Title: CIRCULATING TUMOR CELLS (CTC’S): APOPTOTIC ASSESSMENT IN PROSTATE CANCER PATIENTS

Abstract: A method for detecting and enumerating apoptotic cancer cells in a mixed cell population by obtaining a biological specimen, the presence of said cancer cells in said population being indicative of a diseases state, is provided. The method comprises obtaining a biological specimen from a test subject, said specimen comprising a mixed cell population suspected of containing said cancer cells; preparing an immunomagnetic sample wherein said specimen is mixed with magnetic particles coupled to a biospecific ligand which reacts specifically with said cancer cells, to the substantial exclusion of other sample components; contacting said immunomagnetic sample with at least one biospecific reagent which labels said cancer cells; and characterizing said cancer cells into one or more apoptotic states from the group consisting of enzymatic analysis, biochemical analysis, morphological analysis and combination thereof, the presence and number of said apoptotic state being indicative of said disease state.
Title: Circulating Tumor Cells (CTC's): Apoptotic Assessment in Prostate Cancer Patients.

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Cross Reference to Related Applications
This application is a continuation-in-part of U.S. Application No. 10/269,579, filed on 11 October 2002.

Background

• Field of the Invention
This invention relates generally to the field of diagnostic testing, and more particularly to diagnostics in the oncology field. The invention is useful in cancer screening, staging, monitoring for chemotherapy treatment responses, cancer recurrence or the like. More specifically, the present invention provides reagents, methods and test kits that facilitate analysis and enumeration of tumor cells, or other rare cells isolated from biological samples. The invention also provides materials and methods for assessing tumor diathesis associated molecules, such as nucleic acids, proteins and carbohydrates, thereby aiding the clinician in the design of therapeutic treatment strategies.

• Background Art
Each year in the United States, approximately 600,000 new cases of cancer are diagnosed; one out of every five people in this country will die from cancer or from complications associated with its treatment. Considerable efforts are continually directed at improving treatment and diagnosis of this disease.

Most cancer patients are not killed by their primary tumor. They succumb instead to metastases: multiple widespread tumor colonies established by malignant cells that detach themselves from the original tumor and travel through the body, often to distant sites. If a primary tumor is
detected at an early stage, it can often be eliminated by surgery, radiation, or chemotherapy or some combination of these treatments. Unfortunately, metastatic colonies are frequently more difficult to detect and eliminate and it is often impossible to treat all of them successfully. Therefore, from a clinical point of view, metastasis can be considered the penultimate event in the natural progression of cancer. Moreover, the ability to metastasize is the property that uniquely characterizes a malignant tumor.

Cancer metastasis comprises a complex series of sequential events. These are: 1) extension from the primary locus into surrounding tissues; 2) penetration into body cavities and vessels; 3) release of tumor cells for transport through the circulatory system to distant sites; 4) reinvasion of tissue at the site of arrest; and 5) adaptation to the new environment so as to promote tumor cell survival, vascularization and tumor growth.

Based on the complexity of cancer and cancer metastasis and the frustration in treating cancer patients over the years, many attempts have been made to develop diagnostic tests to guide treatment and monitor the effects of such treatment on metastasis or relapse. Such tests presumably could also be used for cancer screening, replacing relatively crude tests such as mammography for breast tumors or digital rectal exams for prostate cancers. Towards that goal, a number of tests have been developed over the last 20 years. One of the first attempts was the formulation of an immunoassay for carcinoembryonic antigen [CEA]. This antigen appears on fetal cells and reappears on tumor cells in certain cancers. Extensive efforts have been made to evaluate the usefulness of testing for CEA as well as many other tumor antigens, such as PSA, CA 15.3, CA125, PSMA, and CA27.29. However, the appearance of such antigens in blood has not been generally predictive and is often detected when there is little hope for the patient. In the last few years, however, one test has proven to be useful in the early detection of cancer, viz., Prostate Specific Antigen [PSA] for prostate cancers. When used with follow-up physical examination and biopsy, the PSA test has played a remarkable role in detecting prostate cancer early, at the time when it is best treated.

Despite the success of PSA testing, the test leaves much to be desired. For example, high levels of PSA do not always correlate with cancer
nor do they appear to be an indication of the metastatic potential of the tumor. This may be due in part to the fact that PSA is a component of normal prostate tissue as well as other unknown factors. Moreover, it is becoming clear that a large percentage of prostate cancer patients will continue to have localized disease which is not life threatening. Based on the desire to obtain better concordance between those patients with cancers that will metastasize and those that won't, attempts have been made to determine whether or not prostate cells are in the circulation. When added to high PSA levels and biopsy data, the existence of circulating tumor cells might give indications as to how vigorously the patient should be treated.

One approach for determining the presence of circulating prostate tumor cells has been to test for the expression of messenger RNA for PSA in blood. This is being done through the laborious procedure of density separation of mononuclear cells from a blood sample, followed by isolating all of the mRNA from these cells, and performing reverse transcriptase PCR for PSA. As of this date, (Gomella LG. J of Urology. 158:326-337(1997)) no good correlation exists between the presence of such cells in blood and the ability to predict which patients are in need of vigorous treatment. It is noteworthy that PCR is difficult, if not impossible in many situations, to perform quantitatively, i.e., determine number of tumor cells per unit volume of biological sample. Additionally false positives are often observed using this technique. An added drawback is that there is a finite and practical limit to the sensitivity of this technique based on the sample size examined. Typically, the test is performed on $10^5$ to $10^6$ cells purified away from interfering red blood cells. This corresponds to a practical lower limit of sensitivity of one tumor cell/ 0.1 ml of blood. Hence, approximately 10 tumor cells in a ml of blood must be present before signal is detectable. As a further consideration, tumor cells are often genetically unstable. Accordingly, cancer cells having genetic rearrangements and sequence changes may be missed in a PCR assay as the requisite sequence complementarity between PCR primers and target sequences can be lost.

In summary, a useful diagnostic test needs to be very sensitive and reliably quantitative. If a blood test can be developed where the presence of a single tumor cell can be detected in 1ml of blood, that would correspond on
average to 3000-4000 total cells in circulation. Innoculum studies for establishing tumors in animals show that injection of 3000-4000 of cells can indeed lead to the establishment of a tumor. Further if 3000-4000 circulating cells represent 0.01% of the total cells in a tumor, then it would contain about $4 \times 10^7$ total cells. A tumor containing that number of cells would not be visible by any technique currently in existence. Hence, if tumor cells are shed in the early stages of cancer, a test with the sensitivity mentioned above would detect the cancer. If tumor cells are shed in some functional relationship with tumor size, then a quantitative test would be beneficial to assess tumor burden. Heretofore there has been no information regarding the existence of circulating tumor cells in very early cancers. Further, there are considerable doubts in the medical literature regarding the existence of such cells and the potential of such information. The general view is that tumors are initially well confined and hence there will be few if any circulating cells in early stages of disease. Also, there are doubts that the ability to detect cancer cells early on will provide useful information.

Based on the above, it is apparent that a method for identifying those cells in circulation with metastatic potential prior to establishment of a secondary tumor is highly desirable, particularly early on in the cancer. To appreciate the advantage such a test would have over conventional immunoassays, consider that a highly sensitive immunoassay has a lower limit of functional sensitivity of $10^{-17}$ moles. If one tumor cell can be captured from a ml of blood and analyzed, the number of moles of surface receptor, assuming 100,000 receptors per cell would be $10^{-19}$ moles. Since about 300 molecules can be detected on a cell such an assay would have a functional sensitivity on the order of $10^{-22}$ moles, which is quite remarkable. To achieve that level of sensitivity in the isolation of such rare cells, and to isolate them in a fashion which does not compromise or interfere with their characterization is a formidable task.

Many laboratory and clinical procedures employ bio-specific affinity reactions for isolating rare cells from biological samples. Such reactions are commonly employed in diagnostic testing, or for the separation of a wide range of target substances, especially biological entities such as cells, proteins, bacteria, viruses, nucleic acid sequences, and the like.
Various methods are available for analyzing or separating the above-mentioned target substances based upon complex formation between the substance of interest and another substance to which the target substance specifically binds. Separation of complexes from unbound material may be accomplished gravitationally, e.g. by settling, or, alternatively, by centrifugation of finely divided particles or beads coupled to the target substance. If desired, such particles or beads may be made magnetic to facilitate the bound/free separation step. Magnetic particles are well known in the art, as is their use in immune and other bio-specific affinity reactions. See, for example, US Patent No. 4,554,088 and Immunoassays for Clinical Chemistry, pp. 147-162, Hunter et al. eds., Churchill Livingston, Edinburgh (1983). Generally, any material that facilitates magnetic or gravitational separation may be employed for this purpose. However, it has become clear that magnetic separation means are the method of choice.

Magnetic particles can be classified on the basis of size; large (1.5 to about 50 microns), small (0.7-1.5 microns), or colloidal (<200nm), which are also referred to as nanoparticles. The third, which are also known as ferrofluids or ferrofluid-like materials and have many of the properties of classical ferrofluids, are sometimes referred to herein as colloidal, superparamagnetic particles.

Small magnetic particles of the type described above are quite useful in analyses involving bio-specific affinity reactions, as they are conveniently coated with biofunctional polymers (e.g., proteins), provide very high surface areas and give reasonable reaction kinetics. Magnetic particles ranging from 0.7-1.5 microns have been described in the patent literature, including, by way of example, US Patent Nos. 3,970,518; 4,018,886; 4,230,685; 4,267,234; 4,452,773; 4,554,088; and 4,659,678. Certain of these particles are disclosed to be useful solid supports for immunological reagents.

The efficiency with which magnetic separations can be done and the recovery and purity of magnetically labeled cells will depend on many factors. These include:

- the number of cells being separated,
- the receptor or epitope density of such cells,
- the magnetic load per cell,
- the non-specific binding (NSB) of the magnetic material,
- the carry-over of entrapped non-target cells,
- the technique employed,
- the nature of the vessel,
- the nature of the vessel surface,
- the viscosity of the medium, and
- the magnetic separation device employed.

If the level of non-specific binding of a system is substantially constant, as is usually the case, then as the target population decreases so will the purity.

As an example, a system with 0.8% NSB that recovers 80% of a population which is at 0.25% in the original mixture will have a purity of 25%. Whereas, if the initial population was at 0.01% (one target cell in $10^6$ bystander cells), and the NSB were 0.001%, then the purity would be 8%. Hence, a high the purity of the target material in the specimen mixture results in a more specific and effective collection of the target material. Extremely low non-specific binding is required or advantageous to facilitate detection and analysis of rare cells, such as epithelial derived tumor cells present in the circulation.

Less obvious is the fact that the smaller the population of a targeted cell, the more difficult it will be to magnetically label and to recover. Furthermore, labeling and recovery will markedly depend on the nature of magnetic particle employed. For example, when cells are incubated with large magnetic particles, such as Dynal beads, cells are labeled through collisions created by mixing of the system, as the beads are too large to diffuse effectively. Thus, if a cell were present in a population at a frequency of 1 cell per ml of blood or even less, as may be the case for tumor cells in very early cancers, then the probability of labeling target cells will be related to the number of magnetic particles added to the system and the length of time of mixing. Since mixing of cells with such particles for substantial periods of time would be deleterious, it becomes necessary to increase particle concentration as much a possible. There is, however, a limit to the quantity of magnetic particle that can be added, as one can substitute a rare cell mixed in
with other blood cells for a rare cell mixed in with large quantities of magnetic particles upon separation. The latter condition does not markedly improve the ability to enumerate the cells of interest or to examine them.

As discussed in U.S. patent 6,365,362 and PCT/US99/03073, high gradient magnetic separation with an external field device employing highly magnetic, low non-specific binding, colloidal magnetic particles is the method of choice for separating a cell subset of interest from a mixed population of eukaryotic cells, particularly if the subset of interest comprises but a small fraction of the entire population. Such materials, because of their diffusive properties, readily find and magnetically label rare events, such as tumor cells in blood. For magnetic separations for tumor cell analysis to be successful, the magnetic particles must be specific for epitopes that are not present on hematopoietic cells.

Summary of the Invention
According to the present invention, a method for detecting and enumerating apoptotic cancer cells in a mixed cell population by obtaining a biological specimen, the presence of said cancer cells in said population being indicative of a disease state, is provided. The method comprises:

a. obtaining a biological specimen from a test subject, said specimen comprising a mixed cell population suspected of containing said cancer cells;

b. preparing an immunomagnetic sample wherein said specimen is mixed with magnetic particles coupled to a biospecific ligand which reacts specifically with said cancer cells, to the substantial exclusion of other sample components;

c. contacting said immunomagnetic sample with at least one biospecific reagent which labels said cancer cells; and

d. characterizing said cancer cells into one or more apoptotic states from the group consisting of enzymatic analysis, biochemical analysis, morphological analysis and combinations thereof, the presence and number of said apoptotic state being indicative of said disease state.
In a preferred embodiment of the invention, a method for assessing a patient for the presence of a malignancy is provided. The method entails obtaining a mixed cell population suspected of containing hematopoietic and non-hematopoietic malignant cells, immediately collected in preservative tubes, containing a stabilizing agent and fixative agent. After enrichment, the sample is then prepared wherein the biological sample is mixed with a detectably labeled ligand which reacts specifically with the malignant cells, to the substantial exclusion of other sample components. The sample is then contacted with at least one reagent which also specifically labels said malignant cells in an apoptotic state. One embodiment of the present invention uses the M30-FITC antibody to detect the apoptotic state. This antibody is specific for apoptosis-induced caspase cleaved cytokeratin 18. Analysis of the sample is then performed to determine the presence and number of labeled cells, detection of said cells indicating the presence of malignancy. Either the greater the number of labeled intact cells present, the greater the number of apoptotic cells present, or a combination of both is suggestive of the severity of the malignancy.

In accordance with the invention, cells are analyzed by a process selected from the group consisting of multiparameter flow cytometry, immunofluorescent microscopy, laser scanning cytometry, bright field base image analysis, capillary volumetry, spectral imaging analysis, manual cell analysis, and automated image analysis.

In a further aspect of the present invention, kits are provided for screening a patient sample for the presence of non-hematopoietic malignant cells.

**Brief Description of the Drawings**

**Figure 1:** Immunomagnetic Enrichment of whole blood sample prior to CTC analysis. Semi-automated fluorescent analysis used CD45-APC, Cytokeratin (CK) PE, and DAPI as staining reagents. Flow cytometry analysis used CD45-PerCP, CK PE, and nucleic acid dye as staining reagents.
Figure 2: Analysis by fluorescent microscopy CTC analysis from a 7.5 ml blood sample from a metastatic cancer patient. The columns of thumbnails correspond to the images from corresponding fields of the nucleus (DAPI), cytokeratin (CK-PE), control, leukocyte (CD45 APC) and false color overlay image of cytokeratin (green) and the nucleus (magenta). The rows of numbered thumbnails present the different locations in the analysis cartridge that contain CTC candidates. The center box of the “cross-hairs” in the lower right corner is 4μm and is used to size individual events during review of the images ensuring a minimal cell size of 4μm.

Figure 3: Flow cytometric CTC analysis of a sample previously analyzed by microscopy. Panel A and B shows all events whereas in Panels C and D only the cytokeratin positive events are shown.

Figure 4: Fluorescent microscopy and flow cytometric CTC analysis in three prostate cancer patients. For patient ten, 53 of 240 intact CTC, 7 of 20 damaged and 19 of 238 fragments are shown in Panel A. Green represents cytokeratin and magenta the nucleus. The corresponding flow cytometric analysis of only the cytokeratin positive events is shown in Panel B. For patient six, 2 of 16 intact CTC, 33 of 40 damaged and 6 of 320 fragments are shown in Panel C and the corresponding flow cytometric analysis is shown in Panel D. For patient five, 2 of 16 intact CTC, 15 of 38 damaged and 49 of 490 fragments are shown in Panel E and the corresponding flow cytometric analysis is shown in Panel F.

Figure 5: Fluorescent microscopy and flow cytometric CTC analysis of LnCaP cells spiked in blood of normal donors. Panel A shows the images of untreated LnCaP cells and Panel B the corresponding flow cytometric analysis of only the cytokeratin positive events. Panel C shows the images of paclitaxel treated LnCaP cells and Panel D the corresponding flow cytometric analysis.
Detailed Description of the Invention

Once tumor cells are identified in circulation, it is desirable to further characterize the isolated cells phenotypically or biochemically. Thus, particular tumor diathesis associated molecules, such as nucleic acid molecules, proteins, or carbohydrates that are associated with the malignant phenotype may be analyzed. Accordingly, methods are provided in accordance with the invention for measuring the level of expression of predetermined tumor diathesis associated molecules present in or on tumor cells identified in the circulation in a blood sample where these molecules have been morphologically and antigenically preserved. Classification of the apoptotic state of the tumor cells provides strict criteria in defining an intact CTC and their possible invasive potential. Further, classification and enumeration of apoptotic tumor cells provides critical information about the disease state. This information assists the clinician in diagnosing the type of cancer and assessing the efficacy of chemotherapeutic intervention strategies.

Another object of this invention is the improved detection of circulating tumor cells as an early prognostic indicator of a patient's disease state.

The method of the invention can be used to assess residual cancer cells in the circulation following medical radiation or surgical treatment to eradicate the tumor. The method can also be performed periodically over the course of years to assess the patient for the presence and number of tumor cells in the circulation, and alterations in tumor diathesis molecules therein as an indicator of occurrence, recurrence and/or progression of disease as previously described (PCT/US04/05848, PCT/US99/03073, and U.S. 6,365,362)

In yet another aspect of the invention, methods are provided for determining alterations in tumor diathesis associated molecules as a means to predict efficacy of therapy. An exemplary method comprises obtaining a sample from a patient; isolating and enumerating circulating malignant cells from said sample if present, and determining the number of at least one predetermined tumor diathesis associated molecule associated with apoptosis on individual cells present in said sample as a means to predict efficacy of therapy. Such methods may also be used to an advantage to assess the
appropriate dosage of a given therapeutic regimen and/or for monitoring the efficacy of therapy over time.

In yet another aspect of the invention, methods for culturing tumor cells isolated from the circulation are provided. Such cells may then be contacted with therapeutic agents to assess their sensitivity thereto. Such cells also provide a source for tumor diathesis associated molecules which may or may not be altered.

An exemplary kit of the invention comprises coated magnetic nanoparticles comprising: i) a preservative ii) a magnetic core material, a protein base coating material, and an antibody that binds specifically to a first characteristic determinant of said malignant cell, the antibody being coupled, directly or indirectly, to said base coating material; iii) at least one antibody having binding specificity for a second characteristic determinant of said malignant cell; iv) a cell specific dye for excluding sample components other than said malignant cells from analysis; v) a sample cartridge used in imaging; and at least one detectably labeled agent recognizing the cleavage of cytokeratin 18 after caspase cleavage. Such kits may optionally comprise an antibody which has binding affinity for non-target cells, a biological buffer, a permeabilization buffer, a protocol and optionally, an information sheet.

While any effective mechanism for isolating, enriching, and analyzing CTCs in blood is appropriate, one method for collecting circulating tumor cells combines immunomagnetic enrichment technology, immunofluorescent labeling technology with an appropriate analytical platform after initial blood draw. The associated test has the sensitivity and specificity to detect these rare cells in a sample of whole blood and to investigate their role in the clinical course of the disease in malignant tumors of epithelial origin. From a sample of whole blood, rare cells are detected with a sensitivity and specificity to allow them to be collected and used in the diagnostic assays of the invention, namely predicting the clinical course of disease in malignant tumors.

With this technology, circulating tumor cells (CTC) have been shown to exist in the blood in detectable amounts. This created a tool to investigate the significance of cells of epithelial origin in the peripheral circulation of cancer patients (Racila E., Euhus D., Weiss A.J., Rao C., McConnell J., Terstappen L.W.M.M. and Uhr J.W., Detection and characterization of carcinoma cells in
the blood, Proc. Natl. Acad. Sci. USA, 95:4589-4594 (1998)). This study demonstrated that these blood-borne cells might have a significant role in the pathophysiology of cancer. Having a detection sensitivity of 1 epithelial cell per 5 ml of blood, the assay incorporates immunomagnetic sample enrichment and fluorescent monoclonal antibody staining followed by flowcytometry for a rapid and sensitive analysis of a sample. The results show that the number of epithelial cells in peripheral blood of eight patients treated for metastatic carcinoma of the breast correlated with disease progression and response to therapy. In 13 of 14 patients with localized disease, 5 of 5 patients with lymph node involvement and 11 of 11 patients with distant metastasis, epithelial cells were found in peripheral blood. The number of epithelial cells was significantly larger in patients with extensive disease.

The assay was further configured to an image cytometric analysis such that the immunomagnetically enriched sample is analyzed by an automated imaging system. This is a fluorescence-based microscope image analysis system, which in contrast with flowcytometric analysis permits the visualization of events and the assessment of morphologic features to further identify objects.

Automated fluorescence imaging provides for automated enumeration of isolated cells from blood. The system contains an integrated computer controlled fluorescence microscope and automated stage with a magnetic yoke assembly that will hold a disposable sample cartridge. The magnetic yoke is designed to enable ferrofluid-labeled candidate tumor cells within the sample chamber to be magnetically localized to the upper viewing surface of the sample cartridge for microscopic viewing. Software detects cancer cells, labeled with an antibody of cytokeratin and having epithelial origin, from blood.

While the actual isolation of tumor cells can be accomplished by any means known in the art, one embodiment uses a stabilizing and fixative process for isolating tumor cells from 7.5 ml of whole blood. Epithelial cell-specific magnetic particles are added and incubated for 20 minutes. After magnetic separation, the cells bound to the immunomagnetic-linked
antibodies are magnetically held at the wall of the tube. Unbound sample is then aspirated and an isotonic solution is added to resuspend the sample. A nucleic acid dye, monoclonal antibodies to cytokeratin (a marker of epithelial cells) and CD 45 (a broad-spectrum leukocyte marker) are incubated with the sample. After magnetic separation, the unbound fraction is again aspirated and the bound and labeled cells are resuspended in 0.2 ml of an isotonic solution. The sample is suspended in a cell presentation chamber and placed in a magnetic device whose field orients the magnetically labeled cells for fluorescence microscopic examination. Cells are identified automatically with control cells enumerated and candidate circulating tumor cells presented to the operator for checklist enumeration. An enumeration checklist consists of predetermined morphologic criteria constituting a complete cell.

The diagnostic potential, together with the use of intact circulating tumor cells as a prognostic factor in cancer survival (Cristofanilli M., Budd G.T., Ellis M.J., Stopeck A., Matera J., Miller M.C., Doyle G.V., Allard W.J., Terstappen L.W.M.M., Hayes D.F. Correlation of Circulating Tumor cells with Progression and Survival in Metastatic Breast Cancer, New England J. of Med., 351: 781-791 (2004)), can provide a rapid and sensitive method for determining appropriate treatment. Accordingly in the present invention, method and kits are provided for the rapid enumeration and characterization of tumor cells shed into the blood in metastatic and primary patients which can readily be assessed for individual cell integrity, based upon apoptotic state. This analysis has potential for prognostic assessment in survival and progression-free survival predictions.

The methods of the invention are useful in assessing a favorable or unfavorable survival, and even preventing unnecessary therapy that could result in harmful side-effects when the prognosis is favorable. Thus, the present invention can be used for prognosis of any of a wide variety of cancers, including without limitation, solid tumors and leukemia’s including: apudoma, choristoma, branchioma, malignant carcinoid syndrome, carcinoid heart disease, carcinoma (i.e. Walker, basal cell, basosquamous, Brown-Pearce, ductal, Ehrlich tumor, Krebs 2, merkel cell, mucinous, non-small cell lung, oat cell, papillary, scirrhus, bronchiolar, bronchogenic, squamous cell,
and transitional cell), histiocytic disorders, leukemia (i.e. B-cell, mixed-cell, null-cell, T-cell, T-cell chronic, HTLV-II-associated, lymphocytic acute, lymphocytic chronic, mast-cell, and myeloid), histiocytosis malignant, Hodgkin’s disease, immunoproliferative small, non-Hodgkin’s lymphoma, plasmacytoma, reticuloendotheliosis, melanoma, chondroblastoma, chondroma, chondrosarcoma, fibroma, fibrosarcoma, giant cell tumors, histiocyte, lipoma, liposarcoma, mesothelioma, myxoma, myosarcoma, osteoma, osteosarcoma, Ewing’s sarcoma, synovioma, adenofibroma, adenolymphoma, carcinosarcoma, chordoma, craniopharyngioma, dysgerminoma, hamartoma, mesenchymoma, mesonephroma, myosarcoma, ameloblastoma, cementoma, odontoma, teratoma, thymoma, trophoblastic tumor, adenocarcinoma, adenoma, cholangioma, cholesteatoma, cylindroma, cystadenocarcinoma, cystadenoma, granulose cell tumor, gynandroblastoma, hepatoma, hidradenoma, islet cell tumor, icydig cell tumor, papilloma, sertoli cell tumor, theca cell tumor, leiomyoma, leiomyosarcoma, myoblastoma, myoma, myosarcoma, rhabdomyoma, rhabdomyosarcoma, ependymoma, ganglioneuroma, glioma, medulloblastoma, meningioma, neurilemmoma, neuroblastoma, neuroepithelioma, neurofibroma, neuroma, paraganglioma, paraganglioma nonchromaffin, angiookeratoma, angiolympoid hyperplasia with eosinophilia, angioma sclerosing, angiomatosis, glomangioma, hemangioendothelioma, hemangioma, hemangiopericytoma, hemangiosarcoma, lymphangioma, lymphangiomyxoma, lymphangiosarcoma, pinealoma, carcinosarcoma, chondrosarcoma, cystosarcoma, phylloides, fibrosarcoma, hemangiosarcoma, leiomyosarcoma, leukosarcoma, liposarcoma, lymphangiosarcoma, myosarcoma, myosarcoma, ovarian carcinoma, rhabdomyosarcoma, sarcoma (i.e. Ewing’s experimental, Kaposi’s and mast-cell), neoplasms (i.e. bone, breast, digestive system, colorectal, liver, pancreatic, pituitary, testicular, orbital, head and neck, central nervous system, acoustic, pelvic, respiratory tract, and urogenital, neurofibromatosis, and cervical dysplasia.

**Enumeration of circulating cytokeratin positive cells**

Cytokeratin positive cells are isolated by stabilizing and fixing the mix population in a 7.5 ml sample of whole blood. Epithelial cell-specific
immunomagnetic fluid is added and incubated for 20 minutes. After magnetic separation for 20 minutes, the cells bound to the immunomagnetic-linked antibodies are magnetically held at the wall of the tube. Unbound sample is then aspirated and an isotonic solution is added to resuspend the sample. A nucleic acid dye, monoclonal antibodies to cytokeratin (a marker of epithelial cells) and CD 45 (a broad-spectrum leukocyte marker) are incubated with the sample for 15 minutes. After magnetic separation, the unbound fraction is again aspirated and the bound and labeled cells are resuspended in 0.2 ml of an isotonic solution. The sample is suspended in a cell presentation chamber and placed in a magnetic device whose field orients the magnetically labeled cells for fluorescence microscopic examination by automated image analysis. Cells are identified automatically; control cells are enumerated, and the candidate circulating tumor cells are presented to the operator for enumeration by fluorescent microscopy (Figure 1).

Figure 2 depicts a typical characterization of CTC by fluorescent microscopy of a blood sample from a patient with metastatic prostate cancer. Regions that potentially contain CTC are displayed in rows of thumbnails. The ruler in the left lower corner of the figure indicates the size of the thumbnails. From right to left these thumbnails represent nuclear (DAPI), cytoplasmic cytokeratin (CK-PE), control (no reagent) and surface CD45 (CD45-APC) staining. The composite images are displayed a false color overlay of nuclear (DAPI) and cytoplasmic (CK-PE) staining. The check box beside the composite image allows the user to confirm that the images represented in the row are consistent with a tumor cell and the check box beside the CD45-APC image is used to identify a leukocyte or tumor cell that stained non-specifically. In this patient sample, 2761 rows of thumbnails were detected by the software that demonstrated staining consistent with tumor cells. Of the 2761 rows, 12 are shown in Figure 2 and labeled 1632-1638 and 1869-1873. Rows numbered 1636, 1638 and 1873 are checked off and display features of intact CTC defined as a size greater than 4μm, the presence of a nucleus surrounded by cytoplasmic cytokeratin staining and absence of control and CD45 staining. Note the difference in appearance of the tumor cells, the cell in row 1638 is large and the one in row 1636 is
significantly smaller. The immunophenotype of the event in row 1869 is consistent with a tumor cell but the morphology shows a large nucleus with speckled cytoplasm due to retraction of cytoskeletal proteins. The thumbnail in row 1634 shows a damaged cell that appears to extrude its nucleus. The thumbnail shown in row 1632 shows a cell that stains both with cytokeratin as well as CD45 and is either a tumor cell non-specifically binding to CD45 or a leukocyte non specifically staining with cytokeratin. In this instance, the morphology of the cell closely resembles that of a lymphocyte. The thumbnails shown in rows 1633, 1635, 1637, 1870 and 1872 shows cytokeratin staining objects that are larger that 4 μm but have no resemblance to cells. The cytokeratin positive objects in thumbnails 1637 and 1872 are in close proximity of a leukocyte. Based on the observation of images of CTC candidates in several patient samples, CTC were classified into three categories, intact CTC, damaged CTC and CTC fragments none of which stained with CD45 nor appeared in the control channel.

Figure 3 shows an example of the flow cytometric analysis of a blood sample from a patient with metastatic prostate cancer that was previously analyzed by fluorescent microscopy. Panel A shows the CD45-APC and CK-PE staining of all events in the sample and Panel B the DAPI and CK-PE staining. The darker events that stain with CD45-APC and have a normal DNA content as evidenced by their presence in the lower right hand quadrant of Panel B are intact leukocytes. The events that are positive for cytokeratin and are present in the upper right quadrant of Panel B are CTC with normal DNA content. The events that are positive for cytokeratin are CTC with insufficient DNA content, shown in the upper left quadrant of Panel B. All other events are depicted gray. Panels C and D are from the same experiments but only the cytokeratin positive events are shown. The CK-PE positive and CD45 APC negative events were subdivided based on staining with M30 FITC and DAPI, as shown in Panel D. Events that were positive for DAPI and negative for M30 are shown in the upper left portion of Panel C and the lower right quadrant of Panel D. Events that were positive for DAPI and M30 are shown in the upper right quadrant of Panel D. Events that were negative for DAPI and positive for M30 are shown in upper left quadrant of
Panel D. Events that were negative for DAPI and M30 are shown in the lower left quadrant of panel D. The arrow in Panel D indicates the apoptotic pathway of CTC.

**Assessing apoptosis of circulating tumor cells in prostate cancer patients by Fluorescent Microscopy and Flow Cytometry**

Figure 4 exemplifies the analysis of CTC by fluorescent microscopy and flow cytometry from blood samples. Individual thumbnails from the CTC images are shown as a composite and divided into three morphological distinct categories separated by arrows from left to right, intact CTC, damaged CTC and CTC fragments. The flow cytometry plot of DAPI versus M30 FITC staining of the cytokeratin positive events of the same sample is shown to the right of the collection of images. The number and relative frequency of the DAPI$^{\text{pos}}$, M30$^{\text{neg}}$; DAPI$^{\text{pos}}$, M30$^{\text{pos}}$; DAPI$^{\text{dim}}$, M30$^{\text{pos}}$ and DAPI$^{\text{dim}}$, M30$^{\text{neg}}$ events are provided in the figure.

In the blood of patient 10 shown in Panel A, the majority of CTC are intact and defined as objects larger than 4 μm, with a relatively smooth cytoplasmic membrane, cytoskeletal proteins throughout the cytoplasm and an intact nucleus encompassed within the cytoplasm. This population most likely correlates with the DAPI$^{\text{pos}}$, M30$^{\text{neg}}$ and DAPI$^{\text{pos}}$, M30$^{\text{pos}}$ regions on the corresponding flow plot in Panel B. Fewer CTC were identified as damaged or cell fragments.

In the blood of patient 6 shown in Panel C, the majority of CTC found by microscopy consisted of damaged CTC defined as objects larger than 4 μm, with speckled cytokeratin staining or ragged cytoplasmic membrane and a nucleus associated with the cytokeratin. The speckled cytokeratin staining is most likely the result of cytoskeletal retraction and degradation associated with the apoptotic cascade. Consistent with this is the finding by flow cytometry that the majority (74%) of events are DAPI$^{\text{dim}}$ and M30$^{\text{pos}}$ indicating that these cells are in the process of apoptosis, Panel D.

Finally, in the blood of patient 5 shown in Panel E, the majority of CTC found by microscopy were cell fragments defined as round cytokeratin positive objects at least 4 μm with or without association of nuclear material.
that had no morphological resemblance to a cell. The morphology of the objects are consistent with cell fragments likely representing apoptotic bodies at the end of the apoptosis process. This is consistent with this finding that the majority of events by flow cytometry (86%) are DAPI$^{\text{dim}}$ and M30$^{\text{neg}}$, see Panel F.

Table 1 summarizes the findings obtained by microscopy and flow cytometry of each of the 10 patients. In addition the analysis of blood samples from healthy men is shown in the table. In these samples 0 events classify as intact CTC by microscopy and no more than two were detected by flow cytometry. In some of the healthy donors, few events were found that classified as damaged or CTC fragments. The correlation between both analysis methods to detect CTC in patient samples was $R^2=0.71$ indicating that although the same samples were analyzed, not the same numbers of CTC were detected. For detection of intact CTC, the correlation improved to $R^2=0.95$. The number of intact CTC detected by flow cytometry (FC) was significantly greater ($p=0.0247$), this may however be contributed to the inclusion of CTC expressing M30. Only a small percent of all cytokeratin positive events are classified as intact CTC by microscopy (17%) as well as by flow cytometry (10%, M30$^{\text{neg}}$ and 7%, M30$^{\text{pos}}$) with the majority representing CTC fragments. The variation in the relative proportion of intact CTC between patients was large ranging from 3% to 76% by microscopy. No or poor correlation was found in detection of damaged CTC and CTC fragments between flow cytometry and microscopy.

**Induction of apoptosis in LnCaP cells**

To investigate the effect of apoptosis induced by cytotoxic agents on flow cytometric and fluorescent microscopic analysis of CTC, cells from the prostate cell line LnCaP were cultured in the presence or absence of 40nM paclitaxel for 72 hours. Viability assessed by trypan blue exclusion following incubation of untreated LnCaP cells was 91% and 35% for the paclitaxel treated cells. The treated and untreated LnCaP cells were spiked into blood of healthy donors. After immunomagnetic selection and fluorescent labeling the cells were analyzed by fluorescent microscopy. Greater than 90% of the LnCaP cells that were not treated with paclitaxel were classified as intact
tumor cells as illustrated in Figure 5A whereas the majority of the paclitaxel treated cells showed features consistent with apoptosis, Figure 5C. After microscopic analysis the cells were retrieved from the cartridge, stained with M30 FITC and analyzed by flow cytometry. The correlative display of the DAPI and M30 FITC staining by flow cytometry of the untreated cells that express cytokeratin is shown in Figure 5B and of the treated cells in Figure 5D. The majority of cells by flow cytometry have intact DNA content (75%) and 24% of these cells express M30 indicating that they have started the process of apoptosis. In contrast to the untreated LnCaP cells, the paclitaxel treated cells displayed speckled cytokeratin staining and irregular morphology. By flow cytometry, the predominant population showed a decreased in DAPI staining (87%) and 26% of these cells express M30. The apoptotic pattern of CTC in the patient samples closely resembles that of the paclitaxel treated LnCaP cells.
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y = 0.80x + 6.4
R² = 0.71

y = 0.96x + 12
R² = 0.95

y = 1.56x + 52
R² = 0.25

y = 0.58x + 20
R² = 0.57
We claim:

1. A method for detecting and enumerating apoptotic cancer cells in a mixed cell population, the presence of said cancer cells in said population being indicative of a disease state, comprising:
   a. obtaining a biological specimen from a test subject, said specimen comprising a mixed cell population suspected of containing said cancer cells;
   b. preparing an immunomagnetic sample wherein said specimen is mixed with magnetic particles coupled to a biospecific ligand which reacts specifically with said cancer cells, to the substantial exclusion of other sample components;
   c. contacting said immunomagnetic sample with at least one biospecific reagent which labels said cancer cells; and
   d. characterizing said cancer cells into one or more apoptotic states from the group consisting of enzymatic analysis, biochemical analysis, morphological analysis and combinations thereof, the presence and number of said apoptotic state being indicative of said disease state.

2. The method of claim 1 wherein said biological specimen is exposed to a preservative prior to preparing said immunomagnetic mixture.

3. The method of claim 1 wherein as an intermediate step between preparing said immunomagnetic sample and contacting said immunomagnetic sample with said at least one biospecific reagent, said immunomagnetic sample is subjected to a magnetic field to produce an enriched suspension of said cancer cells as said immunomagnetic sample.

4. The method of claim 3, wherein said magnetic field is an externally applied high gradient magnetic field.

5 The method of claim 1, wherein said magnetic particles are colloidal in size.
6. The method of claim 1, wherein said ligand is a monoclonal antibody specific for at least one cancer cell determinant, and said at least one labeling reagent comprises at least one additional monoclonal antibody specific for said apoptotic state of said cancer cell and said method further comprises adding to said labeled cancer cell-containing fraction a third monoclonal antibody specific for an antigen present on a non-tumor cell, and a cell-specific dye.

7. The method of claim 6, wherein said method further comprises the step of assessing said enriched suspension from a group consisting of intact CTC, apoptotic CTC, tumor cell fragments, tumor cell debris, and combinations thereof.

8. The method of claim 6, wherein said test specimen is obtained periodically and assessed as an indicator of progression of said disease.

9. The method of claim 6, wherein said ligand binds specifically to an epithelial cell adhesion molecule.

10. The method of claim 6, wherein said at least one labeling reagent binds specifically to an intracellular cytokeratin.

11. A method in claim 6, wherein said apoptotic state is determined by caspase cleavage of cytokeratin 18 in said cancer cells.

12. A method as claimed in claim 6, wherein said apoptotic state is determined by M30 antibody recognition of cytokeratin 18.

13. A method as claimed in claim 6, wherein said test subject has a cancer selected from a group consisting of prostate cancer, breast cancer, and colon cancer.

14. A kit for screening a patient sample for the presence of circulating apoptotic tumor cells, comprising:
a. coated colloidal size magnetic particles comprising a magnetic core material, a protein base coating material, and anti-EpCAM couples, directly or indirectly, to said base coating material;
b. at least one additional monoclonal antibody specific for said apoptotic tumor cell;
c. cell specific dye for excluding sample components other than said tumor cells; and
d. an antibody which has binding affinity for non-tumor cells.

15. A kit as claimed in claim 14, said kit further containing a biological buffer, a permeabilization buffer, a protocol and optionally, an information sheet.

16. An improved method for determining the likelihood of cancer recurrence in a human subject previously treated for cancer by obtaining a blood sample, which comprises determining a number of epithelial cells in said sample, comparing said number with a statistically determined number of epithelial cells from a group of tumor-free patient controls, and assigning a likelihood of cancer recurrence when said number exceeds a predetermined value based on statistical averages of circulating epithelial cells from healthy subjects compared with statistical averages of circulating epithelial cells from cancer patients, and wherein said improvement comprises a further assessment of said epithelial cells based upon an apoptotic state.

17. The improved method of claim 15, wherein said number of epithelial cells are from a group consisting of intact cells, apoptotic cells, and combinations thereof.
Figure 5

A

B

286

D\`{A}PI

735

M30 FITC

D

M30 FITC

D\`{A}PI
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
   IPC(7) : G01N 33/553, 33/545, 33/537, 33/574, 33/53; C07K 16/00; C12P 21/08
   US Cl. : 436/526, 532, 538; 435/7.1, 975; 530/388.1, 387.7
   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)
   U.S. : 436/526, 532, 538; 435/7.1, 975; 530/388.1, 387.7
   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 practicable, search terms used)
   Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tr>
<td>A</td>
<td>US 6,008,002 A (BODEY) 28 December 1999 (28.12.1999), especially abstract, summary of invention and claims 1-2.</td>
<td>1-9, 13 and 16-17</td>
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☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
  "A" document defining the general state of the art which is not considered to be of particular relevance
  "E" earlier application or patent 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
27 October 2005 (27.10.2005)

Date of mailing of the international search report
04 November 2005

Authorized officer
Shahin Huq
Telephone No. 571-272-1600

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Facsimile No. (571) 273-3201

Form PCT/ISA/210 (second sheet) (April 2005)
Continuation of B. FIELDS SEARCHED Item 3:
APS, CAPLUS
Search terms: immunodetection, immunoassay, cancer cells, magnetic particle, immunomagnetic, antibody, cytokeratin, apoptotic.