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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 September 2012 (13.09.2012)

WIPO | PCT

(10) International Publication Number
WO 2012/120377 A2

(51) International Patent Classification:
C12Q 1/68 (2006.01)

(21) International Application Number:
PCT/IB2012/000552

(22) International Filing Date:
8 March 2012 (08.03.2012)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/450,212 8 March 2011 (08.03.2011) US

(71) Applicant *(for all designated States except US)*: **KING ABDULLAH UNIVERSITY OF SCIENCE AND TECHNOLOGY** [SA/SA]; P.o. Box 55455, Jeddah, 21534 (SA).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **BAJIC, Vladimir** [ZA/SA]; King Abdullah University Of, Science And Technology, Thuwal, 23955-6900 (SA). **KAUR, Mandeep** [IN/SA]; 4700 King Abdullah University Of, Science And Technology, Thuwal, 23955-6900 (SA).

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

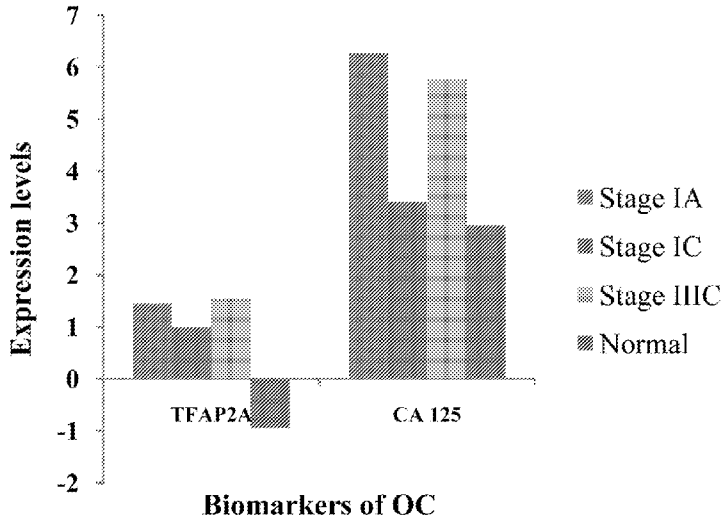
(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR,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

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

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

(54) **Title**: MOLECULAR BIOMARKER SET FOR EARLY DETECTION OF OVARIAN CANCER



(57) **Abstract**: Embodiments of the present invention concern methods and compositions related to detection of ovarian cancer, including detection of the stage of ovarian cancer, in some cases. In particular, the invention encompasses use of expression of TFAP2A and in some embodiments CA125 and/or E2F5 to identify ovarian cancer, including detecting mRNA and/or protein levels of the respective gene products. Kits for detection of ovarian cancer are also described.

WO 2012/120377 A2

MOLECULAR BIOMARKER SET FOR EARLY DETECTION OF OVARIAN CANCER

[0001] This application claims priority to U.S. Provisional Patent Application Serial No. 61/450,212, filed March 8, 2011, which is incorporated by reference herein in its entirety.

TECHNICAL FIELD

[0002] The present invention generally concerns at least the fields of molecular biology, cell biology, and medicine. In particular aspects, the field concerns detection of cancer, including ovarian cancer.

BACKGROUND OF THE INVENTION

[0003] Ovarian cancer is the leading cause of death among gynecological malignancies and represents the fifth leading cause of cancer-related deaths in women. The disease is diagnosed at a stage when cancer has already metastasized beyond the ovary in approximately 70% of patients and only 30% of these patients with this advanced-stage ovarian cancer survive 5 years after initial diagnosis. Early diagnosis greatly enhances the chances of successful cancer treatment. To this date, very few early-detection approaches have shown promise for routine clinical use. However, the most commonly used marker of ovarian cancer is CA125, but it is only expressed in 50-60% of patients during early stages of the disease.

BRIEF SUMMARY OF THE INVENTION

[0004] The present invention is directed to methods and compositions that regard a biomarker set for detection of ovarian cancer in both early and late stages. In some embodiments, the present invention concerns identification of the increased risk for an individual to develop ovarian cancer. In embodiments of the invention, the level of TFAP2A, CA125, and E2F5 (or TFAP2A alone or in other combinations) is assayed in an individual for the detection of ovarian cancer or other cancers. The individual may be suspected of having ovarian cancer (as an example), based on, for example, family history, testing, and/or symptoms that persist, for example, for two or more weeks (for example, abdominal swelling/bloating; abdominal/pelvic pain or pressure or feeling "full"; gastrointestinal symptoms (such as gas, indigestion, nausea, or changes in bowel movements); vaginal bleeding or discharge; urinary problems (urgency,

burning, or spasms); fatigue and/or fever; pain during intercourse; back pain; and/or difficulty breathing). In some embodiments the individual has no symptoms of cancer, including ovarian cancer, yet the individual is subjected to methods of the invention as a part of routine testing.

[0005] Individuals with an increased risk of developing ovarian cancer may be subjected to methods of the present invention. In specific cases, an individual with an increased risk is of a particular age (such as over about 40, over about 45, over about 50, over about 55, over about 60, or over about 65); has reached menopause; is obese; has no biological children; has taken fertility drugs or androgens; has had estrogen therapy; has a family history of ovarian and/or breast cancer; has a personal history of breast cancer; or uses talcum powder on the genitalia, for example. In some embodiments, the individual has no personal or family history of ovarian cancer and is not necessarily considered at risk but undergoes testing at least by methods of the invention as routine testing in preventative health care (similar to periodic Pap smear testing for cervical cancer, for example).

[0006] In some embodiments, the levels of TFAP2A and optionally one or both of CA125 and E2F5 are assayed to monitor the effectiveness of an ovarian cancer treatment. Exemplary ovarian cancer treatments include surgery, radiation, and/or primary therapy with drugs that contain platinum and taxane compounds (*e.g.*, cisplatin, carboplatin, paclitaxel); however, other drugs, such as "mustards" (*e.g.*, melphalan) and anthracyclines (*e.g.*, doxorubicin) are also useful for first-line activity in ovarian cancer. Other drugs include altretamine, 5-fluorouracil, topotecan, ifosamide, and/or etoposide, for example. The regimen of ovarian chemotherapies are determined by a variety of factors, including the type and/or stage of ovarian cancer, health status, *etc.* In some embodiments, the levels of TFAP2A and optionally CA125, and/or E2F5 are utilized for determination of a treatment regimen for ovarian cancer. In some embodiments of the invention, the methods are employed to determine if an individual should be given a particular cancer therapy, for example whether an individual would be resistant to a particular cancer drug.

[0007] The present invention allows detection of any type of ovarian cancer, in particular embodiments. Thus, in certain embodiments the present invention may detect ovarian cancers of the three main types of ovarian tumors, including epithelial ovarian tumors (the most common), which are derived from the cells on the surface of the ovary; germ cell ovarian tumors, which are derived from the egg-producing cells within the ovarian body; and sex cord stromal

ovarian tumors, which are a type that often produces steroid hormones. Epithelial tumors are further subdivided into (a) benign, (b) borderline (low-malignant potential [LMP] or atypical proliferative), and (c) invasive carcinoma, and the present invention determines any of these types, in specific embodiments. The present invention may be utilized to detect any stage of ovarian cancer, including those identified in the American Joint Committee on Cancer (AJCC) TNM (Tumor size, Lymph Nodes affected, Metastases) system for ovarian cancer: Stage IA, IB, IC, IIA, IIB, IIC, IIIA, IIIB, IIIC, or IV, although in specific embodiments the detection occurs for early stage ovarian cancer, such as Stage IA, IB, or IC.

[0008] Levels of TFAP2A and optionally CA125 and/or E2F5 may be determined by any suitable means, but in specific embodiments the levels are determined for protein or mRNA, or both. The levels may be determined from a sample from the individual, including from a fluid or tissue, for example. In specific embodiments, the sample is blood or ovarian tissue, such as from a biopsy. The blood may be further fractionated before analysis, in specific embodiments. Samples used in the invention may be subjected to methods of the invention directly from the individual, or the samples may be stored in a suitable storage means, such as under refrigeration, and the sample may be transported from the individual to a separate facility for analysis, for example. The person that extracts the sample may or may not be the person that performs the method(s) of the invention, and the facility in which the person that extracts the sample is located may not be the facility in which the method(s) of the invention is performed.

[0009] In some methods of the invention, the method further comprises the step(s) of obtaining a sample from the individual, isolating nucleic acid (including mRNA) and/or protein from the sample; and analyzing the levels of TFAP2A and optionally CA125 and/or E2F5 in the sample.

[0010] The same sample may be used for processing of each of TFAP2A and optionally CA125 and/or E2F5, although in other embodiments different samples from the same area or type from the same individual are used to analyze TFAP2A, CA125, and/or E2F5, respectively. The analysis of each of TFAP2A, CA125, and/or E2F5 may be performed substantially concomitantly or may be performed successively. In some embodiments, a sample is analyzed for one or more of TFAP2A and optionally CA125 and/or E2F5, and upon determination of a particular outcome of such analysis, the one or more of TFAP2A, CA125, and/or E2F5, respectively, that were not originally analyzed are thereafter analyzed.

[0011] In one embodiment of the invention, there is a method of evaluating the probability of the presence of ovarian cancer in a subject, the method comprising measuring the amounts of TFAP2A and optionally CA125 and E2F5 in a biological sample from the subject; comparing the measured amounts of TFAP2A and optionally CA125 and E2F5 in the biological sample to a standard for TFAP2A and optionally CA125 and E2F5, respectively, wherein the standard is a level of TFAP2A, CA125, and E2F5, respectively, obtained from a sample of a member of the group consisting of a healthy subject or a subject with normal or benign ovarian tissue, and identifying an increase in the amount of the respective TFAP2A, CA125, and E2F5 in the biological sample as compared to the standard, wherein the increase is indicative of the presence of or the probability of the presence of malignant or pre-malignant cells of ovarian cancer.

[0012] In one embodiment of the invention, there is a method of identifying ovarian cancer in an individual or the risk of developing ovarian cancer in an individual, comprising the step of comparing the level of TFAP2A and optionally CA125 and/or E2F5 from the individual with the expression level of the respective TFAP2A, CA125, and E2F5 from a control. In a specific embodiment of the invention, when the levels of the respective TFAP2A, CA125, and E2F5 are higher in the individual compared to the control, the individual has ovarian cancer or has a risk of developing ovarian cancer. In particular aspects of the invention, the protein level of TFAP2A and optionally CA125 and/or E2F5 from the individual is determined, such as determined from the blood of the individual. The protein level of TFAP2A, CA125, and/or E2F5 may be determined with an antibody, such as a monoclonal antibody, for example.

[0013] In some aspects of the invention, the mRNA level of TFAP2A and optionally CA125 and E2F5 from the individual is determined, such as determined from ovarian tissue from the individual. The mRNA level may be determined by microarray, Northern, or RT-PCR, for example.

[0014] In specific embodiments of the invention, the control is from blood or tissue from one or more normal individuals. In particular aspects, the method detects the stage of ovarian cancer in the individual. In certain embodiments, the stage is stage IA, IC, IIIC, or a combination thereof. In some embodiments, the control is from normal tissue from the individual being screened.

[0015] In some embodiments of the invention, the method further comprises the step of performing an additional ovarian cancer detection method, such as one selected from the group consisting of palpitation, ultrasound, magnetic resonance imaging, X-ray, CT scan, blood testing, and biopsy, for example.

[0016] In some embodiments, the method further comprises the step of administering treatment for ovarian cancer. Exemplary treatments for ovarian cancer include surgery, radiation, and/or primary therapy with drugs that contain platinum and taxane compounds (*e.g.*, cisplatin, carboplatin, paclitaxel); "mustards" (*e.g.*, melphalan), anthracyclines (*e.g.*, doxorubicin), altretamine, 5-fluorouracil, topotecan, ifosamide, and etoposide.

[0017] In specific aspects, the method further comprises the step of obtaining a sample from the individual. The sample may be obtained by routine methods in the art.

[0018] In some cases, the method further comprises the step of isolating TFAP2A, CA125, and E2F5 protein and/or mRNA from the sample. In specific aspects, the method further comprises the steps of obtaining a sample from the individual and isolating TFAP2A, CA125, and E2F5 protein and/or mRNA from the sample.

[0019] In one embodiment of the invention, there is a kit comprising one or more detection reagents for TFAP2A, CA125, and E2F5, said reagents housed in a suitable container, and in some cases the reagent is selected from the group consisting of antibody, microarray, oligonucleotide, polymerase, deoxyribonucleotides, buffer, or a combination thereof. In specific aspects, the method further comprises an apparatus for obtaining a sample from an individual, such as drawing blood or taking a biopsy from an individual.

[0020] In one embodiment, there is a method of identifying ovarian cancer in an individual or the risk of developing ovarian cancer in an individual, comprising the step of comparing the level of TFAP2A from the individual with the expression level of TFAP2A from a control. In a specific embodiment, the method further comprises the step of comparing the level of CA125 or E2F5 or both from the individual with the expression level of CA125 or E2F5 or both, respectively, from a control.

[0021] In a particular embodiment, there is a method of identifying ovarian cancer in an individual or the risk of developing ovarian cancer in an individual, comprising the step of

comparing the level of TFAP2A and one or both of CA125 or E2F5 from the individual with the expression level of TFAP2A and one or both of CA125 or E2F5, respectively, from a control.

[0022] In some embodiments, there are methods of identifying ovarian cancer in an individual or the risk of developing ovarian cancer in an individual, comprising the steps of a) obtaining a sample from the individual, b) determining the expression level of TFAP2A from the sample, and c) comparing the expression level of TFAP2A from the individual with the expression level of TFAP2A from a control, wherein when the level of TFAP2A is higher in the individual compared to the control, the individual has ovarian cancer or has a risk of developing ovarian cancer.

[0023] In some embodiments, there are methods of identifying ovarian cancer in an individual or the risk of developing ovarian cancer in an individual, comprising the steps of a) obtaining a sample from the individual, b) determining the expression level of TFAP2A from the sample, c) determining the expression level of CA125 and/or E2F5 from the sample, d) comparing the expression level of TFAP2A from the individual with the expression level of TFAP2A from a control, and e) comparing the expression level of CA125 and/or E2F5 from the individual with the expression level of CA125 and/or E2F5 from a control, wherein when the level of TFAP2A and CA125 or E2F5 is higher in the individual compared to the control, the individual has ovarian cancer or has a risk of developing ovarian cancer.

[0024] The foregoing has outlined rather broadly the features and technical advantages of the present invention in order that the detailed description of the invention that follows may be better understood. Additional features and advantages of the invention will be described hereinafter which form the subject of the claims of the invention. It should be appreciated by those skilled in the art that the conception and specific embodiment disclosed may be readily utilized as a basis for modifying or designing other structures for carrying out the same purposes of the present invention. It should also be realized by those skilled in the art that such equivalent constructions do not depart from the spirit and scope of the invention as set forth in the appended claims. The novel features which are believed to be characteristic of the invention, both as to its organization and method of operation, together with further objects and advantages will be better understood from the following description when considered in connection with the accompanying figures. It is to be expressly understood, however, that each

of the figures is provided for the purpose of illustration and description only and is not intended as a definition of the limits of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] For a more complete understanding of the present invention, reference is now made to the following descriptions taken in conjunction with the accompanying drawing.

[0026] FIG. 1 shows the comparison of the expression levels of TFAP2A and CA125 in randomly chosen ovarian serous adenocarcinoma tissue samples categorized at stages IA, IC, and IIIC in comparison to normal tissue based on an ovarian cancer study by Lu *et al.* (2004).

[0027] FIG. 2 shows a bar graph representing the expression pattern of three biomarkers (TFAP2A, CA125 and E2F5) in normal samples.

[0028] FIG. 3 shows the expression pattern of three biomarkers (TFAP2A, CA125 and E2F5) in breast cancer samples.

[0029] FIG. 4 shows the expression pattern of three biomarkers (TFAP2A, CA125 and E2F5) in cervix cancer samples.

[0030] FIG. 5 demonstrates the expression pattern of three biomarkers (TFAP2A, CA125 and E2F5) in uterus cancer samples.

DETAILED DESCRIPTION OF THE INVENTION

[0031] Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. For purposes of the present invention, the following terms are defined below.

[0032] As used herein, the use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one." Some embodiments of the invention may consist of or consist essentially of one or more elements, method steps, and/or methods of the invention. It is contemplated that any method or

composition described herein can be implemented with respect to any other method or composition described herein.

[0033] As used herein, the term "biomarker" refers to a marker (expressed gene, including mRNA and/or protein) that allows detection of disease in an individual when compared to a healthy individual, including detection of disease in its early stages. In specific embodiments, the expression level of the biomarker as determined by mRNA and/or protein levels in tissue or biological material from an individual to be tested is compared with respective levels in normal ovarian tissue or biological material from the same individual or another healthy individual.

[0034] As used herein, the term "control" refers to any entity used in comparison of the biomarkers expression. For example, in the cases when disease biomarkers are in question, a control could be the expression pattern of the biomarkers in an individual not affected by the disease; it could be the averaged expression pattern of the biomarkers from a group of individuals not affected by the disease; it could be the expression of another gene/protein in the same individual; for one biomarker it could be the expression of another biomarker in the same individual; it could be a threshold on the score produced by a mathematical model that uses the expressions of biomarkers and possibly expression of other genes/proteins so that scores for disease-affected individuals and for individuals not affected by the disease significantly differ (this for example includes all model-based classifiers of disease-affected and disease-non-affected cases). The expression and the expression pattern could be either absolute or relative, *i.e.* determined relative to the expression of some other gene(s)/protein(s). In specific embodiments, the control is derived at least in part from the level of expression of a reference gene/protein from a single individual without ovarian cancer. One of skill in the art recognizes that the control expression level may be normalized by standard means in the art. The normalization may include standardization to a reference protein (such as a housekeeping gene including GAPDH and RN18S1), for example (see also Tunbridge *et al.*, 2011; Bär *et al.*, 2009). In certain embodiments, the identification of ovarian cancer (or another respective type of cancer) is achieved when the level of expression of a biomarker is above a normalized threshold compared to a control. In some cases, the identification of ovarian cancer (or another respective type of cancer) is achieved when the level of expression of a biomarker is below a normalized threshold compared to a control.

[0035] In specific embodiments of the invention, the control is from blood or tissue from one or more normal individuals or is from blood or tissue from the individual being screened. In specific aspects, the control is derived at least in part from the average level of expression of a reference gene/protein in a collection of individuals that do not have ovarian cancer, although the individuals in the collection may or may not have another type of cancer.

I. General Embodiments of the Invention

[0036] The present invention concerns detection of ovarian cancer in an individual or identifying an individual with an increased risk of developing ovarian cancer. In some embodiments, the invention offers diagnostic benefits for patients with ovarian cancer, for example by helping medical care providers diagnose ovarian cancer in very early stages that can substantially improve the survival rate in these patients. The invention is useful for the health care industry in embodiments where diagnostic kits are routinely used, in specific cases.

[0037] Using computational analysis, the inventors have identified TFAP2A, which controls the expression of several ovarian cancer genes in the cell, as a new biomarker for early diagnosis of ovarian cancer. The computation analysis described in this disclosure shows that TFAP2A is useful as a biomarker for detection of early stages of ovarian cancer, because the data indicate that it is expressed in patients with early stages of ovarian cancer, but no expression is seen in normal individuals. For the same set of patients, the analysis shows that CA125, a known biomarker of ovarian cancer, has detectable expression level in both normal as well as ovarian cancer individuals. Therefore, CA125 does not have the power to differentiate the cancer from normal states in many cases, whereas the TFAP2A has clearly detectable higher expression levels in ovarian cancer patients as compared to normal. A combination of biomarkers TFAP2A, E2F5 and CA125 is a more accurate means for diagnosing ovarian cancer.

[0038] In certain embodiments of the invention, the technology is based on a unique combination of three biomarkers for use as a diagnostic tool for ovarian cancer. All three biomarkers were evaluated individually as well as in certain combinations for use in diagnosis of cancer. In certain embodiments, the invention concerns identification of a new set of a combination of biomarkers for use in simultaneous diagnosis and early detection of ovarian cancer. The biomarker set consists of three gene products: TFAP2A, E2F5, and CA125. In specific aspects, the presence of these biomarkers in blood, for example, is an indicator of ovarian cancer, although other fluids or tissues may be assayed. Different combinations of these

biomarkers result in different sensitivity and specificity of ovarian cancer detection. That is, the CA125 is in use as a standard biomarker for detection of ovarian cancer, but it does not have good accuracy of ovarian cancer detection (Sasaroli *et al.*, 2009). Another biomarker, E2F5, has been recently discovered (Kothandaraman *et al.*, 2010) where it has been shown that in combination with CA125 there was significant improvement of the accuracy of ovarian cancer detection and ability to detect ovarian cancer in early stages. The third biomarker for use in combination with CA125 and E2F5 is TFAP2A that shows expression in early ovarian cancer stages but not in normal individuals.

[0039] In some embodiments of the invention, there is a single biomarker used as a diagnostic tool for cancer, including ovarian cancer. In specific aspects, the presence of this biomarker in blood, for example, is an indicator of ovarian cancer, although other fluids or tissues may be assayed.

[0040] Thus, in embodiments of the invention, presence of TFAP2A, E2F5, and CA125 in different combinations in human blood samples improves the accuracy of diagnosis of ovarian cancer in clinical settings and enhances the capability to detect ovarian cancer, for example in early stages.

[0041] In some embodiments, the mere presence or absence of a marker, without quantifying the amount of marker, is useful and can be correlated with a diagnosis of ovarian cancer or increased risk of developing ovarian cancer. For example, TFAP2A, E2F5, and CA125 can be more frequently detected in human ovarian cancer patients than in normal subjects. Thus, a detected presence of these markers in a subject being tested indicates that the subject has ovarian cancer or has a higher probability of having ovarian cancer.

[0042] In other embodiments, the measurement of markers can involve quantifying the markers to correlate the detection of markers with a probable diagnosis of ovarian cancer. Thus, if the amount of the markers detected in a subject being tested is different compared to a control amount (*i.e.*, higher or lower than the control, depending on the marker), then the subject being tested has a higher probability of having ovarian cancer.

[0043] The correlation may take into account the amount of the marker or markers in the sample compared to a control amount of the marker or markers (up or down regulation of the marker or markers) (*e.g.*, in normal subjects in whom human cancer is undetectable). A

control can be, *e.g.*, the average or median amount of marker present in comparable samples of normal subjects in whom human cancer is undetectable. The control amount is measured under the same or substantially similar experimental conditions as in measuring the test amount. The correlation may take into account the presence or absence of the markers in a test sample and the frequency of detection of the same markers in a control. The correlation may take into account both of such factors to facilitate determination of ovarian cancer status.

[0044] In certain embodiments of the methods of qualifying ovarian cancer status, the methods further comprise managing subject treatment based on the status. As aforesaid, such management describes the actions of the physician or clinician subsequent to determining ovarian cancer status. For example, if the result of the methods of the present invention is inconclusive or there is reason that confirmation of status is necessary, the physician may order more tests. Alternatively, if the status indicates that surgery is appropriate, the physician may schedule the patient for surgery. In other instances, the patient may receive chemotherapy or radiation treatments, either in lieu of, or in addition to, surgery. Likewise, if the result is negative, no further action may be warranted. Furthermore, if the results show that treatment has been successful, no further management may be necessary.

[0045] The invention also provides for such methods where the biomarkers (or specific combination of biomarkers) are measured again after subject management. In these cases, the methods are used to monitor the status of the cancer, *e.g.*, response to cancer treatment, remission of the disease or progression of the disease. Because of the ease of use of the methods and the lack of invasiveness at least in certain embodiments of the methods, the methods can be repeated after each treatment the patient receives. This allows the physician to follow the effectiveness of the course of treatment. If the results show that the treatment is not effective, the course of treatment can be altered accordingly. This enables the physician to be flexible in the treatment options.

[0046] The methods of the present invention have other applications as well. For example, the markers can be used to screen for compounds that modulate the expression of the markers *in vitro* or *in vivo*, which compounds in turn may be useful in treating or preventing ovarian cancer in patients. In another example, the markers can be used to monitor the response to treatments for ovarian cancer. In yet another example, the markers can be used in heredity studies to determine if the subject is at risk for developing ovarian cancer. For instance, certain

markers may be genetically linked. This can be determined by, *e.g.*, analyzing samples from a population of ovarian cancer patients whose families have a history of ovarian cancer. The results can then be compared with data obtained from, *e.g.*, ovarian cancer patients whose families do not have a history of ovarian cancer. The markers that are genetically linked may be used as a tool to determine if a subject whose family has a history of ovarian cancer is predisposed to having ovarian cancer.

II. Gene Products Used in Embodiments of the Invention

[0047] The present invention employs methods and reagents related to assaying for expression levels of one or more particular genes, including TFAP2A and, in embodiments of the invention, also of CA125 and E2F5. The expression level may be determined by measuring levels of mRNA and/or protein, in specific embodiments.

[0048] TFAP2A is also referred to as transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha); RP1-290I10.1; AP-2; AP-2alpha; AP2TF; BOFS; FLJ51761; TFAP2; AP-2 transcription factor; AP2-alpha; OTTHUMP00000214240; OTTHUMP00000214243; activating enhancer-binding protein 2-alpha; and activator protein 2; for example. Exemplary GenBank® TFAP2A mRNA sequences are provided in NM_001032280.2; NM_001042425.1; and NM_003220.2 (SEQ ID NO:1) (the three of which correspond to isoforms b, c, and a, respectively), all of which sequences are incorporated by reference herein. Exemplary GenBank® TFAP2A protein sequences are provided in NP_001027451.1; NP_001035890.1; and NP_003211.1 (SEQ ID NO:2) (the three of which correspond to isoforms b, c, and a, respectively), all of which sequences are incorporated by reference herein.

[0049] E2F5 is also referred to as E2F transcription factor 5, E2F-5, and p130-binding. Exemplary GenBank® E2F5 mRNA sequences are provided in NM_001083588.1; NM_001083589.1; and NM_001951.3 (SEQ ID NO:3) (the three of which correspond to isoforms 2, 3, and 1, respectively), all of which sequences are incorporated by reference herein. Exemplary GenBank®E2F5 protein sequences are provided in NP_001077057.1; NP_001077058.1; and NP_001942.2 (SEQ ID NO:4) (the three of which correspond to isoforms 2, 3, and 1, respectively), all of which sequences are incorporated by reference herein.

[0050] CA125 is also referred to as cancer antigen 125; carbohydrate antigen 125; MUC16; mucin 16, cell surface associated; FLJ14303; CA-125; CA125 ovarian cancer antigen; MUC-16; mucin-16; ovarian cancer-related tumor marker CA125; and ovarian carcinoma antigen CA125. Exemplary GenBank® CA125 mRNA sequence is provided in NM_024690.2 (SEQ ID NO:5), which is incorporated by reference herein. Exemplary GenBank® CA125 protein sequence is provided in NP_078966.2 (SEQ ID NO:6), which is incorporated by reference herein.

Other Markers

[0051] In some embodiments of the invention, additional markers to TFAP2A, E2F5, and CA125 are utilized in the invention. In exemplary embodiments, ovarian cancer markers described in U.S. Patent No. 7,605,003, which is incorporated herein by reference, are employed in the invention. Exemplary markers include CA15-3, CA19-9, CA72-4, CA 195, TATI, CEA, PLAP, Sialyl TN, galactosyltransferase, M-CSF, CSF-1, LPA, p110EGFR, tissue kallikreins, prostasin, HE4, CKB, LASA, HER-2/neu, urinary gonadotropin peptide, Dianon NB 70/K, TPA, osteopontin and haptoglobin, and protein variants (*e.g.*, cleavage forms, isoforms) of the markers; in specific embodiments, the levels of these markers are different in individuals with cancer compared to normal individuals. For example, CA 19-9, CA 72.4, CA 195, TATI, inhibin and PLAP, and others, are known to be elevated in the blood of women with ovarian cancer.

[0052] Other markers that may be used in the methods of the invention include those described in U.S. Patent No. 7,745,149, which is incorporated by reference herein, and encompasses galectin-1, cathepsin B, MHC class I antigen, heat shock protein (HSP) 27, ubiquitin carboxy-termal esterase L1, plasma retinol-binding protein (PRBP), transthyretin, SH3 binding glutamate-rich protein, tubulin-specific chaperone A, RNA binding protein regulatory subunit, γ-actin, tropomyosin and calcium/calmodulin-stimulated cyclic nucleotide phosphatase.

[0053] Additional markers that may be used in the methods of the invention include hK10 and/or hK6, described in U.S. Patent No. 7,741,019, which is incorporated by reference herein.

III. Test Samples

A. Subject Types

[0054] Samples are collected from subjects, *e.g.*, women, who want to establish ovarian cancer status. The subjects may be women who have been determined to have a high risk of ovarian cancer, for example based on their family history. Other patients include women who have ovarian cancer and the test is being used to determine the effectiveness of therapy or treatment they are receiving. Also, patients could include healthy women who are having a test as part of a routine examination, or to establish baseline levels of the biomarkers. Samples may be collected from women who had been diagnosed with ovarian cancer and received treatment to eliminate the cancer, or perhaps are in remission.

B. Types of Sample and Preparation of the Sample

[0055] The markers can be measured in different types of biological samples. The sample is preferably a biological fluid sample. Examples of a biological fluid sample useful in this invention include blood, blood serum, plasma, vaginal secretions, urine, tears, saliva, *etc.* Blood is a preferred sample source for certain embodiments of the invention because of its non-invasiveness. In alternative embodiments, the sample is from biopsy.

[0056] If desired, the sample can be prepared to enhance detectability of the markers. For example, to increase the detectability of markers, a blood serum sample from the subject can be preferably fractionated by, *e.g.*, Cibacron blue agarose chromatography and single stranded DNA affinity chromatography, anion exchange chromatography, affinity chromatography (*e.g.*, with antibodies) and the like. The method of fractionation depends on the type of detection method used. Any method that enriches for the protein of interest can be used. Sample preparations, such as pre-fractionation protocols, are optional and may not be necessary to enhance detectability of markers depending on the methods of detection used, in some embodiments. For example, sample preparation may be unnecessary if antibodies that specifically bind markers are used to detect the presence of markers in a sample.

[0057] Typically, sample preparation involves fractionation of the sample and collection of fractions determined to contain the biomarkers. Methods of pre-fractionation include, for example, size exclusion chromatography, ion exchange chromatography, heparin chromatography, affinity chromatography, sequential extraction, gel electrophoresis and liquid

chromatography. The analytes also may be modified prior to detection. These methods are useful to simplify the sample for further analysis. For example, it can be useful to remove high abundance proteins, such as albumin, from blood before analysis. Examples of methods of fractionation are described in PCT/US03/00531 (incorporated herein in its entirety).

[0058] In some cases, the sample is pre-fractionated by anion exchange chromatography. Anion exchange chromatography allows pre-fractionation of the proteins in a sample roughly according to their charge characteristics. For example, a Q anion-exchange resin can be used (*e.g.*, Q HyperD F, Biosepra), and a sample can be sequentially eluted with eluants having different pH's. Anion exchange chromatography allows separation of biomolecules in a sample that are more negatively charged from other types of biomolecules. Proteins that are eluted with an eluant having a high pH is likely to be weakly negatively charged, and a fraction that is eluted with an eluant having a low pH is likely to be strongly negatively charged. Thus, in addition to reducing complexity of a sample, anion exchange chromatography separates proteins according to their binding characteristics.

[0059] In certain embodiments, the serum samples are fractionated *via* anion exchange chromatography. Signal suppression of lower abundance proteins by high abundance proteins presents a significant challenge to SELDI mass spectrometry. Fractionation of a sample reduces the complexity of the constituents of each fraction. This method can also be used to attempt to isolate high abundance proteins into a fraction, and thereby reduce its signal suppression effect on lower abundance proteins. Anion exchange fractionation separates proteins by their isoelectric point (pI). Proteins are comprised of amino acids, which are ambivalent-their charge changes based on the pH of the environment to which they are exposed. A protein's pI is the pH at which the protein has no net charge. A protein assumes a neutral charge when the pH of the environment is equivalent to pI of the protein. When the pH rises above the pI of the protein, the protein assumes a net negative charge. Similarly, when the pH of the environment falls below the pH of the protein, the protein has a net positive charge. The serum samples were fractionated according to the protocol set forth in the Examples below to obtain the markers described herein.

[0060] After capture on anion exchange, proteins were eluted in a series of step washes at pH 9, pH 7, pH 5, pH 4 and pH 3. A panel of three potential biomarkers was discovered by UMSA analysis of profiling data of three fractions (pH 9/flow through, pH 4, and

organic solvent). Two of the peaks were from fraction pH 4 at m/z of 12828 and 28043, both down-regulated in the cancer group, and the third was from fraction pH 9/flow through at m/z of 3272, up-regulated in the cancer group. All bound to the immobilized metal affinity chromatography array charged with copper ions (IMAC3-Cu) (spectra in FIG. 1).

[0061] Biomolecules in a sample can also be separated by high-resolution electrophoresis, *e.g.*, one or two-dimensional gel electrophoresis. A fraction containing a marker can be isolated and further analyzed by gas phase ion spectrometry. Preferably, two-dimensional gel electrophoresis is used to generate two-dimensional array of spots of biomolecules, including one or more markers. See, *e.g.*, Jungblut and Thiede, Mass Specir. Rev. 16:145-162 (1997).

[0062] The two-dimensional gel electrophoresis can be performed using methods known in the art. See, *e.g.*, Deutscher ed., Methods In Enzymology vol. 182. Typically, biomolecules in a sample are separated by, *e.g.*, isoelectric focusing, during which biomolecules in a sample are separated in a pH gradient until they reach a spot where their net charge is zero (*i.e.*, isoelectric point). This first separation step results in one-dimensional array of biomolecules. The biomolecules in one-dimensional array is further separated using a technique generally distinct from that used in the first separation step. For example, in the second dimension, biomolecules separated by isoelectric focusing are further separated using a polyacrylamide gel, such as polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). SDS-PAGE gel allows further separation based on molecular mass of biomolecules. Typically, two-dimensional gel electrophoresis can separate chemically different biomolecules in the molecular mass range from 1000-200,000 Da within complex mixtures. The pI range of these gels is about 3-10 (wide range gels).

[0063] Biomolecules in the two-dimensional array can be detected using any suitable methods known in the art. For example, biomolecules in a gel can be labeled or stained (*e.g.*, Coomassie Blue or silver staining). If gel electrophoresis generates spots that correspond to the molecular weight of one or more markers of the invention, the spot can be further analyzed by gas phase ion spectrometry. For example, spots can be excised from the gel and analyzed by gas phase ion spectrometry. Alternatively, the gel containing biomolecules can be transferred to an inert membrane by applying an electric field. Then a spot on the membrane that approximately corresponds to the molecular weight of a marker can be analyzed by gas phase ion

spectrometry. In gas phase ion spectrometry, the spots can be analyzed using any suitable techniques, such as MALDI or SELDI (*e.g.*, using ProteinChip® array) as described herein.

[0064] Prior to gas phase ion spectrometry analysis, it may be desirable to cleave biomolecules in the spot into smaller fragments using cleaving reagents, such as proteases (*e.g.*, trypsin). The digestion of biomolecules into small fragments provides a mass fingerprint of the biomolecules in the spot, which can be used to determine the identity of markers if desired.

[0065] High performance liquid chromatography (HPLC) can also be used to separate a mixture of biomolecules in a sample based on their different physical properties, such as polarity, charge and size. HPLC instruments typically consist of a reservoir of mobile phase, a pump, an injector, a separation column, and a detector. Biomolecules in a sample are separated by injecting an aliquot of the sample onto the column. Different biomolecules in the mixture pass through the column at different rates due to differences in their partitioning behavior between the mobile liquid phase and the stationary phase. A fraction that corresponds to the molecular weight and/or physical properties of one or more markers can be collected. The fraction can then be analyzed by gas phase ion spectrometry to detect markers. For example, the spots can be analyzed using either MALDI or SELDI (*e.g.*, using ProteinChip® array) as described herein.

[0066] Optionally, a marker can be modified before analysis to improve its resolution or to determine its identity. For example, the markers may be subject to proteolytic digestion before analysis. Any protease can be used. Proteases, such as trypsin, that are likely to cleave the markers into a discrete number of fragments are particularly useful. The fragments that result from digestion function as a fingerprint for the markers, thereby enabling their detection indirectly. This is particularly useful where there are markers with similar molecular masses that might be confused for the marker in question. Also, proteolytic fragmentation is useful for high molecular weight markers because smaller markers are more easily resolved by mass spectrometry. In another example, biomolecules can be modified to improve detection resolution. For instance, neuraminidase can be used to remove terminal sialic acid residues from glycoproteins to improve binding to an anionic adsorbent (*e.g.*, cationic exchange ProteinChip® arrays) and to improve detection resolution. In another example, the markers can be modified by the attachment of a tag of particular molecular weight that specifically bind to molecular markers, further distinguishing them. Optionally, after detecting such modified markers, the

identity of the markers can be further determined by matching the physical and chemical characteristics of the modified markers in a protein database (*e.g.*, SwissProt).

C. Capture of Markers

[0067] Biomarkers may be captured with capture reagents immobilized to a solid support, such as any biochip described herein, a multiwell microtiter plate or a resin. In particular, the biomarkers of this invention are preferably captured on SELDI protein biochips. Capture can be on a chromatographic surface or a biospecific surface. Any of the SELDI protein biochips comprising reactive surfaces can be used to capture and detect the biomarkers of this invention. However, the biomarkers of this invention bind well to immobilized metal chelates. The IMAC-3 and IMAC 30 biochips, which nitriloacetic acid functionalities that adsorb transition metal ions, such as Cu++ and Ni++, by chelation, are the preferred SELDI biochips for capturing the biomarkers of this invention. Any of the SELDI protein biochips comprising reactive surfaces can be used to capture and detect the biomarkers of this invention. These biochips can be derivatized with the antibodies that specifically capture the biomarkers, or they can be derivatized with capture reagents, such as protein A or protein G that bind immunoglobulins. Then the biomarkers can be captured in solution using specific antibodies and the captured markers isolated on chip through the capture reagent.

[0068] In general, a sample containing the biomarkers, such as blood or serum, is placed on the active surface of a biochip for a sufficient time to allow binding. Then, unbound molecules are washed from the surface using a suitable eluant, such as phosphate buffered saline. In general, the more stringent the eluant, the more tightly the proteins must be bound to be retained after the wash. The retained protein biomarkers now can be detected by appropriate means.

D. Detection and Measurement of Markers

[0069] Once captured on a substrate, *e.g.*, biochip or antibody, any suitable method can be used to measure a marker or markers in a sample. For example, markers can be detected and/or measured by a variety of detection methods including for example, gas phase ion spectrometry methods, optical methods, electrochemical methods, atomic force microscopy and radio frequency methods. Using these methods, one or more markers can be detected.

1. SELDI

[0070] One preferred method of detection and/or measurement of the biomarkers uses mass spectrometry and, in particular, "Surface-enhanced laser desorption/ionization" or "SELDI". SELDI refers to a method of desorption/ionization gas phase ion spectrometry (*e.g.*, mass spectrometry) in which the analyte is captured on the surface of a SELDI probe that engages the probe interface. In "SELDI MS," the gas phase ion spectrometer is a mass spectrometer. SELDI technology is described in more detail above. ApoA1, transthyretin .DELTA.N10 and IAIH4 fragment are detected as peaks at m/z of 28043, m/z of about 12870.9, and m/z of 3272, respectively.

2. **Immunoassay**

[0071] In another embodiment, an immunoassay can be used to detect and analyze markers in a sample. This method comprises: (a) providing an antibody that specifically binds to a marker; (b) contacting a sample with the antibody; and (c) detecting the presence of a complex of the antibody bound to the marker in the sample.

[0072] An immunoassay is an assay that uses an antibody to specifically bind an antigen (*e.g.*, a marker). The immunoassay is characterized by the use of specific binding properties of a particular antibody to isolate, target, and/or quantify the antigen. The phrase "specifically (or selectively) binds" to an antibody or "specifically (or selectively) immunoreactive with," when referring to a protein or peptide, refers to a binding reaction that is determinative of the presence of the protein in a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein at least two times the background and do not substantially bind in a significant amount to other proteins present in the sample. Specific binding to an antibody under such conditions may require an antibody that is selected for its specificity for a particular protein. For example, polyclonal antibodies raised to a marker from specific species such as rat, mouse, or human can be selected to obtain only those polyclonal antibodies that are specifically immunoreactive with that marker and not with other proteins, except for polymorphic variants and alleles of the marker. This selection may be achieved by subtracting out antibodies that cross-react with the marker molecules from other species.

[0073] Using the purified markers or their nucleic acid sequences, antibodies that specifically bind to a marker can be prepared using any suitable methods known in the art. See, *e.g.*, Coligan, Current Protocols in Immunology (1991); Harlow & Lane, Antibodies: A

Laboratory Manual (1988); Goding, Monoclonal Antibodies: Principles and Practice (2d ed. 1986); and Kohler & Milstein, Nature 256:495-497 (1975). Such techniques include, but are not limited to, antibody preparation by selection of antibodies from libraries of recombinant antibodies in phage or similar vectors, as well as preparation of polyclonal and monoclonal antibodies by immunizing rabbits or mice (see, *e.g.*, Huse *et al.*, Science 246:1275-1281 (1989); Ward *et al.*, Nature 341:544-546 (1989)). Typically a specific or selective reaction will be at least twice background signal or noise and more typically more than 10 to 100 times background.

[0074] Generally, a sample obtained from a subject can be contacted with the antibody that specifically binds the marker. Optionally, the antibody can be fixed to a solid support to facilitate washing and subsequent isolation of the complex, prior to contacting the antibody with a sample. Examples of solid supports include glass or plastic in the form of, *e.g.*, a microtiter plate, a stick, a bead, or a microbead. Antibodies can also be attached to a probe substrate or ProteinChip® array described above. The sample is preferably a biological fluid sample taken from a subject. Examples of biological fluid samples include blood, serum, plasma, nipple aspirate, urine, tears, saliva *etc.* In a preferred embodiment, the biological fluid comprises blood serum. The sample can be diluted with a suitable eluant before contacting the sample to the antibody.

[0075] After incubating the sample with antibodies, the mixture is washed and the antibody-marker complex formed can be detected. This can be accomplished by incubating the washed mixture with a detection reagent. This detection reagent may be, *e.g.*, a second antibody which is labeled with a detectable label. Exemplary detectable labels include magnetic beads (*e.g.*, DYNABEADS^{TM}), fluorescent dyes, radiolabels, enzymes (*e.g.*, horse radish peroxide, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic beads. Alternatively, the marker in the sample can be detected using an indirect assay, wherein, for example, a second, labeled antibody is used to detect bound marker-specific antibody, and/or in a competition or inhibition assay wherein, for example, a monoclonal antibody which binds to a distinct epitope of the marker is incubated simultaneously with the mixture.

[0076] Methods for measuring the amount of, or presence of, antibody-marker complex include, for example, detection of fluorescence, luminescence, chemiluminescence, absorbance, reflectance, transmittance, birefringence or refractive index (*e.g.*, surface plasmon

resonance, ellipsometry, a resonant mirror method, a grating coupler waveguide method or interferometry). Optical methods include microscopy (both confocal and non-confocal), imaging methods and non-imaging methods. Electrochemical methods include voltametry and amperometry methods. Radio frequency methods include multipolar resonance spectroscopy. Methods for performing these assays are readily known in the art. Useful assays include, for example, an enzyme immune assay (EIA) such as enzyme-linked immunosorbent assay (ELISA), a radioimmune assay (RIA), a Western blot assay, or a slot blot assay. These methods are also described in, *e.g.*, Methods in Cell Biology: Antibodies in Cell Biology, volume 37 (Asai, ed. 1993); Basic and Clinical Immunology (Stites & Terr, eds., 7th ed. 1991); and Harlow & Lane, supra.

[0077] Throughout the assays, incubation and/or washing steps may be required after each combination of reagents. Incubation steps can vary from about 5 seconds to several hours, preferably from about 5 minutes to about 24 hours. However, the incubation time will depend upon the assay format, marker, volume of solution, concentrations and the like. Usually the assays will be carried out at ambient temperature, although they can be conducted over a range of temperatures, such as 10°C to 40°C.

[0078] Immunoassays can be used to determine presence or absence of a marker in a sample as well as the quantity of a marker in a sample. The amount of an antibody-marker complex can be determined by comparing to a standard. A standard can be, *e.g.*, a known compound or another protein known to be present in a sample. As noted above, the test amount of marker need not be measured in absolute units, as long as the unit of measurement can be compared to a control.

[0079] The methods for detecting these markers in a sample have many applications. For example, one or more markers can be measured to aid human cancer diagnosis or prognosis. In another example, the methods for detection of the markers can be used to monitor responses in a subject to cancer treatment. In another example, the methods for detecting markers can be used to assay for and to identify compounds that modulate expression of these markers *in vivo* or *in vitro*. In a preferred example, the biomarkers are used to differentiate between the different stages of tumor progression, thus aiding in determining appropriate treatment and extent of metastasis of the tumor.

IV. Combination Methods for Detection of Ovarian Cancer

[0080] In embodiments of the invention, the results of the inventive detection methods are employed with other detection methods or information to provide a diagnosis for an individual. Exemplary ovarian cancer detection methods include analysis of blood, urine or a biopsy of a suspicious area. Examples of blood and urine tests used in combination with methods of the invention include, for example, complete blood count (CBC); urine cytology; blood protein testing (for example, to detect certain abnormal immune system proteins (immunoglobulins); and tumor marker tests. In some embodiments of the invention, an individual with a pelvic mass is subjected to one or more methods of the invention.

[0081] Although in some embodiments of the invention the gene product levels are used to monitor patients with a known cancer, for example to determine the stage of cancer or to monitor effectiveness of a cancer therapy, in other embodiments the methods are employed as one of several tests in the workup of an individual suspected of having a tumor.

[0082] Thus, in individuals who are known to have a malignancy, such as ovarian cancer, the TFAP2A, E2F5, and CA 125 levels can be monitored periodically, for example. A changed level generally may indicate that therapy, including chemotherapy, has been effective, a level changed in the opposite direction may indicate tumor recurrence, while a stagnant level may indicate lack of effectiveness.

V. Nucleic Acid Detection

[0083] In some embodiments of the invention, the expression of TFAP2A, E2F5, and CA125 nucleic acid sequences disclosed herein is determined for ovarian cancer detection or propensity for developing ovarian cancer.

A. Hybridization

[0084] In some embodiments, hybridization of respective probes to TFAP2A, E2F5, and CA125 mRNAs are employed in the invention. The use of a probe or primer of between 13 and 100 nucleotides, preferably between 17 and 100 nucleotides in length, or in some aspects of the invention up to 1-2 kilobases or more in length, allows the formation of a duplex molecule that is both stable and selective. Molecules having complementary sequences over contiguous stretches greater than 20 bases in length are generally preferred, to increase

stability and/or selectivity of the hybrid molecules obtained. One will generally prefer to design nucleic acid molecules for hybridization having one or more complementary sequences of 20 to 30 nucleotides, or even longer where desired. Such fragments may be readily prepared, for example, by directly synthesizing the fragment by chemical means or by introducing selected sequences into recombinant vectors for recombinant production.

[0085] Accordingly, the nucleotide sequences of the invention may be used for their ability to selectively form duplex molecules with complementary stretches of DNAs and/or RNAs or to provide primers for amplification of DNA or RNA from samples. Depending on the application envisioned, one would desire to employ varying conditions of hybridization to achieve varying degrees of selectivity of the probe or primers for the target sequence.

[0086] For applications requiring high selectivity, one will typically desire to employ relatively high stringency conditions to form the hybrids. For example, relatively low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.10 M NaCl at temperatures of about 50°C to about 70°C. Such high stringency conditions tolerate little, if any, mismatch between the probe or primers and the template or target strand and would be particularly suitable for isolating specific genes or for detecting specific mRNA transcripts. It is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide.

[0087] For certain applications, for example, site-directed mutagenesis, it is appreciated that lower stringency conditions are preferred. Under these conditions, hybridization may occur even though the sequences of the hybridizing strands are not perfectly complementary, but are mismatched at one or more positions. Conditions may be rendered less stringent by increasing salt concentration and/or decreasing temperature. For example, a medium stringency condition could be provided by about 0.1 to 0.25 M NaCl at temperatures of about 37°C to about 55°C, while a low stringency condition could be provided by about 0.15 M to about 0.9 M salt, at temperatures ranging from about 20°C to about 55°C. Hybridization conditions can be readily manipulated depending on the desired results.

[0088] In other embodiments, hybridization may be achieved under conditions of, for example, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 1.0 mM dithiothreitol, at temperatures between approximately 20°C to about 37°C. Other hybridization conditions

utilized could include approximately 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, at temperatures ranging from approximately 40°C to about 72°C.

[0089] In certain embodiments, it will be advantageous to employ nucleic acids of defined sequences of the present invention in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator means are known in the art, including fluorescent, radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of being detected. In preferred embodiments, one may desire to employ a fluorescent label or an enzyme tag such as urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmentally undesirable reagents. In the case of enzyme tags, colorimetric indicator substrates are known that can be employed to provide a detection means that is visibly or spectrophotometrically detectable, to identify specific hybridization with complementary nucleic acid containing samples.

[0090] In general, it is envisioned that the probes or primers described herein will be useful as reagents in solution hybridization, as in PCR®, for detection of expression of corresponding genes, as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. This fixed, single-stranded nucleic acid is then subjected to hybridization with selected probes under desired conditions. The conditions selected will depend on the particular circumstances (depending, for example, on the G+C content, type of target nucleic acid, source of nucleic acid, size of hybridization probe, *etc.*). Optimization of hybridization conditions for the particular application of interest is well known to those of skill in the art. After washing of the hybridized molecules to remove non-specifically bound probe molecules, hybridization is detected, and/or quantified, by determining the amount of bound label. Representative solid phase hybridization methods are disclosed in U.S. Patent Nos. 5,843,663, 5,900,481 and 5,919,626. Other methods of hybridization that may be used in the practice of the present invention are disclosed in U.S. Patent Nos. 5,849,481, 5,849,486 and 5,851,772. The relevant portions of these and other references identified in this section of the Specification are incorporated herein by reference.

B. Amplification of Nucleic Acids

[0091] Nucleic acids used as a template for amplification may be isolated from cells, tissues or other samples according to standard methodologies (Sambrook *et al.*, 1989). In

certain embodiments, analysis is performed on whole cell or tissue homogenates or biological fluid samples without substantial purification of the template nucleic acid. The nucleic acid may be genomic DNA or fractionated or whole cell RNA. Where RNA is used, it may be desired to first convert the RNA to a complementary DNA.

[0092] The term "primer," as used herein, is meant to encompass any nucleic acid that is capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Typically, primers are oligonucleotides from ten to twenty and/or thirty base pairs in length, but longer sequences can be employed. Primers may be provided in double-stranded and/or single-stranded form, although the single-stranded form is preferred.

[0093] Pairs of primers designed to selectively hybridize to nucleic acids corresponding to TFAP2A, E2F5, and CA125 are contacted with the template nucleic acid under conditions that permit selective hybridization. Depending upon the desired application, high stringency hybridization conditions may be selected that will only allow hybridization to sequences that are completely complementary to the primers. In other embodiments, hybridization may occur under reduced stringency to allow for amplification of nucleic acids contain one or more mismatches with the primer sequences. Once hybridized, the template-primer complex is contacted with one or more enzymes that facilitate template-dependent nucleic acid synthesis. Multiple rounds of amplification, also referred to as "cycles," are conducted until a sufficient amount of amplification product is produced.

[0094] The amplification product may be detected or quantified. In certain applications, the detection may be performed by visual means. Alternatively, the detection may involve indirect identification of the product *via* chemiluminescence, radioactive scintigraphy of incorporated radiolabel or fluorescent label or even *via* a system using electrical and/or thermal impulse signals (Affymax technology; Bellus, 1994).

[0095] A number of template dependent processes are available to amplify the oligonucleotide sequences present in a given template sample. One of the best known amplification methods is the polymerase chain reaction (referred to as PCR™) which is described in detail in U.S. Patent Nos. 4,683,195, 4,683,202 and 4,800,159, and in Innis *et al.*, 1988, each of which is incorporated herein by reference in their entirety.

[0096] A reverse transcriptase PCR™ amplification procedure may be performed to quantify the amount of mRNA amplified. Methods of reverse transcribing RNA into cDNA are well known (see Sambrook *et al.*, 1989). Alternative methods for reverse transcription utilize thermostable DNA polymerases. These methods are described in WO 90/07641. Polymerase chain reaction methodologies are well known in the art. Representative methods of RT-PCR are described in U.S. Patent No. 5,882,864.

[0097] Another method for amplification is ligase chain reaction ("LCR"), disclosed in European Application No. 320 308, incorporated herein by reference in its entirety. U.S. Patent 4,883,750 describes a method similar to LCR for binding probe pairs to a target sequence. A method based on PCR™ and oligonucleotide ligase assy (OLA), disclosed in U.S. Patent 5,912,148, may also be used.

[0098] Alternative methods for amplification of target nucleic acid sequences that may be used in the practice of the present invention are disclosed in U.S. Patent Nos. 5,843,650, 5,846,709, 5,846,783, 5,849,546, 5,849,497, 5,849,547, 5,858,652, 5,866,366, 5,916,776, 5,922,574, 5,928,905, 5,928,906, 5,932,451, 5,935,825, 5,939,291 and 5,942,391, GB Application No. 2 202 328, and in PCT Application No. PCT/US89/01025, each of which is incorporated herein by reference in its entirety.

[0099] Qbeta Replicase, described in PCT Application No. PCT/US87/00880, may also be used as an amplification method in the present invention. In this method, a replicative sequence of RNA that has a region complementary to that of a target is added to a sample in the presence of an RNA polymerase. The polymerase will copy the replicative sequence which may then be detected.

[0100] An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of target molecules that contain nucleotide 5'-[alpha-thio]-triphosphates in one strand of a restriction site may also be useful in the amplification of nucleic acids in the present invention (Walker *et al.*, 1992). Strand Displacement Amplification (SDA), disclosed in U.S. Patent No. 5,916,779, is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, *i.e.*, nick translation.

[0101] Other nucleic acid amplification procedures include transcription-based amplification systems (TAS), including nucleic acid sequence based amplification (NASBA) and 3SR (Kwoh *et al.*, 1989; Gingeras *et al.*, PCT Application WO 88/10315, incorporated herein by reference in their entirety). European Application No. 329 822 disclose a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may be used in accordance with the present invention.

[0102] PCT Application WO 89/06700 (incorporated herein by reference in its entirety) disclose a nucleic acid sequence amplification scheme based on the hybridization of a promoter region/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This scheme is not cyclic, *i.e.*, new templates are not produced from the resultant RNA transcripts. Other amplification methods include "race" and "one-sided PCR" (Frohman, 1990; Ohara *et al.*, 1989).

C. Detection of Nucleic Acids

[0103] Following any amplification for TFAP2A, E2F5, or CA125, it may be desirable to separate the amplification product from the template and/or the excess primer. In one embodiment, amplification products are separated by agarose, agarose-acrylamide or polyacrylamide gel electrophoresis using standard methods (Sambrook *et al.*, 1989). Separated amplification products may be cut out and eluted from the gel for further manipulation. Using low melting point agarose gels, the separated band may be removed by heating the gel, followed by extraction of the nucleic acid.

[0104] Separation of nucleic acids may also be effected by chromatographic techniques known in art. There are many kinds of chromatography which may be used in the practice of the present invention, including adsorption, partition, ion-exchange, hydroxylapatite, molecular sieve, reverse-phase, column, paper, thin-layer, and gas chromatography as well as HPLC.

[0105] In certain embodiments, the amplification products are visualized. A typical visualization method involves staining of a gel with ethidium bromide and visualization of bands under UV light. Alternatively, if the amplification products are integrally labeled with

radio- or fluorometrically-labeled nucleotides, the separated amplification products can be exposed to x-ray film or visualized under the appropriate excitatory spectra.

[0106] In one embodiment, following separation of amplification products, a labeled nucleic acid probe is brought into contact with the amplified marker sequence. The probe preferably is conjugated to a chromophore but may be radiolabeled. In another embodiment, the probe is conjugated to a binding partner, such as an antibody or biotin, or another binding partner carrying a detectable moiety.

[0107] In particular embodiments, detection is by Southern blotting and hybridization with a labeled probe. The techniques involved in Southern blotting are well known to those of skill in the art (see Sambrook *et al.*, 1989). One example of the foregoing is described in U.S. Patent No. 5,279,721, incorporated by reference herein, which discloses an apparatus and method for the automated electrophoresis and transfer of nucleic acids. The apparatus permits electrophoresis and blotting without external manipulation of the gel and is ideally suited to carrying out methods according to the present invention.

[0108] Other methods of nucleic acid detection that may be used in the practice of the instant invention are disclosed in U.S. Patent Nos. 5,840,873, 5,843,640, 5,843,651, 5,846,708, 5,846,717, 5,846,726, 5,846,729, 5,849,487, 5,853,990, 5,853,992, 5,853,993, 5,856,092, 5,861,244, 5,863,732, 5,863,753, 5,866,331, 5,905,024, 5,910,407, 5,912,124, 5,912,145, 5,919,630, 5,925,517, 5,928,862, 5,928,869, 5,929,227, 5,932,413 and 5,935,791, each of which is incorporated herein by reference.

VI. Methods of Measuring Protein Expression

[0109] In certain embodiments, immunodetection methods are used to measure expression levels, such as increased expression levels of TFAP2A, E2F5, and CA125. Examples of particular immunodetection methods include enzyme linked immunosorbent assay (ELISA), radioimmunoassay (RIA), immunoradiometric assay, immunohistochemistry, fluoroimmunoassay, chemiluminescent assay, bioluminescent assay, Western blot, and so forth. The steps of various useful immunodetection methods have been described in the scientific literature, such as, *e.g.*, Doolittle and Ben-Zeev, 1999; Gulbis and Galand, 1993; De Jager *et al.*, 1993; Nakamura *et al.*, 1987, each incorporated herein by reference.

[0110] In general, the immunobinding methods involve measurement of the formation of immunocomplexes. Other methods include methods for isolating and purifying the TFAP2A, E2F5, and CA125 protein from a cell, tissue or organism's samples (such as blood, for example). In these instances, the antibody removes the antigenic TFAP2A, E2F5, and CA125 protein or message from a sample. The antibody will preferably be linked to a solid support, such as in the form of a column matrix, and the sample suspected of containing the message, protein, polypeptide and/or peptide antigenic component will be applied to the immobilized antibody. The unwanted components will be washed from the column, leaving the antigen immunocomplexed to the immobilized antibody to be eluted.

[0111] The immunobinding methods also include methods for detecting and quantifying the amount of an antigen component in a sample and the detection and quantification of any immune complexes formed during the binding process. Here, one would obtain a sample suspected of containing an antigen, and contact the sample with an antibody against the TFAP2A, E2F5, and CA125 protein, and then detect and quantify the amount of immune complexes formed under the specific conditions.

[0112] In terms of antigen detection, the biological sample analyzed may be any sample that is suspected of containing an antigen, such as, for example, a tissue section or specimen, a homogenized tissue extract, a cell, an organelle, separated and/or purified forms of any of the above antigen-containing compositions, or even any biological fluid that comes into contact with the cell or tissue, including blood and/or serum, although tissue samples or extracts are preferred.

[0113] Contacting the chosen biological sample with the antibody under effective conditions and for a period of time sufficient to allow the formation of immune complexes (primary immune complexes) is generally a matter of simply adding the antibody composition to the sample and incubating the mixture for a period of time long enough for the antibodies to form immune complexes with, *i.e.*, to bind to, any TFAP2A, E2F5, and CA125 antigens present. After this time, the sample-antibody composition, such as a tissue section, ELISA plate, dot blot or western blot, will generally be washed to remove any non-specifically bound antibody species, allowing only those antibodies specifically bound within the primary immune complexes to be detected.

[0114] In general, the detection of immunocomplex formation is well known in the art and may be achieved through the application of numerous approaches. These methods are generally based upon the detection of a label or marker, such as any of those radioactive, fluorescent, biological and enzymatic tags. U.S. Patents concerning the use of such labels include 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241, each incorporated herein by reference. Of course, one may find additional advantages through the use of a secondary binding ligand such as a second antibody and/or a biotin/avidin ligand binding arrangement, as is known in the art.

[0115] Any TFAP2A, E2F5, and CA125 antibody employed in the detection may itself be linked to a detectable label, wherein one would then simply detect this label, thereby allowing the amount of the primary immune complexes in the composition to be determined. Alternatively, the first antibody that becomes bound within the primary immune complexes may be detected by means of a second binding ligand that has binding affinity for the antibody. In these cases, the second binding ligand may be linked to a detectable label. The second binding ligand is itself often an antibody, which may thus be termed a "secondary" antibody. The primary immune complexes are contacted with the labeled, secondary binding ligand, or antibody, under effective conditions and for a period of time sufficient to allow the formation of secondary immune complexes. The secondary immune complexes are then generally washed to remove any non-specifically bound labeled secondary antibodies or ligands, and the remaining label in the secondary immune complexes is then detected.

[0116] Further methods include the detection of primary immune complexes by a two step approach. A second binding ligand, such as an antibody, that has binding affinity for the antibody is used to form secondary immune complexes, as described above. After washing, the secondary immune complexes are contacted with a third binding ligand or antibody that has binding affinity for the second antibody, again under effective conditions and for a period of time sufficient to allow the formation of immune complexes (tertiary immune complexes). The third ligand or antibody is linked to a detectable label, allowing detection of the tertiary immune complexes thus formed. This system may provide for signal amplification if this is desired.

[0117] One method of immunodetection designed by Charles Cantor uses two different antibodies. A first step biotinylated, monoclonal or polyclonal antibody is used to detect the target antigen(s), and a second step antibody is then used to detect the biotin attached

to the complexed biotin. In that method the sample to be tested is first incubated in a solution containing the first step antibody. If the target antigen is present, some of the antibody binds to the antigen to form a biotinylated antibody/antigen complex. The antibody/antigen complex is then amplified by incubation in successive solutions of streptavidin (or avidin), biotinylated DNA, and/or complementary biotinylated DNA, with each step adding additional biotin sites to the antibody/antigen complex. The amplification steps are repeated until a suitable level of amplification is achieved, at which point the sample is incubated in a solution containing the second step antibody against biotin. This second step antibody is labeled, as for example with an enzyme that can be used to detect the presence of the antibody/antigen complex by histoenzymology using a chromogen substrate. With suitable amplification, a conjugate can be produced which is macroscopically visible.

[0118] Another known method of immunodetection takes advantage of the immuno-PCR (Polymerase Chain Reaction) methodology. The PCR method is similar to the Cantor method up to the incubation with biotinylated DNA, however, instead of using multiple rounds of streptavidin and biotinylated DNA incubation, the DNA/biotin/streptavidin/antibody complex is washed out with a low pH or high salt buffer that releases the antibody. The resulting wash solution is then used to carry out a PCR reaction with suitable primers with appropriate controls. At least in theory, the enormous amplification capability and specificity of PCR can be utilized to detect a single antigen molecule.

A. Western Blot Analysis

[0119] Western blot analysis is an established technique that is commonly employed for analyzing and identifying proteins. The proteins are first separated by electrophoresis in polyacrylamide gel, then transferred ("blotted") onto a nitrocellulose membrane or treated paper, ,where they bind in the same pattern as they formed in the gel. The antigen is overlaid first with antibody, then with anti-immunoglobulin or protein A labeled with a radioisotope, fluorescent dye, or enzyme. One of ordinary skill in the art would be familiar with this commonly used technique for quantifying protein in a sample.

B. ELISAs

[0120] As detailed above, immunoassays, in their most simple and/or direct sense, are binding assays. Certain preferred immunoassays are the various types of enzyme linked immunosorbent assays (ELISAs) and/or radioimmunoassays (RIA) known in the art.

Immunohistochemical detection using tissue sections is also particularly useful. However, it will be readily appreciated that detection is not limited to such techniques, and/or western blotting, dot blotting, FACS analyses, and/or the like may also be used. One of ordinary skill in the art would be familiar with use of ELISAs and other immunohistochemical assays.

C. Immunohistochemistry

[0121] The antibodies of the present invention may also be used in conjunction with both fresh-frozen and/or formalin-fixed, paraffin-embedded tissue blocks, such as blocks prepared from a tumor biopsy, prepared for study by immunohistochemistry (IHC). The method of preparing tissue blocks from these particulate specimens has been successfully used in previous IHC studies of various prognostic factors, and/or is well known to those of skill in the art (Brown *et al.*, 1990; Abbondanzo *et al.*, 1999; Allred *et al.*, 1990).

[0122] Briefly, frozen-sections may be prepared by rehydrating 50 ng of frozen "pulverized" tissue at room temperature in phosphate buffered saline (PBS) in small plastic capsules; pelleting the particles by centrifugation; resuspending them in a viscous embedding medium (OCT); inverting the capsule and/or pelleting again by centrifugation; snap-freezing in 70°C isopentane; cutting the plastic capsule and/or removing the frozen cylinder of tissue; securing the tissue cylinder on a cryostat microtome chuck; and/or cutting 25-50 serial sections.

[0123] Permanent-sections may be prepared by a similar method involving rehydration of the 50 mg sample in a plastic microfuge tube; pelleting; resuspending in 10% formalin for 4 hours fixation; washing/pelleting; resuspending in warm 2.5% agar; pelleting; cooling in ice water to harden the agar; removing the tissue/agar block from the tube; infiltrating and/or embedding the block in paraffin; and/or cutting up to 50 serial permanent sections.

D. Protein Array Technology

[0124] Protein array technology allows high-throughput screening for gene expression and molecular interactions. Protein arrays appear as new and versatile tools in functional genomics, enabling the translation of gene expression patterns of normal and diseased tissues into protein product catalog. Protein function, such as enzyme activity, antibody specificity, and other ligand–receptor interactions and binding of nucleic acids or small molecules can be analyzed on a whole-genome level.

1. Protein Biochip Assays

[0125] These arrays, which contain thousands of different proteins or antibodies spotted onto glass slides or immobilized in tiny wells, allow one to examine the biochemical activities and binding profiles of a large number of proteins at once. To examine protein interactions with such an array, a labeled protein is incubated with each of the target proteins immobilized on the slide, and then one determines which of the many proteins the labeled molecule binds.

[0126] The basic construction of protein chips has some similarities to DNA chips, such as the use of a glass or plastic surface dotted with an array of molecules. These molecules can be DNA or antibodies that are designed to capture proteins. Defined quantities of proteins are immobilized on each spot, while retaining some activity of the protein. With fluorescent markers or other methods of detection revealing the spots that have captured these proteins, protein microarrays are being used as powerful tools in high-throughput proteomics and drug discovery.

[0127] Glass slides are still widely used, since they are inexpensive and compatible with standard microarrayer and detection equipment. However, their limitations include multiple-based reactions, high evaporation rates, and possible cross-contamination.

[0128] Matrix slides offer a number of advantages, such as reduced evaporation and no possibility of cross-contamination, but they are expensive. Nanochips for proteomics have the same advantages, in addition to reduced cost and the capability of multiple-component reactions.

[0129] The earliest and best-known protein chip is the ProteinChip by Ciphergen Biosystems Inc. (Fremont, CA). The ProteinChip is based on the surface-enhanced laser desorption and ionization (SELDI) process. Known proteins are analyzed using functional assays that are on the chip. For example, chip surfaces can contain enzymes, receptor proteins, or antibodies that enable researchers to conduct protein-protein interaction studies, ligand binding studies, or immunoassays. With state-of-the-art ion optic and laser optic technologies, the ProteinChip system detects proteins ranging from small peptides of less than 1000 Da up to proteins of 300 kDa and calculates the mass based on time-of-flight (TOF).

[0130] The ProteinChip biomarker system is the first protein biochip–based system that enables biomarker pattern recognition analysis to be done. This system allows researchers to

address important clinical questions by investigating the proteome from a range of crude clinical samples (*i.e.*, laser capture microdissected cells, biopsies, tissue, urine, and serum). The system also utilizes biomarker pattern software that automates pattern recognition–based statistical analysis methods to correlate protein expression patterns from clinical samples with disease phenotypes.

[0131] Some systems can perform biomarker discovery in days and validation of large sample sets within weeks. Its robotics system accessory automates sample processing, allowing hundreds of samples to be run per week and enabling a sufficient number of samples to be run, which provides high statistical confidence in comprehensive studies for marker discovery and validation.

2. Microfluidic Chip-Based Immunoassays

[0132] Microfluidics is one of the most important innovations in biochip technology. Since microfluidic chips can be combined with mass spectrometric analysis, a microfluidic device has been devised in which an electrospray interface to a mass spectrometer is integrated with a capillary electrophoresis channel, an injector, and a protein digestion bed on a monolithic substrate (Wang *et al.*, 2000). This chip thus provides a convenient platform for automated sample processing in proteomics applications.

[0133] These chips can also analyze expression levels of serum proteins with detection limits comparable to commercial enzyme-linked immunosorbent assays, with the advantage that the required volume sample is markedly lower compared with conventional technologies.

[0134] Biosite (San Diego) manufactures the Triage protein chip that simultaneously measures 100 different proteins by immunoassays. The Triage protein chip immunoassays are performed in a microfluidic plastic chip, and the results are achieved in 15 minutes with picomolar sensitivities. Microfluidic fluid flow is controlled in the protein chip by the surface architecture and surface hydrophobicity in the microcapillaries. The immunoassays utilize high-affinity antibodies and a near-infrared fluorescent label, which is read by a fluorometer.

3. Tissue Microarray Technology

[0135] Tissue microarray technology provides a high-throughput approach for linking genes and gene products with normal and disease tissues at the cellular level in a parallel fashion. Compared with classical in situ technologies in molecular pathology that are very time-consuming, tissue microarrays provide increased throughput in two ways: up to 1000 tissue specimens can be analyzed in a single experiment, either at the DNA, RNA, or protein level; and tens of thousands of replicate tissue microarrays can be generated from a set of tissues. This process provides a template for analyzing many more biomarkers than has ever been possible previously in a clinical setting, even using archival, formalin-fixed specimens.

4. Nanoscale Protein Analysis

[0136] Most current protocols including protein purification and automated identification schemes yield low recoveries that limit the overall process in terms of sensitivity and speed. Such low protein yields and proteins that can only be isolated from limited source material (*e.g.*, biopsies) can be subjected to nanoscale protein analysis: a nanocapture of specific proteins and complexes, and optimization of all subsequent sample-handling steps, leading to a mass analysis of peptide fragments. This focused approach, also termed targeted proteomics, involves examining subsets of the proteome (*e.g.*, those proteins that are specifically modified, bind to a particular DNA sequence, or exist as members of higher-order complexes or any combination thereof). This approach is used to identify genetic determinants of cancer that alter cellular physiology and respond to agonists.

[0137] A new detection technique called multiphoton detection, by Biotrace Inc. (Cincinnati), can quantify subzeptomole amounts of proteins and will be used for diagnostic proteomics, particularly for cytokines and other low-abundance proteins. Biotrace is also developing supersensitive protein biochips to detect concentrations of proteins as low as 5 fg/ml (0.2 attomole/ml), thereby permitting sensitivity that is 1000 times greater than current protein biochips.

VII. Kits of the Invention

[0138] All of the essential materials and/or reagents required for detecting TFA2P2A, E2F5, and/or CA125 in a sample may be assembled together in a kit. In particular embodiments, the kit comprises respective antibodies to TFA2P2A, E2F5, and/or CA125. This may comprise a probe or primers designed to hybridize specifically to individual nucleic acids of

interest in the practice of the present invention, including TFA2P2A, E2F5, and/or CA125, in some cases. Also included may be enzymes suitable for amplifying nucleic acids, including various polymerases (reverse transcriptase, *Taq, etc.*), deoxynucleotides and buffers to provide the necessary reaction mixture for amplification. Such kits may also include enzymes and other reagents suitable for detection of specific nucleic acids or amplification products. Such kits generally will comprise, in suitable means, distinct containers for each individual reagent or enzyme as well as for each probe or primer pair.

[0139] Any of the compositions described herein may be comprised in a kit. In a non-limiting example, a composition of the invention may be comprised in a kit. The components of the kits may be packaged either in aqueous media or in lyophilized form. The container means of the kits will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which a component may be placed, and preferably, suitably aliquoted. Where there is more than one component in the kit, the kit also will generally contain a second, third or other additional container into which the additional components may be separately placed. However, various combinations of components may be comprised in a vial. The kits of the present invention also will typically include a means for containing the composition and any other reagent containers in close confinement for commercial sale. Such containers may include injection or blow molded plastic containers into which the desired vials are retained, for example.

[0140] When the components of the kit are provided in one and/or more liquid solutions, the liquid solution is an aqueous solution, with a sterile aqueous solution being particularly preferred. In specific cases, the container means may itself be a syringe, pipette, and/or other such like apparatus, from which the formulation may be applied to the pancreas, for example. However, the components of the kit may be provided as dried powder(s). When reagents and/or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. It is envisioned that the solvent may also be provided in another container means.

VIII. TFAP2A as a Marker for Other Cancers

[0141] In some embodiments of the invention, TFAP2A level is assayed for detection of a cancer other than ovarian cancer. Although TFAP2A may be indicative of having or being at greater risk for having any cancer other than ovarian cancer, in specific embodiments,

the cancer is of the uterus, breast, lung, prostate, colon, brain, bone, liver, pancreas, cervix, testes, spleen, skin, gall bladder, esophagus, bladder, kidney, thyroid, blood, and so forth. In specific embodiments, level of TFAP2A protein and/or mRNA is increased or decreased in a cancer other than ovarian cancer when compared to a control. In specific embodiments, markers in addition to TFAP2A are employed with TFAP2A to detect the presence or increased risk of having a particular type of cancer. Exemplary other tumor markers include prostate-specific antigen (PSA) for prostate cancer, calcitonin for medullary thyroid cancer, alpha-fetoprotein (AFP) for liver cancer and human chorionic gonadotropin (HCG) for germ cell tumors, such as testicular cancer and ovarian cancer.

EXAMPLES

[0142] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

MOLECULAR BIOMARKER SET FOR DETECTION OF OVARIAN CANCER

[0143] The inventors compared the expression levels of genes in cancerous tissues categorized as stage IA, IC, and IIIC. FIG. 1 demonstrates that the expression of *TFAP2A* gene is higher in early stages (stages IA and IC) as well as advanced stage (IIIC) of the ovarian cancer as compared to the normal tissue (FIG. 1).

[0144] Kothandaraman *et al.* (2010) described E2F5 as a biomarker of ovarian cancer and they showed that the use of E2F5 in combination with CA125 increased sensitivity of ovarian cancer detection to 97.9% (an increase from 87.5% if only CA125 is used) in the case when the status of any of E2F5 or CA125 is confirmed. If the status of both E2F5 and CA125 is confirmed, the specificity of ovarian cancer detection increases to 72.5% (an increase from 55% if only CA125 is used) in a subset of patients. This study is the evidence that the combination markers can be more useful in diagnosing ovarian cancer. To further enhance the accuracy of

ovarian cancer detection at early stages, the inventions set forth that the combination of TFAP2A, E2F5, and CA125 are useful for increased accuracy in detection of ovarian cancer. Two of these biomarkers *i.e.* E2F5 and CA125 have already been published and the computational analysis has detected TFAP2A as a biomarker of ovarian cancer.

[0145] The inventors describe here the identification of a new biomarker using computational methods that is useful for diagnosis of ovarian cancer. The results are based on a set A of 323 experimentally validated OC implicated genes compiled from literature. For this gene set, the inventors determined putative transcription factors (TFs) that control gene activation. The inventors ranked these TFs based on the number of genes they control in set A . In this way, the inventors selected top-ranked TFs as potential biomarkers for set A. Comparing the expression of top-ranked TFs in OC and normal cases based on published data (Lu *et al.*, 2004, Hendrix *et al.*, 2006, Adib *et al.*, 2004), we identified TF named TFAP2A as a new biomarker for detection of ovarian cancer. The newly identified biomarker TFAP2A was compared with known marker CA125 in terms of expression in cancer patients in relevant published datasets.

EXAMPLE 2

EXEMPLARY CLINICAL APPLICATION OF THE INVENTION

[0146] In certain embodiments of the invention, an individual that is suspected of having ovarian cancer (for example, because of one or more test results and/or symptoms of ovarian cancer; an individual that is at risk for developing ovarian cancer (for example, because of a family history); an individual presently or previously on therapy for ovarian cancer; or an individual practicing routine health checks for ovarian cancer is subjected to methods of the invention for determining expression levels of TFAP2A, E2F5, and/or CA125.

[0147] The individual provides a sample to be tested. The sample may be processed by the same person or organization that obtains the sample, or the sample may be forwarded to another party for assaying or may be stored appropriately. The sample may be a biological fluid sample such as a blood sample, although in some cases the sample is an ovarian biopsy. The sample may be further processed, for example, blood may be fractionated, or ovarian tissue may have nucleic acid extracted. The respective mRNA or protein may be obtained from the sample by routine methods in the art, and is level is obtained and compared to

a control, which may also be referred to as a standard. The control may comprise levels of TFAP2A, E2F5, and CA125 that are obtained from one or more normal individuals (persons without the respective cancer), and these levels may be obtained from the respective fluid or tissue in the normal individual(s). In the case of ovarian biopsy, for example, the level may be obtained from ovarian tissue in a region of the ovary being tested that detectably lacks cancerous cells and/or may be from the second ovary of the individual than the one being tested. In other ovarian biopsy cases, the level is obtained from one or more normal individuals.

EXAMPLE 3

COMBINATIONS OF BIOMARKERS FOR DETECTION OF OVARIAN CANCER

[0148] The present example provides exemplary methods demonstrating detection of at least one ovarian cancer markers.

[0149] Samples

[0150] The RNA samples for 100 individuals were obtained from a commercial supplier and the distribution of samples is as follows:

[0151] Table 1. Distribution of tissue samples used for gene expression study

Sample detail	Sample Numbers
Ovarian Cancer (OC)	60
Breast cancer	10
Cervix cancer	10
Uterus cancer	10
Non-cancerous ovary	10

[0152] The RNA was extracted from the respective tissues by standard means in the art.

[0153] Exemplary experimental procedures

[0154] The 100 RNA samples underwent quality control (QC) using a bioanalyzer prior to cDNA synthesis with the ABI high capacity cDNA synthesis kit. The cDNA was then analyzed against five commercially available *TaqMan*® gene expression assays (Table 2) with the ABI *TaqMan*® gene expression protocol using the *7900HT RT-PCR* instrument.

[0155] Table 2: *TaqMan*® Assays used in gene expression study

	Inventory Code	Gene Name	Gene Classification
1	Hs01029413_m1	TFAP2A	Transcription Factor AP-2 α
2	Hs00231092_m1	E2F5	E2F Transcription Factor 5, p130 binding
3	Hs03928990_g1	RN18S1	RNA, 18S ribosomal 1 (exemplary housekeeping gene)
4	Hs03929097_g1	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase (exemplary housekeeping gene)
5	Hs01065189_m1	MUC16 (CA125)	Mucin 16, cell surface associated

[0156] cDNA synthesis and QC

[0157] cDNA synthesis was performed according to the manufacturer's instructions using the *ABI High Capacity cDNA synthesis kits* and QC was carried out using the *Nanodrop* instrument. Good quality cDNA was produced with an average cDNA yield of ~1490 ng/µl per sample. The 260/280 and 260/230 ratios were within the optimal range with average values of 1.8 and 2.0, respectively.

[0158] qRT-PCR QC and Analysis

[0159] A 1 µl aliquot from each of the 100 samples was pooled to create a standard sample. The standard sample was further subjected to QC with the nanodrop and diluted to make up a dilution series for standard curve analysis. A standard curve was generated for each gene and the *TaqMan*® assays were all found to be within 100 ± 10% efficiency (Table 3), where a slope of -3.32 indicates an assay with 100% efficiency.

[0160] Table 3: Standard Curve efficiencies for the five genes under investigation

Detector Name	Slope	R^2
Hs01029413_m1 TFAPZA	-3.6017125	0.998070
Hs00231092_m1 E2F5	-3.5165527	0.9947431
Hs03928990_g1 RN1851	-3.56650	0.9986217
Hs03929097_g1 GAPDH	-3.3637228	0.9978328
Hs01065189_m1 MUC16 (CA125)	-3.5434833	0.99704236

[0161] For the experimental procedure, each sample was assayed in triplicate to limit technical variation. The assay comprised of cDNA samples mixed together with a cocktail containing the primer and probe pairs of the specific gene to be investigated. Samples were

loaded onto a 384-well opti-clear PCR plate and the assay was performed under the *ABI* prescribed conditions on the *7900HT RT-PCR*. Primary data analysis was executed with the use of the *ABI SDS v2.3* software package.

[0162] Results

[0163] Average Ct values for each gene were provided for further analysis. Ct refers to cycle threshold and is defined as the number of cycles required for a fluorescent signal to cross a threshold. The threshold is the point where signal exceeds background level. The amount of amplified nucleic acid in the sample is inversely proportional to Ct (*i.e.* the lower the Ct level the greater the amount of target nucleic acid in the sample).

[0164] The inventors used ΔΔCt method to calculate the expression value for each gene. The method is summarized as follows:

[0165] Step 1:

[0166] $\Delta Ct_{(\text{Target gene})} = Ct_{(\text{Target gene})} - Ct_{(\text{housekeeping gene})}$

[0167] Step 2:

[0168] $\Delta\Delta Ct_{(OC)} = \Delta Ct_{(\text{Target gene in OC})} - \Delta Ct_{(\text{Target gene in non-OC (average)})}$

[0169] $\Delta\Delta Ct_{(\text{non-OC})} = \Delta Ct_{(\text{Target gene in non-OC})} - \Delta Ct_{(\text{Target gene in non-OC (average)})}$

[0170] Determining an accurate combination of biomarkers:

[0171] The expression data of target genes (TFAP2A, E2F5 and CA125) normalized against RN18S1 reference gene (control) (according to Steps 1 and 2 above) was used for predicting the accuracy of the diagnosis of OC using biomarkers in different combinations. Table 4 represents the results of this analysis. The terms "se", "sp" and "acc" denote sensitivity, specificity and accuracy, respectively. These parameters are calculated as follows:

[0172] se = tp/(tp+fn),

[0173] sp = tn/(tn+fp),

[0174] acc = (tp+tn)/(tp+fn+tn+fp),

[0175] where tp, tn, fp, fn stand for true positive, true negative, false positive and false negative outcome of diagnoses.

[0176] tp means that the OC case is diagnosed as being OC;

[0177] tn means that the non-OC case is diagnosed as being non-OC;

[0178] fp means that the non-OC case is diagnosed falsely as being OC;

[0179] fn means that the OC case is falsely diagnosed as being non-OC.

[0180] The columns in Table 4 represent the following:

[0181] 'max se-(1-sp)' is a measure that shows how well OC cases are diagnosed relative to non-OC cases. (1-sp) shows the proportion of the non-OC cases diagnosed wrongly as being OC. Thus the difference between se and (1-sp) is a good indicator of the quality of diagnosis. The higher the value of 'se-(1-sp)', the more useful are the biomarkers.

[0182] Threshold for TFAP2A: the threshold th. In specific embodiments, $\Delta\Delta Ct_{(TFAP2A)} > th$ in different combinations of biomarkers is useful for diagnosis of OC;

[0183] Threshold for E2F5: the threshold th. In specific embodiments, $\Delta\Delta Ct_{(E2F5)} > th$ in different combinations of biomarkers is useful for diagnosis of OC;

[0184] Threshold for CA125: the threshold th. In specific embodiments, $\Delta\Delta Ct_{(CA125)} > th$ in different combinations of biomarkers is useful for diagnosis of OC;

[0185] For different combinations of biomarkers (see Table 4), the threshold values were obtained by searching for the combination of thresholds that will maximize 'se-(1-sp)' subject to condition that this threshold has value between -10 and 10. The inventors used Direct Search optimization method from the Global Optimization Toolbox of Matlab package release 2011b (commercially available from Mathworks, Natick, MA, USA).

[0186] N/A: means that particular biomarker is not used in the test.

[0187] Table 4: The diagnostic value of different combinations of biomarkers were calculated based on threshold of ΔΔCt of each target biomarker.

'&' means logical 'AND'							
'|' means logical 'OR'	**max se-(1-sp)**	**Sensitivity**	**Specificity**	**Accuracy**	**threshold for $\Delta\Delta Ct_{(TFAP2A)}$**	**threshold for $\Delta\Delta Ct_{(E2F5)}$**	**threshold for $\Delta\Delta Ct_{(CA125)}$**
TFAP2A & E2F5 & CA125	0.433333	0.683333	0.75	0.71	-1.59	-1.16	-2.86
TFAP2A | E2F5 & CA125	0.383333	0.883333	0.5	0.73	7.13	-0.85	-2.66
TFAP2A & E2F5 | CA125	0.375	0.65	0.725	0.68	-0.46	-0.58	3.51
TFAP2A & (E2F5 | CA125)	0.341667	0.716667	0.625	0.68	-1.58	9.027	-2.87
TFAP2A | E2F5 | CA125	0.3	0.8	0.5	0.68	-0.38	0.61	3.6
E2F5 & CA125	0.383333	0.883333	0.5	0.73	N/A	-0.85	-2.76
TFAP2A & CA125	0.341667	0.716667	0.625	0.68	-1.62	N/A	-2.87
TFAP2A & E2F5	0.308333	0.633333	0.675	0.65	-1.933	-0.526	N/A
E2F5 | CA125	0.291667	0.866667	0.425	0.69	N/A	-0.526	1.95
TFAP2A | E2F5	0.266667	0.816667	0.45	0.67	8.62	-0.524	N/A
TFAP2A | CA125	0.258333	0.783333	0.475	0.66	-0.75	N/A	3.53
E2F5	0.266667	0.816667	0.45	0.67	N/A	-0.526	N/A
CA125	0.241667	0.916667	0.325	0.68	N/A	N/A	-2.474
TFAP2A	0.183333	0.733333	0.45	0.62	-1.59	N/A	N/A

[0188] Table 4 shows that combination of TFAP2A & E2F5 & CA125 has max value of se-(1-sp) = 0.433 (first data row in Table 4) for the samples analyzed, which is highest than any other combination of biomarkers. This shows that the combination of these three biomarkers provides most confidence in diagnosing correctly 68% of OC cases and identifies

correctly 75% of non-ovarian cases. Individually, each biomarker (last three rows of Table 4) E2F5, CA125 and TFAP2A can only identify 45%, 32% and 45% of non-OC cases, respectively, since the specificity of individual biomarker is very low. For example, CA125, which is a routinely used biomarker, could only classify 32% of cases as non-OC cases, whereas combination of the three exemplary biomarkers could identify 75% of non-OC cases. Therefore, the proposed combination of biomarkers (TFAP2A, CA125 and E2F5) has increased the overall accuracy to detect OC cases as well as non-OC cases as compared to other combinations of biomarkers/individual biomarkers. However, in alternative embodiments, TFAP2A alone is accurate to identify the presence of OC or risk of developing OC, or other cancers.

EXAMPLE 4

ANALYSIS SUMMARY OF EXPRESSION BEHAVIOR OF BIOMARKERS IN BREAST, CERVIX AND UTERUS CANCERS

[0189] This example describes the expression behavior of three biomarkers (TFAP2A, E2F5 and CA125) in samples obtained from breast, cervix and uterine cancers. The sample size for each cancer type and normal is 10. The expression level for each biomarker was assayed and quantitated essentially as described in Example 3. The following graphs represent the expression values observed for normal and cancers calculated as follows:

[0190] Step 1:

[0191] $\Delta Ct_{(\text{Target gene})} = Ct_{(\text{Target gene})} - Ct_{(\text{housekeeping gene})}$

[0192] Step 2:

[0193] $\Delta\Delta Ct_{(\text{cancer})} = \Delta Ct_{(\text{Target gene in cancer})} - \Delta Ct_{(\text{Target gene in normal (average)})}$

[0194] $\Delta\Delta Ct_{(\text{normal})} = \Delta Ct_{(\text{Target gene in normal})} - \Delta Ct_{(\text{Target gene in normal (average)})}$

[0195] The relative expression of each biomarker shown in the figures is calculated as $2^{(-\Delta\Delta Ct)}$ (Arocho *et al.*, 2006)

[0196] **Exemplary Results**

[0197] The results of the analysis are represented in the form of bar graphs for each cancer type and normal samples.

[0198] FIG. 2 shows a bar graph representing the expression pattern of three biomarkers in normal samples. The Y-axis represents the expression levels of biomarkers in each normal sample. The X-axis represents individual samples. The expression of TFAP2A was very low or undetectable in 8/10 samples, the expression of E2F5 was low in most of the normal samples, whereas the expression of CA125 was low or was not detected in 8/10 samples.

[0199] FIG. 3 shows the expression pattern of the three biomarkers in breast cancer samples. The Y-axis represents the expression levels of biomarkers in each breast cancer sample. The X-axis represents individual samples. In breast cancer samples, the expression level of CA125 was higher than the control in 7/10 samples. Zero on the Y-axis is the lowest boundary on expression, *i.e.* it means no expression.

[0200] FIG. 4 shows the expression pattern of the three biomarkers in cervix cancer samples. The Y-axis represents the expression levels of biomarkers in each cervix cancer sample. The X-axis represents individual samples. Six out of 10 cervix cancer samples had higher expression levels than the control for all three biomarkers.

[0201] FIG. 5 demonstrates the expression pattern of the three biomarkers in uterus cancer samples. The Y-axis represents the expression levels of biomarkers in each uterus cancer sample. The X-axis represents individual samples.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

PATENTS AND PATENT APPLICATIONS

European Application No. 320 308

European Application No. 329 822

GB Application No. 2 202 328

PCT Application No. PCT/US87/00880

PCT Application No. PCT/US89/01025

PCT Application No. PCT/US03/00531

PCT Application WO 88/10315

PCT Application WO 89/06700

PCT Application WO 90/07641

U.S. Patent No. 3,817,837

U.S. Patent No. 3,850,752

U.S. Patent No. 3,939,350

U.S. Patent No. 3,996,345

U.S. Patent No. 4,277,437

U.S. Patent No. 4,275,149

U.S. Patent No. 4,366,241

U.S. Patent No. 4,683,195

U.S. Patent No. 4,683,202

U.S. Patent No. 4,800,159

U.S. Patent No. 4,883,750

U.S. Patent No. 4,946,773

U.S. Patent No. 5,279,721

U.S. Patent No. 5,840,873

U.S. Patent No. 5,843,663

U.S. Patent No. 5,843,640

U.S. Patent No. 5,843,650

U.S. Patent No. 5,843,651

U.S. Patent No. 5,843,663

U.S. Patent No. 5,846,708

U.S. Patent No. 5,846,709

U.S. Patent No. 5,846,717

U.S. Patent No. 5,846,726

U.S. Patent No. 5,846,729

U.S. Patent No. 5,846,783

U.S. Patent No. 5,849,481

U.S. Patent No. 5,849,483

U.S. Patent No. 5,849,486

U.S. Patent No. 5,849,487

U.S. Patent No. 5,849,497

U.S. Patent No. 5,849,546

U.S. Patent No. 5,849,547

U.S. Patent No. 5,851,770

U.S. Patent No. 5,851,772

U.S. Patent No. 5,853,990

U.S. Patent No. 5,853,992

U.S. Patent No. 5,853,993

U.S. Patent No. 5,856,092

U.S. Patent No. 5,858,652

U.S. Patent No. 5,861,244

U.S. Patent No. 5,863,732

U.S. Patent No. 5,863,753

U.S. Patent No. 5,866,331

U.S. Patent No. 5,866,337

U.S. Patent No. 5,866,366

U.S. Patent No. 5,882,864

U.S. Patent No. 5,900,481

U.S. Patent No. 5,905,024

U.S. Patent No. 5,910,407

U.S. Patent No. 5,912,124

U.S. Patent No. 5,912,548

U.S. Patent No. 5,912,145

U.S. Patent No. 5,912,148

U.S. Patent No. 5,916,776

U.S. Patent No. 5,916,779

U.S. Patent No. 5,919,626

U.S. Patent No. 5,919,630

U.S. Patent No. 5,922,574

U.S. Patent No. 5,925,517

U.S. Patent No. 5,925,525

U.S. Patent No. 5,928,862

U.S. Patent No. 5,928,869

U.S. Patent No. 5,928,870

U.S. Patent No. 5,928,905

U.S. Patent No. 5,928,906

U.S. Patent No. 5,929,227

U.S. Patent No. 5,932,413

U.S. Patent No. 5,932,451

U.S. Patent No. 5,935,791

U.S. Patent No. 5,935,825

U.S. Patent No. 5,939,291

U.S. Patent No. 5,942,391

U.S. Patent No. 4,683,202

U.S. Patent No. 4,800,159

U.S. Patent No. 5,849,486

U.S. Patent No. 5,851,772

U.S. Patent No. 5,900,481

U.S. Patent No. 5,919,626

U.S. Patent No. 7,605,003

U.S. Patent No. 7,741,019

U.S. Patent No. 7,745,149

PUBLICATIONS

Adib TR, Henderson S, Perrett C, Hewitt D, Bourmpoulia D, Ledermann J, Boshoff C: Predicting biomarkers for ovarian cancer using gene-expression microarrays. Br J Cancer 2004, 90:686-692.

Arocho Alaina, Ladanyi Marc, Pan Qiulu. Validation of the 2-[DELTA][DELTA]Ct Calculation as an Alternate Method of Data Analysis for Quantitative PCR of BCR-ABL P210 Transcripts. Diagnostic Molecular Pathology: March 2006 - Volume 15 - Issue 1 - pp 56-61

Bär M, Bär D, Lehmann B: Selection and validation of candidate housekeeping genes for studies of human keratinocytes--review and recommendations. J Invest Dermatol. 2009, 129(3):535-7. Review.

Frohman, In: PCR PROTOCOLS: A GUIDE TO METHODS AND APPLICATIONS, Academic Press, N.Y., 1990.

Hendrix ND, Wu R, Kuick R, Schwartz DR, Fearon ER, Cho KR: Fibroblast growth factor 9 has oncogenic activity and is a downstream target of Wnt signaling in ovarian endometrioid adenocarcinomas. Cancer Res 2006, 66:1354-1362.

Innis *et al.*, "DNA sequencing with Thermus aquaticus DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA," Proc Natl Acad Sci U S A. 85(24):9436-9440, 1988.

Kothandaraman N, Bajic VB, Brendan PN, Huak CY, Keow PB, Razvi K, Salto-Tellez M, Choolani M. E2F5 status significantly improves malignancy diagnosis of epithelial ovarian cancer. BMC Cancer. 2010 Feb 24; 10:64.

Kwoh *et al.*, "Transcription-based amplification system and detection of amplified human immunodeficiency virus type 1 with a bead-based sandwich hybridization format, Proc Natl Acad Sci U S A. 86(4):1173-1177, 1989.

Lu KH, Patterson AP, Wang L, Marquez RT, Atkinson EN, Bagerly KA, Ramoth LR, Rosen DG, Liu J, Hellstrom I, *et al.*: Selection of potential markers for epithelial ovarian

cancer with gene expression arrays and recursive descent partition analysis. Clin Cancer Res 2004, 10:3291-3300.

Ohara *et al.*, "One-sided polymerase chain reaction: the amplification of cDNA," Proc Natl Acad Sci U S A. 86(15):5673-5677, 1989.

Sasaroli D, Coukos G, Scholler N: Beyond CA125: the coming of age of ovarian cancer biomarkers. Are we there yet? Biomark Med 2009, 3:275-288.

Tunbridge EM, Eastwood SL, Harrison PJ: Changed relative to what? Housekeeping genes and normalization strategies in human brain gene expression studies. Biol Psychiatry. 2011 Jan 15;69(2):173-9. Review

Walker *et al.*, "Strand displacement amplification--an isothermal, *in vitro* DNA amplification technique," Nucleic Acids Res. 20(7):1691-1696, 1992

[0202] Although the present invention and its advantages have been described in detail, it should be understood that various changes, substitutions and alterations can be made herein without departing from the spirit and scope of the invention as defined by the appended claims. Moreover, the scope of the present application is not intended to be limited to the particular embodiments of the process, machine, manufacture, composition of matter, means, methods and steps described in the specification. As one of ordinary skill in the art will readily appreciate from the disclosure of the present invention, processes, machines, manufacture, compositions of matter, means, methods, or steps, presently existing or later to be developed that perform substantially the same function or achieve substantially the same result as the corresponding embodiments described herein may be utilized according to the present invention. Accordingly, the appended claims are intended to include within their scope such processes, machines, manufacture, compositions of matter, means, methods, or steps.

CLAIMS

What is claimed is:

1. A method of identifying ovarian cancer in an individual or the risk of developing ovarian cancer in an individual, comprising the step of assaying for expression of TFAP2A in the individual.

2. A method of identifying ovarian cancer in an individual or the risk of developing ovarian cancer in an individual, comprising the step of comparing the expression level of TFAP2A from the individual with the expression level of TFAP2A from a control.

3. A method of identifying ovarian cancer in an individual or the risk of developing ovarian cancer in an individual, comprising the step of detecting expression of TFAP2A in an individual, wherein expression of TFAP2A indicates the presence of ovarian cancer or the risk of developing ovarian cancer in the individual.

4. A method of identifying ovarian cancer in an individual or the risk of developing ovarian cancer in an individual, comprising the steps:

   a) comparing the expression level of TFAP2A from the individual with the expression level of TFAP2A from a control, and

   b) comparing the expression level of CA125 or E2F5 from the individual with the expression level of CA125 or E2F5 from a control.

5. A method of identifying ovarian cancer in an individual or the risk of developing ovarian cancer in an individual, comprising the step of comparing the expression level of TFAP2A, CA125 and E2F5 from the individual and the expression level of TFAP2A, CA125 and E2F5 from a control.

6. The method of any one of claims 1-5, wherein the protein level of TFAP2A, CA125, and/or E2F5 from the individual is determined.

7. The method of claim 6, wherein the protein level of TFAP2A, CA125, and/or E2F5 is determined from the blood of the individual.

8. The method of claim 6 or 7, wherein the protein level of TFAP2A, CA125, and/or E2F5 is determined with an antibody.

9. The method of claim 8, wherein the antibody is a monoclonal antibody.

10. The method of any one of claims 1-9, wherein the mRNA level of TFAP2A, CA125, and/or E2F5 from the individual is determined.

11. The method of claim 10, wherein the mRNA level of TFAP2A is determined from ovarian tissue or blood from the individual.

12. The method of claim 10 or 11, wherein the mRNA level is determined by microarray, Northern, or RT-PCR.

13. The method of any one of claims 2 or 4-12, wherein the control is tissue or blood from one or more normal individuals or is from tissue or blood from the individual.

14. The method of any one of claims 1-13, wherein the method detects the stage of ovarian cancer in the individual.

15. The method of any one of claims 1-14, wherein the method further comprises a step of detecting the stage of ovarian cancer in the individual.

16. The method of claim 14 or 15, wherein the stage is stage IA, IC, IIIC, or a combination thereof.

17. The method of any one of claims 1-16, further comprising the step of performing an additional ovarian cancer detection method.

18. The method of claim 17, wherein the additional ovarian cancer detection method is selected from the group consisting of

palpitation, ultrasound, magnetic resonance imaging, X-ray, CT scan, blood testing, and biopsy.

19. The method of any one of claims 1-18, further comprising the step of administering treatment for ovarian cancer.

20. The method of any one of claims 1-19, further comprising the step of obtaining a sample from the individual.

21. The method of any one of claims 1-20, further comprising the step of isolating TFAP2A protein and/or mRNA from the sample.

22. The method of any one of claims 1-21, further comprising the steps of obtaining a sample from the individual and isolating TFAP2A protein and/or mRNA from the sample.

23. The method of any one of claims 2-22, further comprising the step of comparing the level of CA125, E2F5, or both from a sample from the individual with the expression level of CA125, E2F5, or both, respectively, from a control.

24. A kit comprising one or more detection reagents for TFAP2A and optionally one or more detection reagents for one or both of CA125 and E2F5, said reagents housed in a suitable container.

25. The kit of claim 24, wherein the reagent is selected from the group consisting of antibody, microarray, oligonucleotide, polymerase, deoxyribonucleotides, buffer, or a combination thereof.

26. The kit of claim 24, further comprising an apparatus for drawing blood or taking a biopsy from an individual.

27. A method of identifying ovarian cancer in an individual or the risk of developing ovarian cancer in an individual, comprising the steps of

a) obtaining a sample from the individual,

b) determining the expression level of TFAP2A from the sample, and

c) comparing the expression level of TFAP2A from the individual with the expression level of TFAP2A from a control.

25. A method of identifying ovarian cancer in an individual or the risk of developing ovarian cancer in an individual, comprising the steps of

a) obtaining a sample from the individual,

b) determining the expression level of TFAP2A from the sample,

c) determining the expression level of CA125 and/or E2F5 from the sample,

d) comparing the expression level of TFAP2A from the individual with the expression level of TFAP2A from a control, and

e) comparing the expression level of CA125 and/or E2F5 from the individual with the expression level of CA125 and/or E2F5 from a control

.

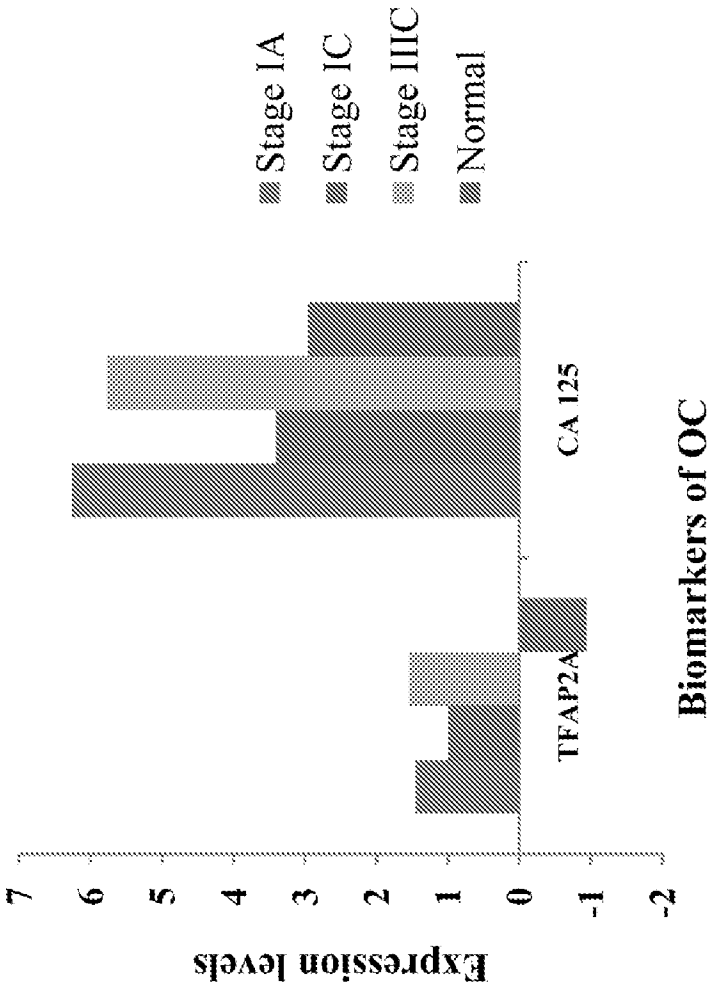

Stage IA
Stage IC
Stage IIIC
Normal
7
6
5
4
3
2
1
0
-1
-2
Expression levels
CA 125
TFAP2A
Biomarkers of OC

FIG. 1

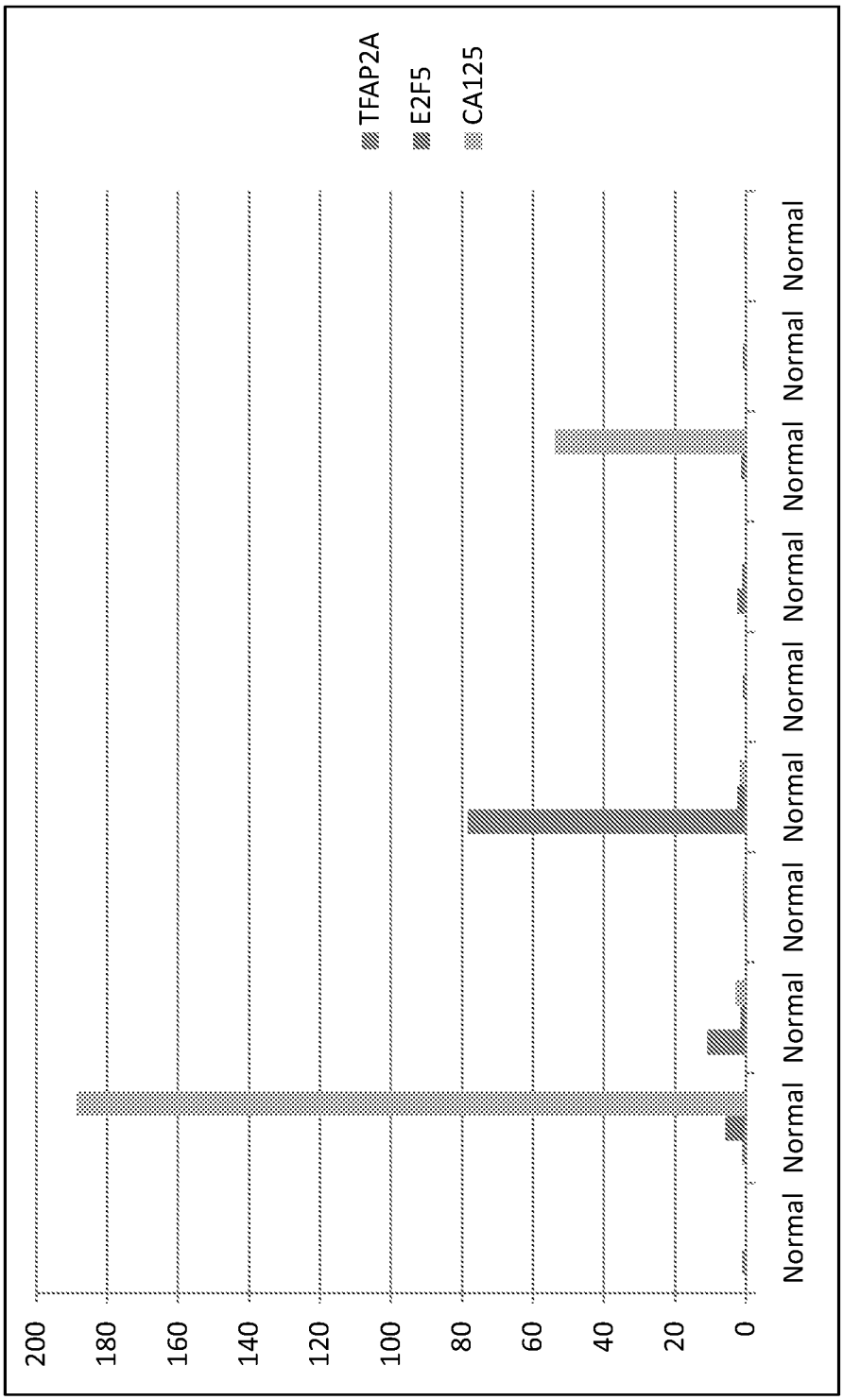

TFAP2A
E2F5
CA125
200
180
160
140
120
100
80
60
40
20
0
Normal Normal Normal Normal Normal Normal Normal Normal Normal Normal

FIG. 2

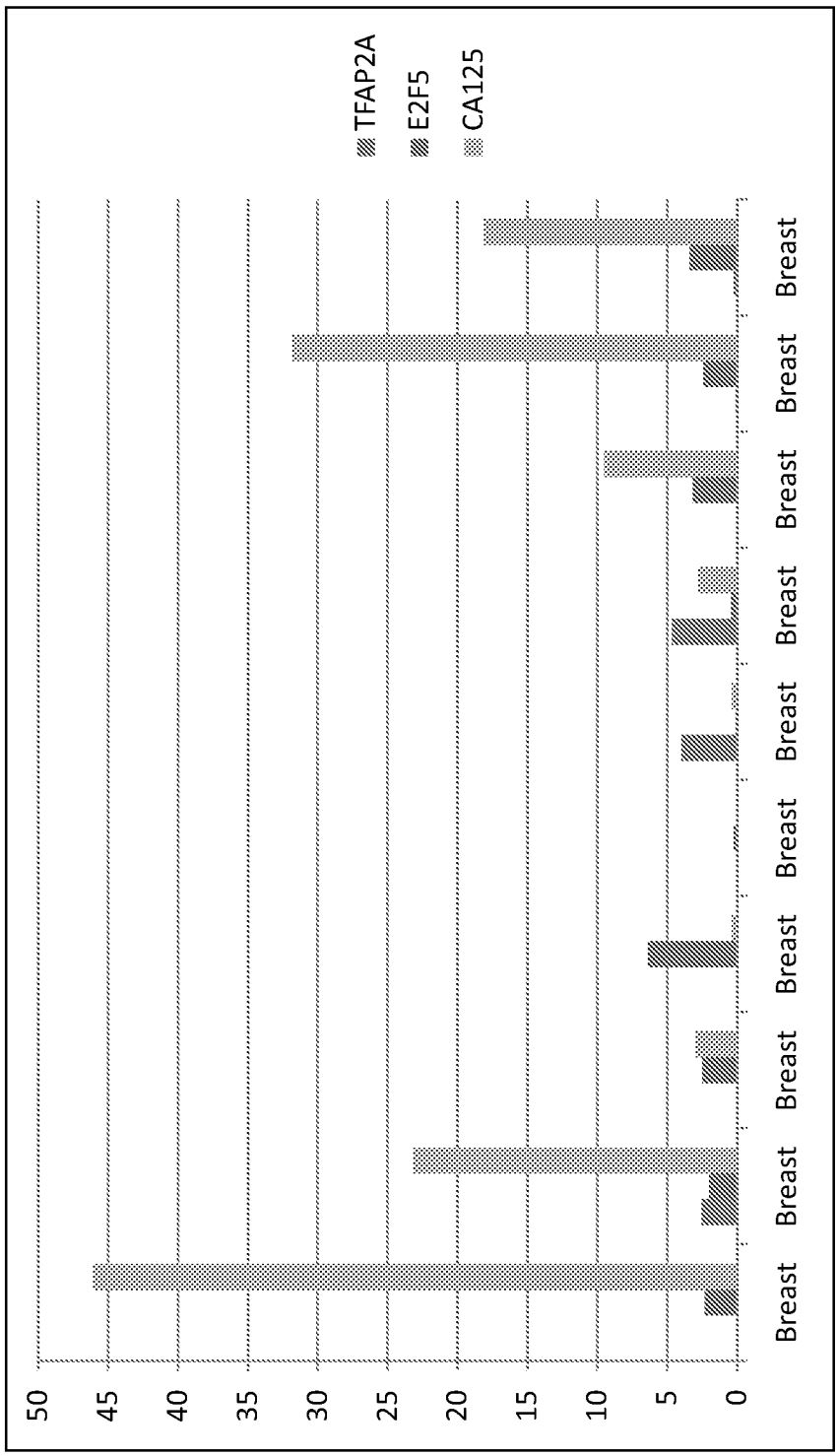

TFAP2A
E2F5
CA125
50
45
40
35
30
25
20
15
10
5
0
Breast
Breast
Breast
Breast
Breast
Breast
Breast
Breast
Breast
Breast
Breast

FIG. 3

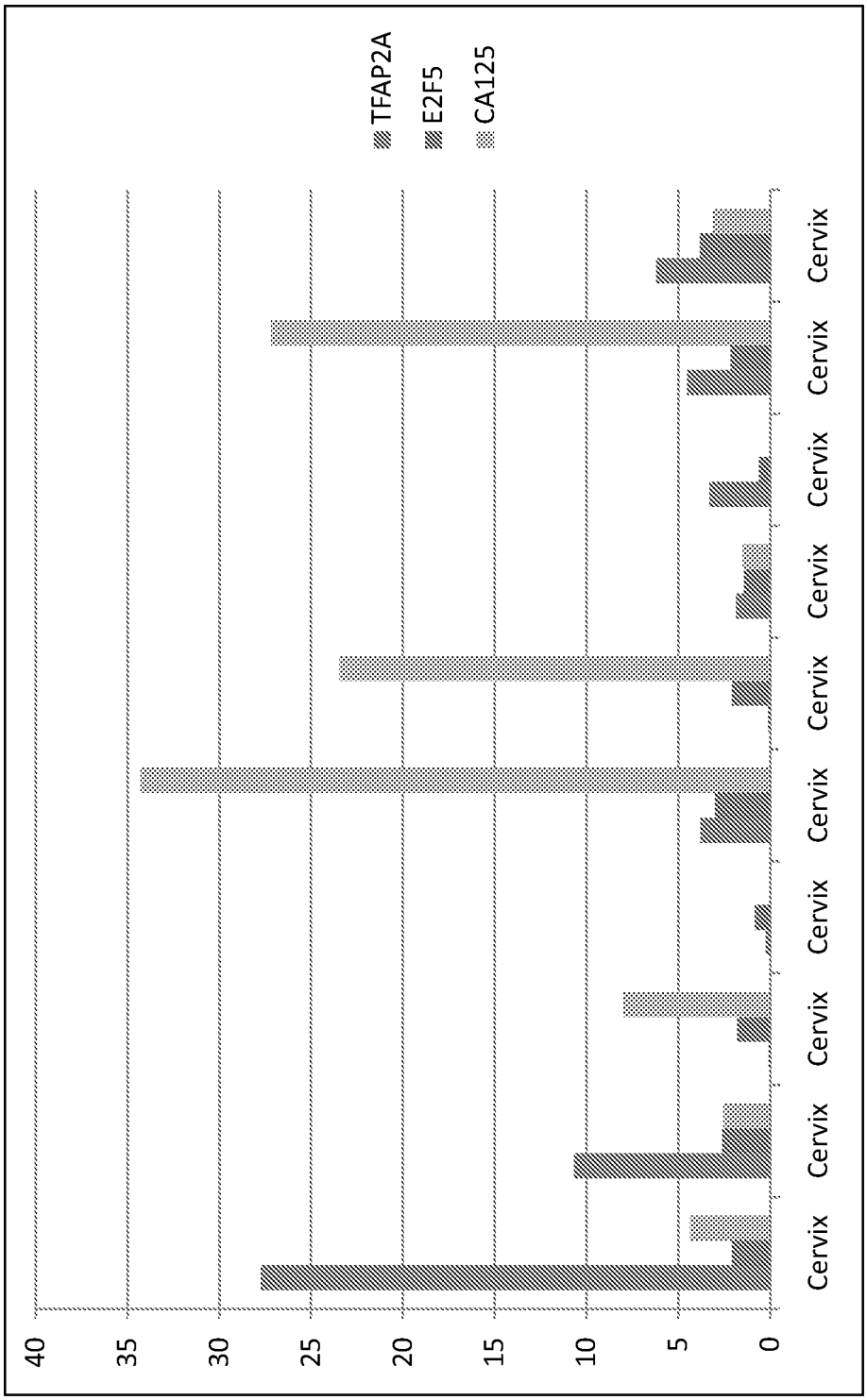

TFAP2A
E2F5
CA125
40
35
30
25
20
15
10
5
0
Cervix
Cervix
Cervix
Cervix
Cervix
Cervix
Cervix
Cervix
Cervix
Cervix
Cervix
Cervix
Cervix
Cervix

FIG. 4

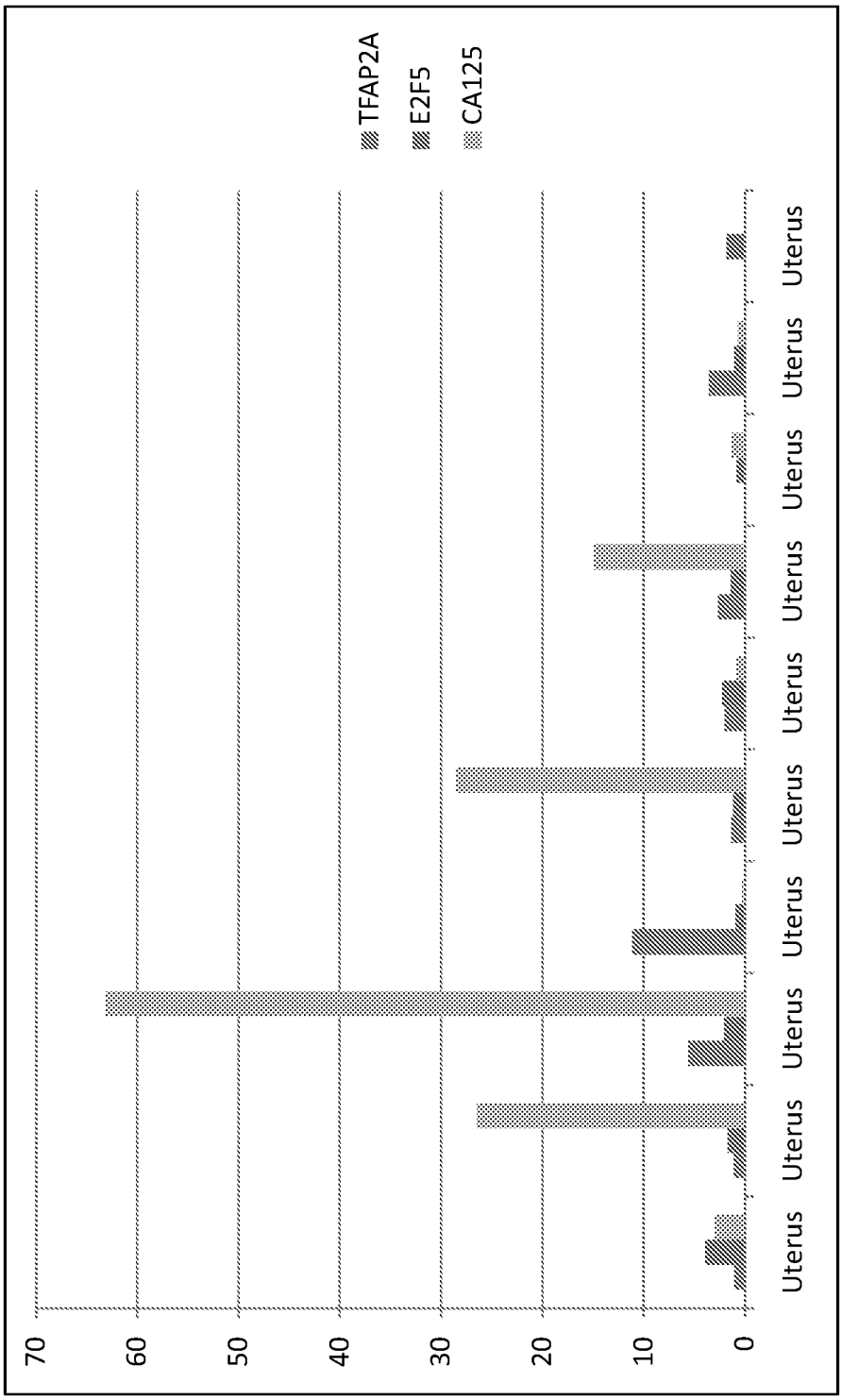

TFAP2A
E2F5
CA125
70
60
50
40
30
20
10
0
Uterus
Uterus
Uterus
Uterus
Uterus
Uterus
Uterus
Uterus
Uterus
Uterus
Uterus
Uterus

FIG. 5