them in an automatic or semi-automatic non-manual way. For this purpose, it is possible to use a dielectrophoretic isolation (DEPArray, using for example, the techniques described in PCT/IB2007/000963 or in PCT/IB2007/000751, or again in *Manaresi et al., IEEE J. Solid-State Circuits*, **38**, 2297-2305 (2003) and in *Romani et al., Proceedings of the International Solid State Circuit*

Conference, **1**, 224-225 (2004)), or else opto-electronic traps or optophoretic isolation or again, laser tweezers (see, for example, *Reichle et al.*, *Electrophoresis*, **22**, 272-82 (2002) or *Fiedler et al.*, *Anal. Chem.*, **70**, 1909-15 (1998)).

The contents of said documents are incorporated herein as regards the relevant parts just for reference.

Identification of the cells of interest can be made, for example, via sensors:
- external sensors;
- optical sensors, such as a fluorescence microscope;
or also
- internal sensors;
- optical sensors, as disclosed in the patents Nos. WO2007049103 and WO2007010367, which describe an integrated method of identification of the fluorescent cells;
- impedentiometric sensors, as disclosed in the patents Nos. WO2007049103 and WO2007010367, for the detection of impedentiometric characteristics of cells.

The signal for selection of the cells can be also indirect, such as, for example, the presence of a microbead coupled to an antibody, which is in turn coupled to the cell. The presence of one or more microbeads coupled to a cell can be detected as already described previously, by means of impedentiometric sensors or optical sensors (in clear field or in fluorescence).

According to the invention, by manipulating individually single tumour cells by means of a microfluidic device, preferably in an automated way, the tumour cells contained in the pre-enriched specimen taken are isolated to constitute a specimen to be analysed having a purity of at least 90%.

Preferably, tumour cells are isolated to constitute a specimen

to be analysed having a purity higher than 95%. Even more preferably, tumour cells are isolated to constitute a specimen to be analysed having a purity of 100%.

Genetic analysis

On the tumour cells recovered according to the invention various types of analysis can be performed that enable genetic or chromosomal characterization thereof at different levels of resolution and sensitivity and according to the diagnostic purpose of the study, in accordance with what has been described previously.

For example, it is possible to proceed to sequencing of CTCs taken from patients presenting metastases in order to detect the presence of mutations of the K-RAS gene.

In the case of prostate cancer, it is possible to evaluate the presence of deletions on CTCs by means of single-cell whole-genome amplification.

Again, in the case of cancers of unknown primary origin (CUPs) it is possible to identify the original tissue via the genetic profile and/or the profile of expression of the CTCs, thus obtaining information that is fundamental for the choice of the most appropriate therapy.

In the case of disseminated tumour cells, then, it is possible to perform analysis of gene expression on single CTCs and/or DTCs in order to identify prognostic indicators and potential therapeutic targets.

Preferred examples of embodiments of the invention

By way of non-limiting example, described in what follows is a preferential embodiment of the method according to the present invention, following the flowchart represented in Figure 1.

Starting specimen

In a specimen of 17 ml of whole blood taken from a healthy male donor there were introduced by spiking 2000 cells belonging to the cell line of breast cancer MCF7.

Preliminary enrichment

The preferential embodiment of the invention envisages a process consisting of successive steps.

(a) FICOLL. The specimen, was treated with an anticoagulant (e.g., EDTA) and transferred, preferably within 8 h, into a 50-ml sterile test-tube made of polypropylene. From this a volume of 50 µl was extracted, which was used for counting white blood cells (WBCs) and red blood cells (RBCs) using a Coulter counter.

The specimen was then diluted 1:4 with PBS and EDTA (2 mM) having a pH of 7.2.

A volume (≤ 30ml) of diluted blood was accurately stratified on 15 ml of Ficoll-Hypaque (density = 1.077 g/ml).

Next, the specimen was centrifuged at 654 g for 30 min at 22°C in a (brakeless) tilt-rotor centrifuge.

The plasma was removed with a pipette (of the Pasteur type) - but an automatic pipettator may also be used - taking care not to interfere with the layer of lymphocytes at the interface. The layer was carefully collected with a drop-counter and transferred into a 50-ml polypropylene sterile conical test-tube.

The test-tube was filled with PBS and EDTA (2mM), shaken and centrifuged at 300g for 10 min at 10°C.

The supernatant portion was rejected.

The test-tube was filled with PBS and EDTA (2mM), and again shaken and centrifuged at 300g for 10 min at 10°C. The supernatant portion was drawn off.

From the specimen the cell pellet (i.e., the complex of the peripheral-bloodflow mononucleated cells, PBMNC) was taken, to be re-suspended in 5 ml of buffer. From this was taken an aliquot of 50 µl to carry out the WBC count with a Coulter.

The test-tube was filled with buffer, shaken and centrifuged at 200g for 10 min at 10°C. The supernatant portion was rejected.

An alternative to Ficoll as first step of pre-enrichment for eliminating the erythrocytes is represented by selective lysis of erythrocytes, which exploits chemical properties of the cells.

(b) CD45 MACS DEPLETION

The cell pellet was re-suspended in 80 µl of buffer for a total of 10^7 cells.

An amount of 20 µl of microbeads of anti-CD45 were added for a total of 10^7 cells. There followed steps of shaking and incubation for 15 min at 4° C.

The cells were washed by filling the test-tube with buffer and centrifuging at 300g for 10 min at 10°C.

The supernatant portion was rejected and the cell pellet was re-suspended in 500 µl of buffer for a total of 10^8 cells.

The MACS column of LS type (Miltenyi) was positioned within the magnetic field of a suitable MACS separator, according to the indications provided by the manufacturer.

A pre-filter was positioned on each column.

Both the pre-filter and the column were prepared by rinsing with 3 ml of buffer.

The cell suspension was applied to the column (or on the pre-filter).

The test-tube that contained the cell suspension, now empty, was filled with 9 ml of buffer.

The non-labelled cells were collected, and the column was washed by adding for three times 3 ml of buffer, taking it each time from the test-tube filled in the previous step.

The column was removed from the separator and set on a test-tube.

An amount of 5 ml of buffer were introduced into the column using a pipette.

The fraction containing the magnetically labelled cells was expelled immediately afterwards by operating the plunger.

To reduce the number of contaminating cells further, it is possible to use a depletion cocktail with further magnetic microbeads functionalized with antibodies that recognize antigens present selectively on cells to be depleted, such as for example, anti-GPA microbeads (to eliminate the residual erythrocytes from the Ficoll).

To reduce the number of contaminating cells even further, it is possible to carry out a second passage in a magnetic column.

(c) FIXING AND LABELLING

The post-MACS cells were centrifuged at 300g for 10 min; the pellet was re-suspended in 40 µl of buffer.

The specimen was transferred into a 1.5-ml test-tube. To this were added 760 µl of 4% paraformaldehyde just prepared, followed by incubation for 20 min at room temperature.

After centrifugation at 0.2 r.c.f. r.p.m. for 5 min in the microcentrifuge, the supernatant fraction was drawn off.

This was followed by washing with 1 ml of PBS, centrifugation at 0.2 r.c.f. r.p.m. for 5 min in the microcentrifuge, and the supernatant fraction was again aspirated.

To this were added 100 µl of PBS/BSA at 3% (blocking buffer).

The specimen was incubated for 10 min at room temperature.

After further centrifugation at 0.2 r.c.f. r.p.m. for 5 min in the microcentrifuge, followed by aspiration of the supernatant fraction, staining was performed with EpCAM-FITC and CD45-PE (Miltenyi, according to the protocol recommended by the manufacturer).

To complete the process, a final washing was performed with 1000 µl of Super Buffer (Hepes 400mM, 1% BSA) and 1 µl of Hoechst 33324 diluted with water (100 µg/ml) and the specimen was vortexed.

The specimen was subjected to a quality control to verify the intensity of fluorescence of the marker and the total cell concentration. A part of the labelled specimen was re-suspended in a minimum volume of specific buffer, useful for dielectrophoretic manipulation, and was loaded on a device for control of quality of the specimen and controlled under the fluorescence microscope: the intensity of fluorescence of the

cells was noted in the different channels and a count was made of the cells labelled in Hoechst 33324 (total nucleated cells). If the cell concentration was higher than the optimal one for proper operation of the device for isolation of the single cells, the next step was dilution of the specimen to obtain the desired concentration.

Isolation

The cells thus obtained were then inserted in the chip DEPArray® CONV600K (manufactured by Silicon BioSystems, see for example, the document No. WO0069525) for dielectrophoretic manipulation and isolation of the tumour cells. The device as a whole is illustrated in Figure 2.

The specimen was subjected to scanning, identification, and selection, sorting, and recovery of the tumour cells.

The caged cells were scanned in an automatic or manual way at the microscope with 3 different fluorescence channels (i.e., in 3 different wavelengths). Observation in the DAPI channel (where by "DAPI channel" is meant UV excitation and emission in the blue, which is hence used also for visualization of Hoechst 33324) enables identification of the (positive) nucleated cells, whilst observation in the channels for EpCAM and CD45 enables differentiation between tumour cells (DAPI+, EpCAM+ and CD45-, with compatible morphology), lymphocytes (DAPI+, EpCAM-, CD45+) and spurious signals (DAPI+, EpCAM+ and CD45+ or also DAPI-, EpCAM+ and CD45+, or DAPI+, EpCAM+ and CD45- with morphology incompatible with tumour cells).

Figures 3A-B-C show images acquired in the course of scanning of the cells of the specimen within the DEPArray® CONV600K. More in particular, Figure 3A shows the images for three tumour cells in the three channels taken into consideration. Figure 3B shows the images for a spurious cell in the three channels taken into consideration. Figure 3C shows the images

for two lymphocytes in the three channels taken into consideration.

Selection of cells was then performed by selecting the cages containing the cells found positive to DAPI (nucleated cells), positive to EpCAM and negative to CD45. Sixteen cells were identified on the chip, some of which were double. Fifteen cells, some of which were double, were finally recovered in a few microlitres (< 40 µl) in a 0.2-ml PCR tube.

The final analysis was performed using an Applied Biosystems Minifiler Kit.

Given in Figures 4A-B-C-D are the results of the analysis of the fragments (QF-PCR) performed using a Minifiler Kit. In the four fluorescence channels taken into consideration, different microsatellites were analysed. It may be noted how in no case were there traces of contamination by alleles coming from the male subject into whose blood the tumour cells were introduced by spiking.

As an alternative to the anti-EpCAM antibody, it is possible to use a different type of antibody, such as, for example, an antibody that recognizes one or more types of cytokeratin, which is not expressed in the blood cells.

Alternatively, it is possible to use an antibody that is more characterizing for cancer (tumour-specific), such as for example:

- for the prostate, the Prostate Specific Antigen (PSA);
- for the lung, the Thyroid Transcription Factor 1 (TTF-1);
- for the breast, the Human Epidermal growth factor Receptor 2 (HER2/neu)

<u>Possibilities of application</u>

Obtaining a specimen of CTC having a purity of 100%, following upon an automatable procedure of selection and isolation, renders feasible diagnostic pathways for a plurality of conditions that, otherwise, could not be analysed accurately, reliably, and precisely.

Example 1 -Non-invasive evaluation of mutations (for example, in the K-RAS gene) in cancer patients.

Described in what follows is the case of isolation of CTCs from peripheral bloodflow of patients affected by metastatic Colon-Rectal Cancer (mCRC).

A specimen of 7.5 ml of peripheral bloodflow was drawn from the patient in Vacutainer tubes with EDTA anticoagulant (Beckton Dickinson). The specimen was analysed with Coulter Counter (Beckman Coulter) and presented 42 x 10^6 leukocytes (WBCs) and 43.95 x 10^9 erythrocytes (RBCs). The PBMCs were then isolated via centrifugation (in Ficoll 1077). The PBMCs recovered (16.5 x 10^6 on the basis of the count with Coulter Counter) were washed in PBS with BSA and EDTA (Running Buffer, Miltenyi) and were selected via depletion with magnetic microbeads conjugated to anti-CD45 and anti-GPA antibodies (Miltenyi) according to the instructions of the manufacturer. The resulting cells (negative fraction for CD45 and GPA) were fixated with 2% PFA in PBS for 20 min at room temperature (RT). This was followed by washing in PBS and incubation in 3% PBS/BSA (blocking buffer), for 10 min at room temperature. After a flushing in PBS, CD45 labelling was performed with 10 µl of PE-conjugated anti-CD45 antibody (Miltenyi) in 100 µl of Miltenyi Running Buffer (RB) for 10 min at 4°C. The reaction was blocked by adding 1 ml of RB, centrifuging and drawing off the supernatant portion. The cells were then permeabilized with 90 µl InsidePerm (Miltenyi) and simultaneously labelled with 10 µl of FITC-conjugated anti-CK antibody for 10 min at room temperature. The reaction was

terminated by adding 1 ml of Inside Perm, centrifuging, and removing the supernatant fraction. The pellet was re-suspended in buffer optimized for manipulation of cells fixated and permeabilized with dielectrophoresis - Hepes (400mM) + BSA (2%) in water (SB) - and DAPI (1 mg/ml) and, finally, washed in SB and re-suspended for injection into the chip DEPArray™ CONV600K (with 100,000 dielectrophoretic cages). Present on the chip were approximately 16,000 cells. After scanning in DAPI/FITC/PE the CTCs were identified. Figure 5 contains images of CTCs (indicated by a white arrow) selected and isolated by means of the method of the invention, said images being appropriately processed so as to highlight the fluorescence in three respective channels: FITC-conjugated anti-CK MoAb (Miltenyi) in the green channel; PE-conjugated anti-CD45 MoAb (Miltenyi) in the red channel; and DAPI labelling of DNA for identification of the nuclei in the blue channel.

It should be noted how it is possible to distinguish clearly between various types of possible events from an analysis of the processed images, whereas in a less refined analysis that is not based upon images, but only upon the intensity of the overall fluorescence, the images could be rejected as false positives.

For example, the cage with ID 382 contains a pair of CTCs. These were evidently already bound at the start, since the probability of two of the three CTCs present ending up in one and the same cage is negligible. Without image analysis, this event could have been rejected since, in general, clusters of cells can give rise to spurious fluorescence signals given that the antibody - for example, anti-CK - gets trapped in a non-specific way. Recovery of the two ID382 cells hence has a purity of 100% (and is compatible with the majority of molecular analyses).

The cage with ID 11439 indicates a DAPI+/CK+/CD45+ event. Without image analysis said event could have been rejected as spurious, but, instead, since the image makes possible detection of the distribution of fluorescence in the event, it is possible to interpret the datum as referring to a DAPI+/CK+/CD45- CTC (indicated by a white arrow) caged together with three other CK- nucleated cells, two of which CD45+. The contents of said cage can be recovered separately. Using a system based upon mobile dielectrophoretic cages, the segregation in single cages of the cells that initially share the same cage can be performed by applying appropriate patterns of cages. If it is not possible to segregate the CTC from the other contaminating cells of the cage, it is possible in any case to recover the contents of the cage, associating to said recovery the information of the presence of contaminants. This is a further advantage of the technique according to the invention. The purity of the cells recovered is noted down and this can be taken into account in the analysis downstream. In the case in point, the cell could hence be recovered together with the other three (a purity of 25%), or else separated and recovered alone to obtain a pure recovery of 100% (if isolated alone), or else of 50% if isolated together with a contaminant, or of 90% if isolated together with a contaminant and a further nine CTCs, without contaminants possibly present in other cages (not in this case).

The cage ID 5007 shows an event which, on the basis of the total fluorescence, would appear similar to that of ID 11439 since it is a DAPI+/CK+/CD45+ event. However, on the basis of the image, it is possible to determine how it is a spurious event in so far as it is linked to a cluster that has trapped anti-CK antibodies.

The cage ID 13103 shows an event that, on the basis of the total fluorescence, would appear similar to that of ID 11439

since it is a DAPI+/CK+/CD45+ event. However, on the basis of the image, it is possible to determine how as it is a spurious event in so far as it is linked to a single positive double cell with CD45+ (non-CTC) signal.

The cages with ID 8614 shows an event CK+/CD45- but DAPI-, not classified as CTC. The cage with ID 7796 shows a control event (two white blood cells) comprising two cells that are not classified as CTCs, because they are CK- and one is also CD45+.

The cells of interest can hence be isolated or otherwise, according to the purposes of the experiment, in terms of purity of the specimen and according to the ease of separation of the cell from an agglomerate of cells within which it is comprised. In this sense, the selection based upon images is particularly effective for exclusion of false positives and false negatives.

As an alternative to Ficoll, it is possible to use a technique based upon selective lysis of red blood cells (RBCs). In this case, in the specimen there remain in the first instance also the granulocytes. After said removal of the RBCs, the tumour cells can be further enriched via positive immuno-magnetic selection (e.g., with magnetic microbeads coupled to anti-EpCAM antibodies), or negative immuno-magnetic selection (e.g., via magnetic microbeads coupled to anti-CD45 antibodies or to a cocktail with anti-CD45 and other antibodies against antigens not present in the CTCs). In any case, the few CTCs present are obtained in a specimen containing tens of thousands or hundreds of thousands of leukocytes.

Isolation of the CTCs from the peripheral bloodflow is likewise compatible with procedures of enrichment and labelling approved by the FDA, such as the Veridex CellSearch™ system. In this case, the CellSearch system can be used also

for carrying out labelling and enrichment in an automated way (with the AutoPrep machine). With this system, the few CTCs present are obtained (with an optimal yield of around 90%) in a specimen with typically only a few thousands of contaminating leukocytes (typical values of 1000-5000). The CellSearch AutoPrep represents an excellent enrichment system, and has as its main limitation the fact that it is based upon an EpCAM-positive immuno-magnetic selection. Consequently, in some types of tumours, where the CTCs do not over-express EpCAM, it would be possible to find only a few CTCs. In such cases, the negative selection is more indicated for depletion of CD45+ cells.

Detection of the mutational status of the K-RAS gene has considerable clinical importance, since it has been demonstrated that some mutations of K-RAS are connected to the inefficacy of therapies based upon monoclinal antibodies directed against the EGFr receptor. Said mutations, in fact, activate, downstream, the mechanism of cell proliferation in spite of the EGFr inhibition upstream. Therapies with Cetuximab and Panitumumab are already indicated only for patients with wild-type K-RAS. The possibility of identifying the mutation in CTCs affords the possibility of not having to resort to a biopsy, in particular in all those circumstances in which a biopsy is not possible or is very complex to perform. Even when tissues removed surgically are available, the possibility of analysing CTCs is in any case interesting. The tumours are intrinsically unstable from the genetic standpoint, and the metastases can derive from cells located in sites distinct from that of the primary tumour; consequently, the CTCs can reflect better the molecular profile of the cells undergoing metastasis.

In order to verify the possibility of detecting mutations of K-RAS from CTCs, CTCs from peripheral bloodflow of patients affected by metastatic cancer of the colon-rectum (mCRC) were

analysed. Following the enrichment procedure described above, CTCs were identified and isolated on the DEPArray™. The purity of 100% enables detection of the possible presence of the mutation via sequencing: after prior amplification of the starting copies, for example with nested PCR, sequencing is carried out using known techniques.

In the case in point, the analysis downstream was performed using the DOP-PCR technique with the Omniplex kit produced by Sigma. After amplification, the product was analysed with a capillary-electrophoresis sequencer manufactured by Applied Biosystems.

Figure 6 shows an example of detection of a mutation in one of the specimens analysed, which corresponds to a mutation from glycine to valine in the codon 12 (shown in Figure 7 is the corresponding negative control).

Example 2 - Evaluation on CTCs of deletions (for example, in prostate cancer) via single-cell whole-genome amplification and CGH array. In this case, there is advantageously performed isolation of the single cells to be recovered in different wells. In this way, the analysis takes into account the heterogeneity of the population, and supplies not only the average, but multiple signals regarding each cell so that it is easier to identify mutations present only in some of the tumour cells.

Example 3 - Non-invasive identification of the original tissue in CUP tumours, via genetic profile and/or profile of expression of the CTCs.

Example 4 - Analysis of gene expression on single disseminated tumour cells (DTCs) for identification of prognostic indicators, and potential therapeutic targets. Also in this case, there is advantageously performed isolation of the

single cells to be recovered in different wells. In this way, the analysis takes into account the heterogeneity of the population, and supplies not only the average, but multiple signals for each cell so that it is easier to identify mutations present only in some of the tumour cells.

Example 5 - As already mentioned, in general, what has been said above regarding "isolation of cells" is to be understood as applying also to "isolation of portions of cell", such as, for example, the nucleus. In fact, this enables in any case, for certain types of biomolecular information, significant information to be obtained (for example, by evaluating the genomic DNA contained in the nucleus).

In the example of isolation of a nucleus, the selection of the cells can be performed via techniques that mark in a distinguishable way the nuclei of tumour cells (CTCs or DTCs) from non-tumour cells, such as for example, FISH. In this case, the presence of multiple signals reveals the presence of duplications of genomic DNA (a characteristic of the tumour cells in general not present in normal cells).

In particular, in the example of breast cancer, it is of interest to evaluate duplication of the genomic region regarding the HER2 gene, normally located on chromosome 17. Hence, two types of probes are applied, one for the centromere of chromosome 17, and one for the HER2 gene. If the gene/chromosome ratio is higher than two the cell is conventionally considered positive to the test. Said information has, for example, implications in the use of drugs such as Trastuzumab (Herceptin) that present efficacy only in the case of over-expression of the corresponding HER2 receptor. Not only counting, but also identifying and isolating said cells in a purified form enables acquisition of further information on the genetic characteristics of the tumour.

Example 6

The presence of copy-number variations (CNVs) in the DNA constitutes a typical characteristic of a tumour. For evaluation of new drugs it is of considerable interest to be able to evaluate the CNVs in order to associate them to the course of the illness and, potentially, to areas of the genome in which the CNVs have a significant influence on the course of the illness (both in the case of favourable prognosis and in the case of unfavourable prognosis). This information can be helpful in identifying genes that could be targets for the pharmacological action. Furthermore, in the stage of diagnosis of cancer it may then be possible to identify in a minimally invasive way the CNV profile of the CTCs of the patient in order to orient the therapy on the basis of the experience acquired.

To demonstrate the possibility of determining CNVs small specimens of CTCs and, accordingly, small negative-control specimens were separated and isolated.

From a patient suffering from metastatic breast cancer (mBrCa), a specimen of 7.5 ml of peripheral blood was taken and put in a CellSave tube (Veridex). The specimen was enriched and labelled with fluorescent antibodies (PE-conjugated anti-CK, APC-conjugated anti-CD45) and DAPI, with the CellSearch AutoPrep, according to the standard procedure. The enriched cells were extracted from the Veridex cartridge, and re-suspended in a reduced volume. Said specimen was injected on the chip DEPArray™ A300K (Silicon Biosystems S.p.A.) - with 307,200 electrodes and a number of programmable cages, typically between 19,200 and 76,800. After scanning, there were isolated two negative controls with five cells, two specimens with five CTCs, one specimen with a single CTC.

Figures 8 and 9 illustrate the cells recovered, respectively, in a first and second recovery with five leukocytes each (DAPI+/CD45+/CK-) as negative control for the CNV detection. Figure 10 illustrates the five CTCs recovered separately, which form the subject of CNV analysis.

After selection and isolation performed using the method according to the invention, and given that a specimen is now available with a degree of purity higher than 90% (in the case in point, 100% for the recovery of CTCs illustrated in Figure 10), it is possible to carry out successfully analysis based upon whole-genome amplification, according to a methodology illustrated in the document No. EP1109938, ligation-mediated PCR, followed by metaphase comparative genomic hybridization (CGH) or CGH array, which, with specimens of lower purity, would be impossible or intrinsically unreliable in so far as the signal detected would be too weak or fuzzy in the presence of non-tumoural cells.

CLAIMS

1. A method for the diagnosis of tumoural conditions and/or of the corresponding state of advance, comprising the steps of:

a. obtaining from a patient a specimen of an organic fluid having a high probability of containing at least one circulating tumour cell or disseminated tumour cell or portions thereof;

b. enriching said specimen of said organic fluid in at least one population of cells comprising at least one type of circulating tumour cells or disseminated tumour cells or portions thereof;

c. isolating at least one cell from amongst said at least one type of circulating tumour cells or disseminated tumour cells or portions thereof obtaining a purified specimen;

d. performing a molecular analysis on said purified specimen, in such a way as to highlight at least one characteristic of said at least one cell suited to enabling said diagnosis;

said method being **characterized in that** said step of isolating at least one cell or portion of cell from amongst said at least one type of circulating tumour cells or disseminated tumour cells or portions thereof is performed by selecting individually single cells or portions of cells in a microfluidic device so as to obtain a purified specimen having a purity of at least 90%.

2. The method according to Claim 1, characterized in that said step of isolating at least one cell or portion of cell from amongst said at least one type of circulating tumour cells or disseminated tumour cells or portions thereof is performed so as to obtain a diagnostic specimen having a purity of at least 95%.

3. The method according to Claim 2, characterized in that

said step of isolating at least one cell or portion of cell from amongst said at least one type of circulating tumour cells or disseminated tumour cells or portions thereof is performed so as to obtain a diagnostic specimen having a purity of 100%.

4. The method according to any one of the preceding claims, characterized in that said microfluidic system isolates said cells in a non-manual way.

5. The method according to claim 4, characterized in that said microfluidic system isolates said cells in an automatic or semi-automatic way.

6. The method according to any one of the preceding claims, characterized in that said individual selection of said cells is made on the basis of parameters of said cells evaluated in the absence of flow of said organic fluid.

7. The method according to any one of the preceding claims, characterized in that said individual selection of said cells is made on the basis of the evaluation of images of said cells.

8. The method according to the claim 7, characterized in that said images comprise fluorescence images.

9. The method according to any one of the preceding claims, characterized in that said individual selection of said cells is made on the basis of impedentiometric properties.

10. The method according to any one of the preceding claims, characterized in that it comprises a step of labelling of the circulating tumour cells or disseminated tumour cells or portions thereof, with at least one type of tracer.

11. The method according to the preceding claim, characterized in that said at least one type of tracer is a FISH probe.

12. The method according to Claim 10, characterized in that said at least one type of tracer is an antibody conjugated with a marker chosen in the group consisting of:

- fluorophors
- chromogens
- (fluorescent or non-fluorescent) microbeads

13. The method according to the preceding claim, characterized in that said at least one type of antibody recognizes an antigen chosen in the group consisting of:

- EpCAM
- Cytokeratin 8
- Cytokeratin 18
- Cytokeratin 19
- Cytokeratin 20
- CD45
- EGFR
- IGFR
- PSA
- TTF1
- HER2/neu
- MUC-1

14. The method according to any one of the preceding claims, characterized in that said step of isolating at least one cell or portion of cell from amongst said at least one type of circulating tumour cells or disseminated tumour cells or portions thereof is performed by capturing single cells, preferably each at a specific site of a plurality of sites of said microfluidic device arranged in the microfluidic device according to an array and, subsequently, by selecting single cells from among the captured cells, on the basis of at least

one parameter that can be detected by means of a sensor internal or external to the microfluidic device.

15. The method according to any one of the preceding claims, characterized in that said step of enriching said specimen of said organic fluid comprises a selection of cells made on the basis of at least one parameter chosen in the group consisting of:

a. mass density;
b. morphology;
c. electrical properties;
d. chemical properties;
e. mechanical properties;
f. expression of surface antigens;
g. expression of intracytosolic antigens;
h. dielectric properties;
i. magnetic properties;
l. optical properties;
m. geometrical properties;

or combinations thereof.

16. The method according to any one of the preceding claims, characterized in that said step of enriching said specimen of said organic fluid in at least one population of cells comprising at least one type of circulating tumour cells or disseminated tumour cells or portions thereof comprises the step of treating said specimen of said organic fluid so as to separate the nucleated cells and enrich it subsequently in nucleated cells.

17. The method according to the preceding claim, characterized in that said step of enriching said specimen of said organic fluid in at least one population of cells comprising at least one type of circulating tumour cells or disseminated tumour cells or portions thereof comprises at least the step of centrifuging said specimen of said organic

fluid in density gradient.

18. The method according to Claim 16, characterized in that said step of enriching said specimen of said organic fluid in at least one population of cells comprising at least one type of circulating tumour cells or disseminated tumour cells or portions thereof comprises at least the step of carrying out a selective lysis of the erythrocytes.

19. The method according to any one of the preceding claims, characterized in that said step of enriching said specimen of said organic fluid in at least one population of cells comprising at least one type of circulating tumour cells or disseminated tumour cells or portions thereof comprises at least the step of carrying out a positive and/or negative selection via magnetic beads coupled to antibodies according to at least one of the following modalities:

a) CD45 negative
b) CD45 and GPA negative
c) CD45 and CD14 negative
d) CD45, CD14 and GPA negative
e) EpCAM positive
f) CK positive

20. The method according to any one of the preceding claims, characterized in that said at least one enrichment step is performed in combination with said isolation step within the same said microfluidic device used for performing the isolation step.

21. The method according to the preceding claim, characterized in that a microfluidic device is used equipped with a plurality of different chambers, separated from one another and hydraulically connected, delimited on at least one face by a single chip or by a plurality of separate chips.

22. The method according to any one of the preceding claims, characterized in that said step of molecular analysis is performed by means of a procedure selected from among:

- sequencing
- microsatellite analysis
- comparative genomic hybridization (CGH)
- array CGH
- end-point PCR
- real-time PCR
- methylation analysis:
  - o quantitative methylation analysis with pyrosequencing
  - o bisulphite-genomic-sequencing PCR (BSP)
  - o methylation-specific PCR (MSP)
  - o Combined Bisulphite Restriction Analysis of DNA (COBRA)
  - o methylation-sensitive single nucleotide primer extension (MS-SNuPE)
- gene-expression analysis
  - o RT-PCR
  - o single-cell gene expression
  - o digital PCR

23. The method according to Claim 22, characterized in that said step of molecular analysis comprises assessing the presence of point (single-nucleotide) mutations.

24. The method according to Claim 23, characterized in that said point (single-nucleotide) mutations regard the K-RAS and/or B-RAF gene and their presence is an index of non-responsivity of the patient to a predetermined therapy.

25. The method according to any one of Claims 1 to 22, characterized in that said step of molecular analysis comprises evaluating the presence of hypermethylation of oncosuppressor genes.

26. The method according to any one of Claims 1 to 22, characterized in that said step of molecular analysis comprises evaluating the presence of deletions and/or duplications.

27. The method according to Claim 26, characterized in that said step of molecular analysis comprises evaluating the presence of deletions in the chromosome region 13q14 in tumour cells in patients with suspected prostate cancer.

28. The method according to any one of Claims 1 to 22, characterized in that said step of molecular analysis comprises evaluating the over-expression of at least one of the genes BCL2, CCND1 and WNT3A in tumour cells in patients with suspected prostate cancer.