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- (54) **METHODS AND COMPOSITIONS FOR THE DIAGNOSIS OF OVARIAN CANCER**
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- CPC ...

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(57) **ABSTRACT**

A diagnostic reagent or device comprises at least one ligand capable of specifically complexing with, binding to, or quantitatively detecting or identifying the biomarker chloride intracellular channel protein 4 (CLIC4) or an isoform, pro-form, modified molecular form including posttranslational modification, or unique peptide fragment or nucleic acid fragment thereof. An alternative diagnostic reagent or device comprises ligand or ligands capable of specifically complexing with, binding to, or quantitatively detecting or identifying multiple tropomyosin biomarkers. Optionally, such reagent or device includes a signaling molecule and/or a substrate on which the ligand is immobilized. Other reagents and methods of diagnosing ovarian cancer include use of CLIC4 ligands and/or multiple tropomyosin ligands with an additional ovarian cancer biomarker. For example, CLIC4 combined with one or more of CLIC1 and/or one or multiple members of the tropomyosin family, e.g., TPM1, TPM2, TPM3 or TPM4, and further optionally including CTSD-30 kDa and/or PRDX-6, among other ovarian cancer biomarkers can form a characteristic diagnostic pattern or profile of expression that is diagnostic of the disease. Still other embodiments are described.

Specification includes a Sequence Listing.

FIG. 1A



FIG. 1B

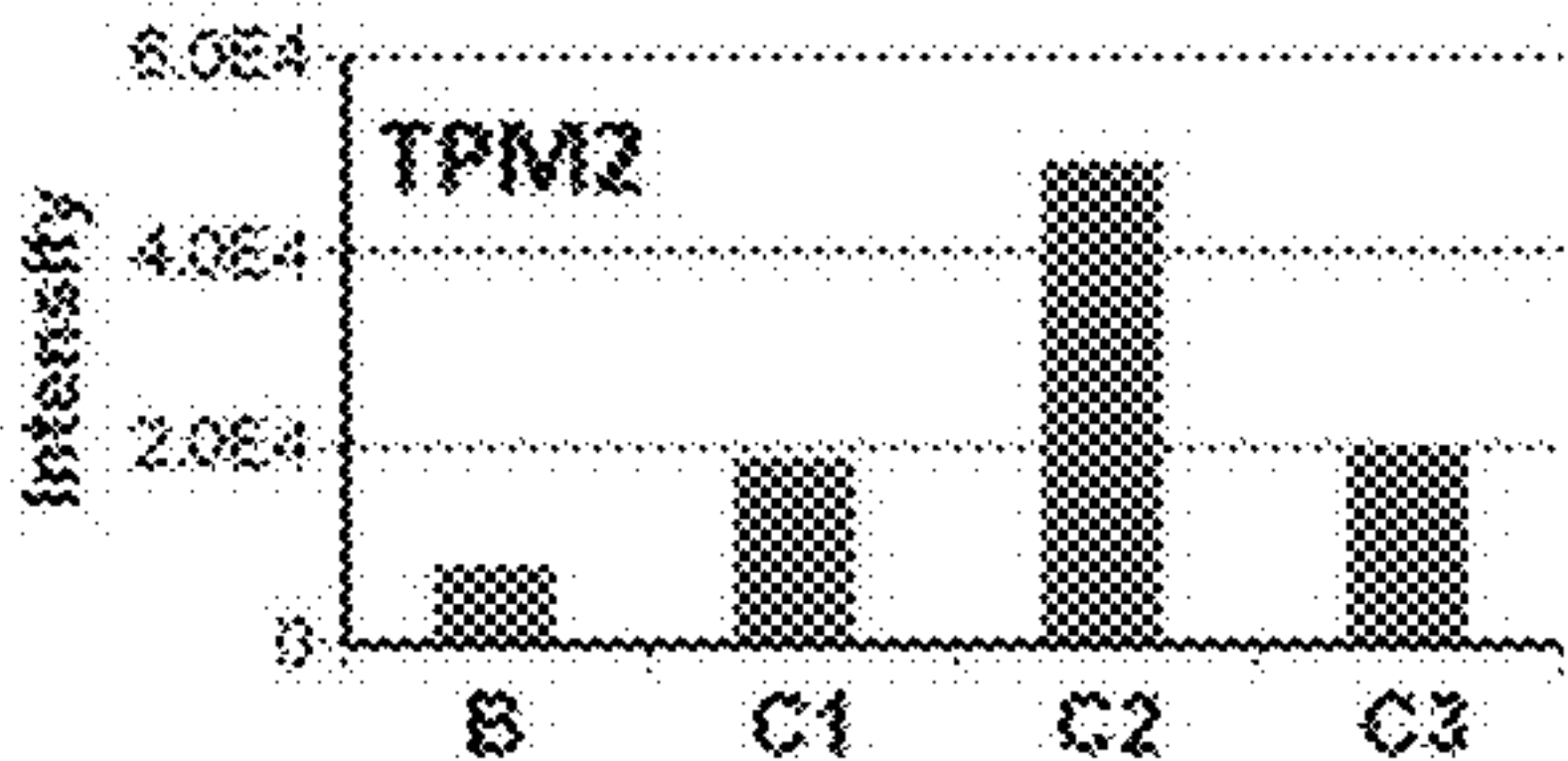


FIG. 1C

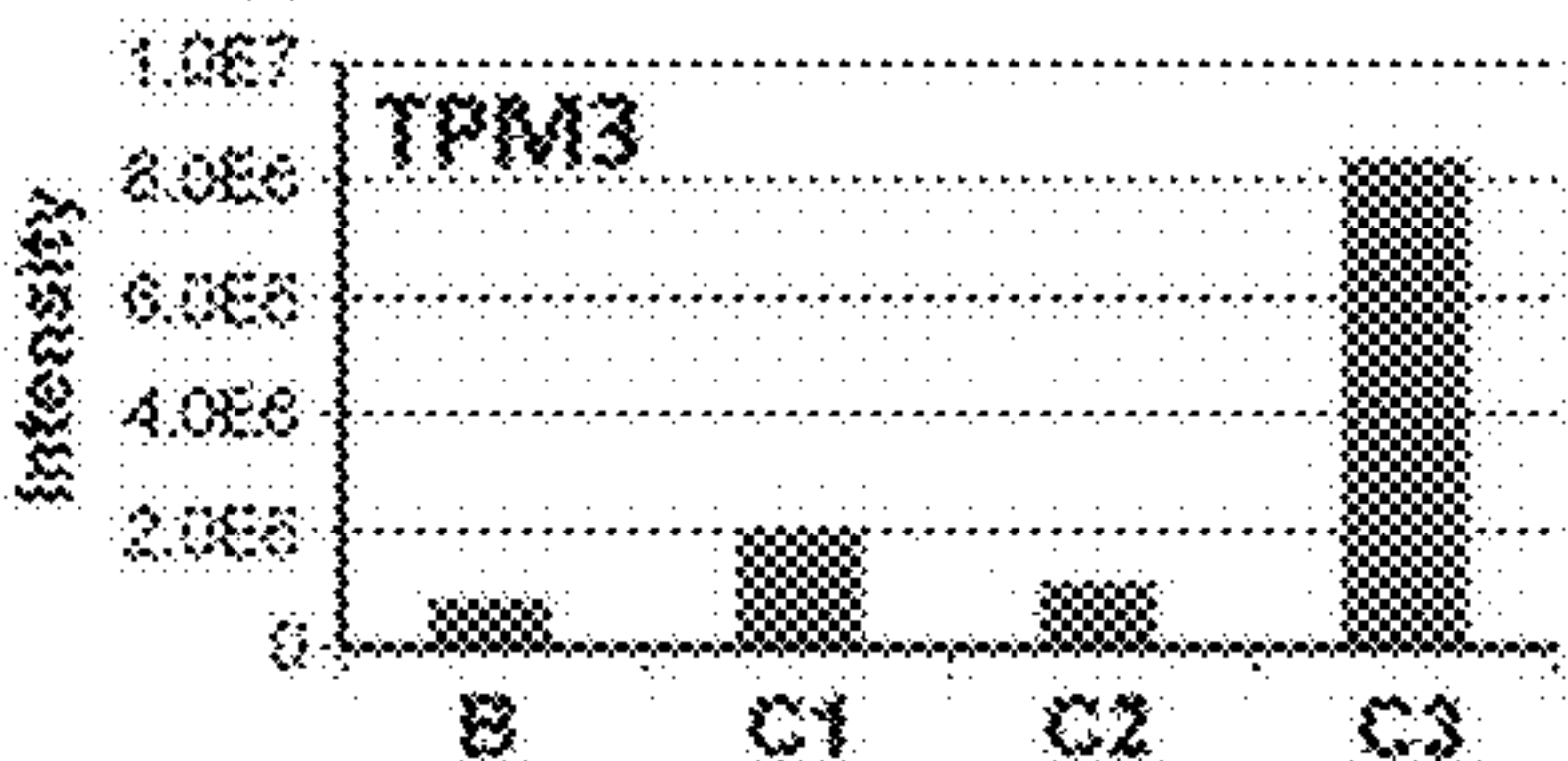


FIG. 1D

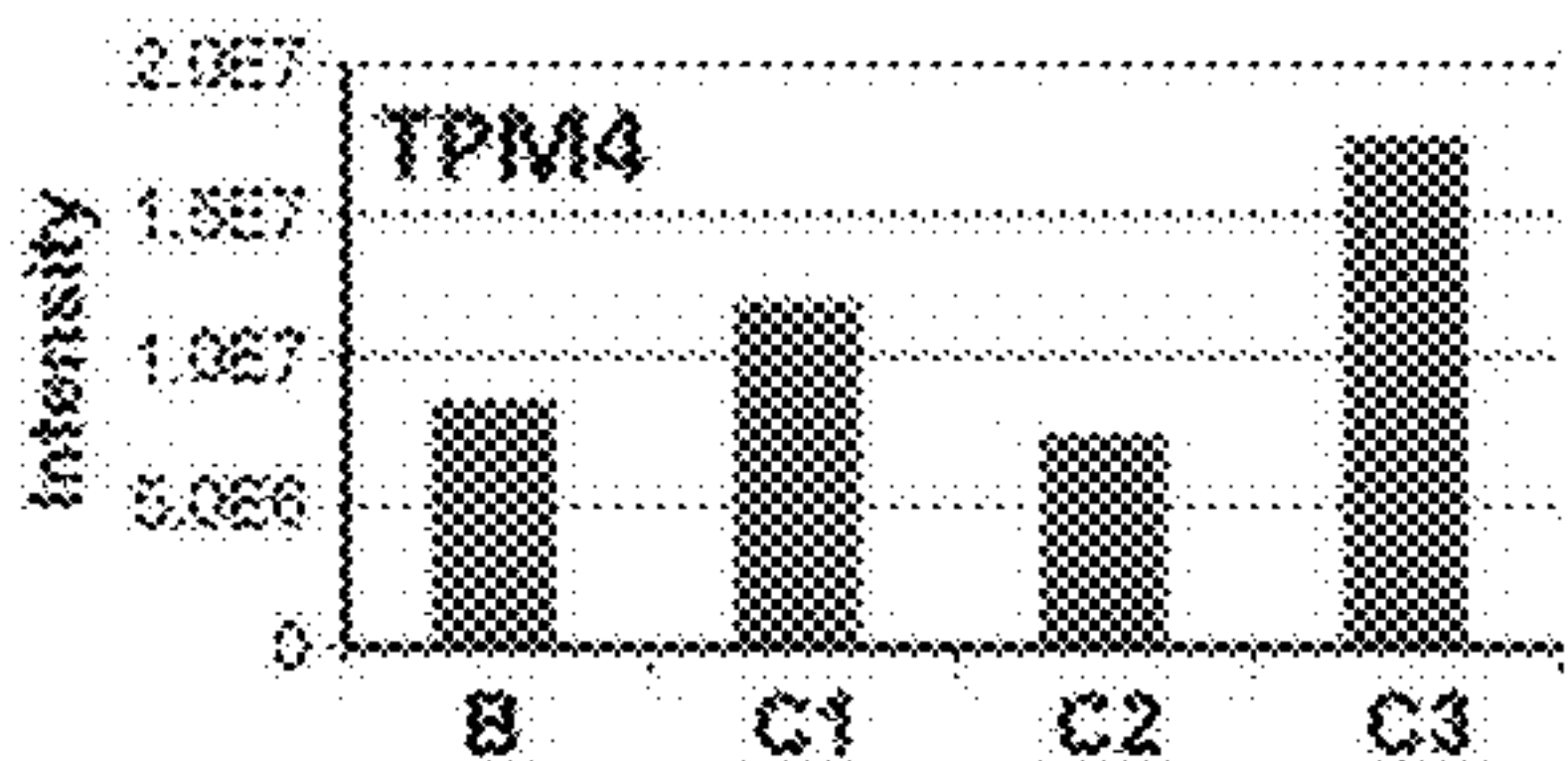


FIG. 1E

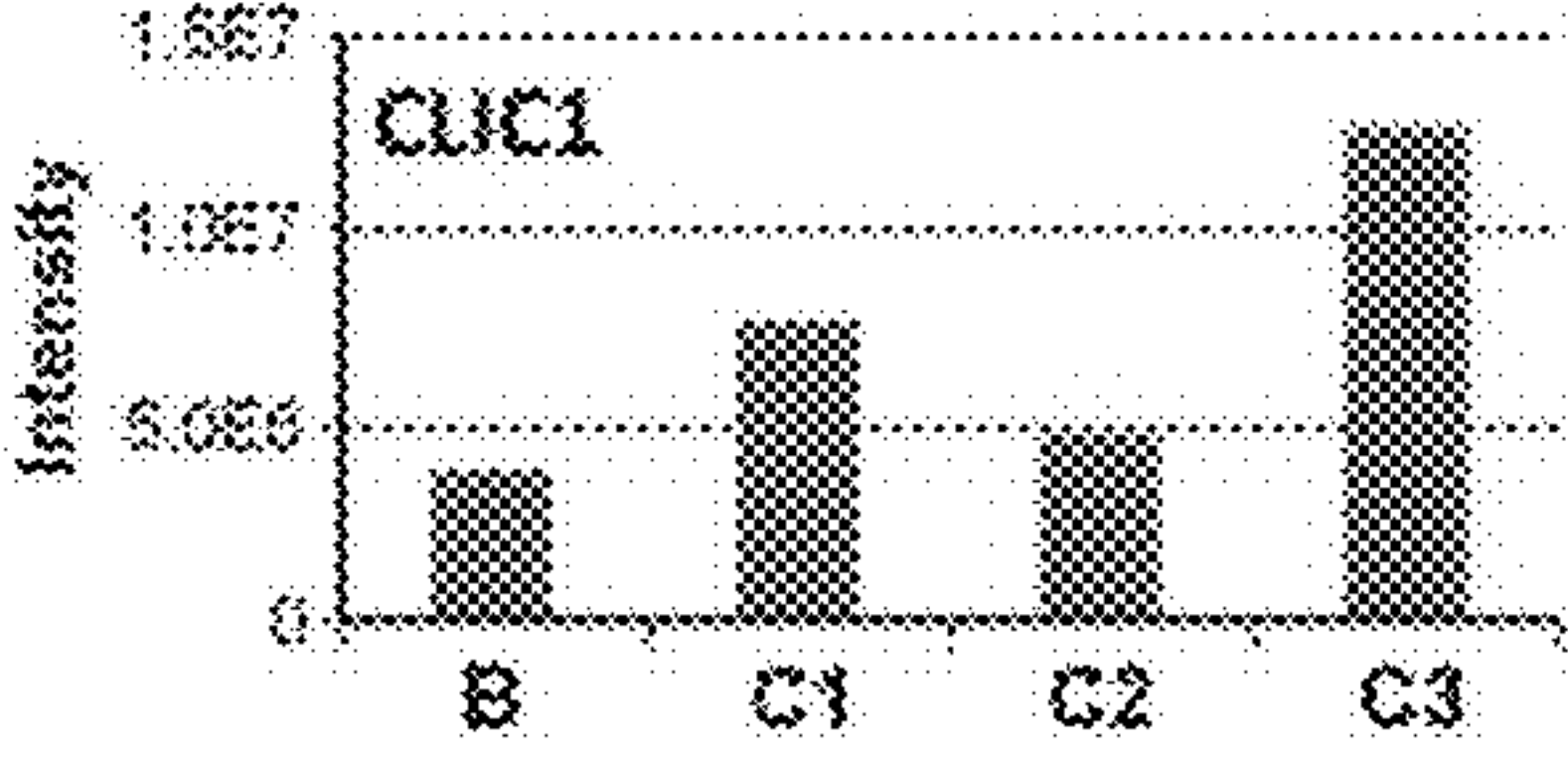


FIG. 1F

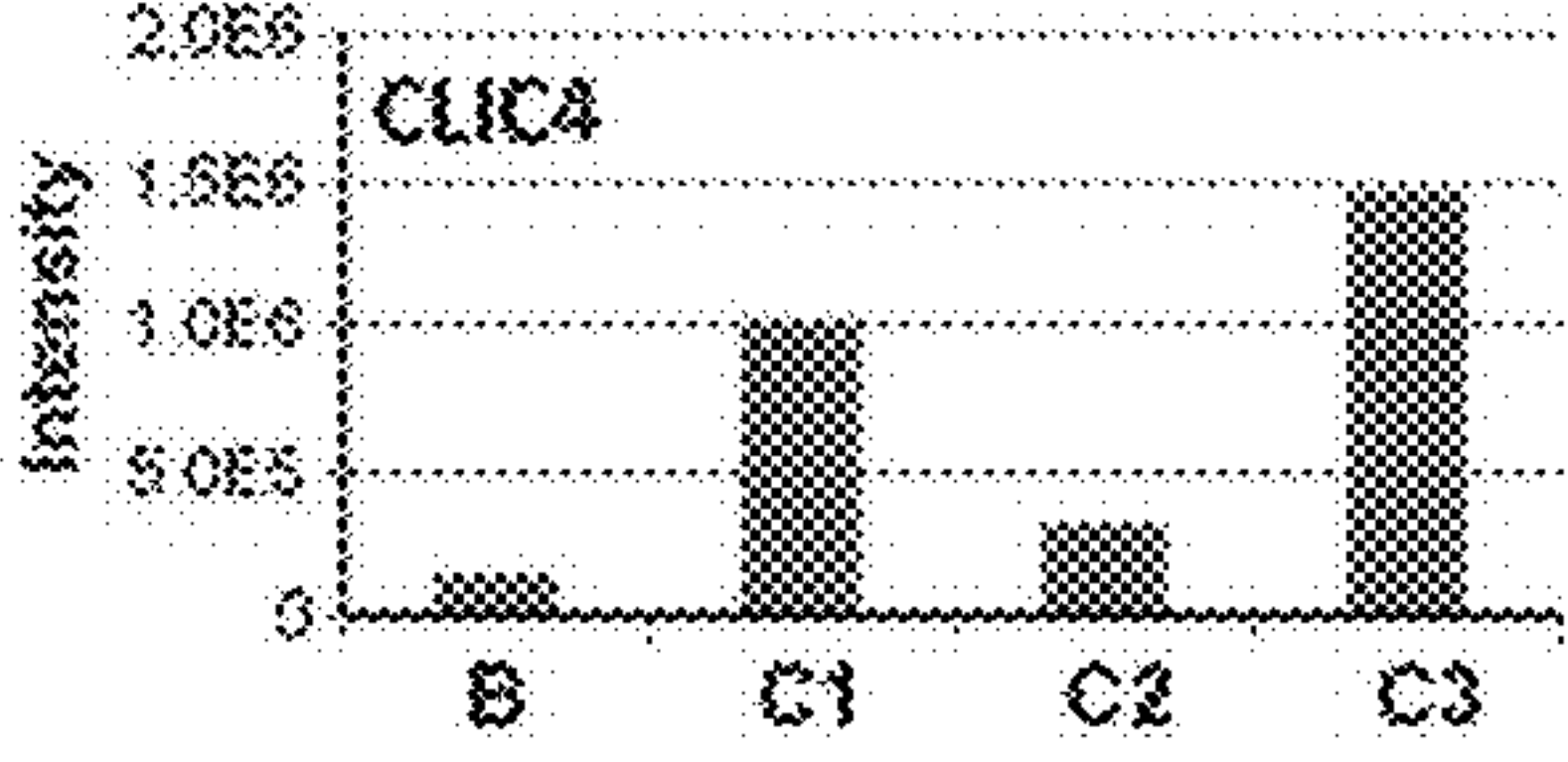


FIG. 2A

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SP|Q9Y696|CLIC4_HUMAN  MALSMPLNGLKEEDKEFLIELEVAGSDGSGSIGNCPFSQRLFMILWLKGVVFESVTTVDLE  60
SP|Q9QYB1|CLIC4_MOUSE  MALSMPLNGLKEEDKEFLIELEVAGSDGSGSIGNCPFSQRLFMILWLKGVVFESVTTVDLE  60
*****

SP|Q9Y696|CLIC4_HUMAN  EEPEDLQHLAPGTHPEFTTENSEVETDVNTEEFLEEVLCPPEYLELSEKSPESHTAGMD  120
SP|Q9QYB1|CLIC4_MOUSE  EEPADLQHLAPGTHPEFTTENSEVETDVNTEEFLEEVLCPPEYLELSEKSPESHTAGMD  120
*****

SP|Q9Y696|CLIC4_HUMAN  IFAKFSAYIKNSPEANEALERGLLXTLOKLDVYLNRPIDPEIDENGMKIKFSTKFLD  180
SP|Q9QYB1|CLIC4_MOUSE  IFAKFSAYIKNSPEANEALERGLLXTLOKLDVYLNRPIDPEIDENGMKIKFSTKFLD  180
*****

SP|Q9Y696|CLIC4_HUMAN  GNENTLADCNILFELHIVGVVAKNYNEDIPKEMTGIVNLYLTNAYSSEDEFNTNCPSEKVEY  240
SP|Q9QYB1|CLIC4_MOUSE  GDEMTLADCNILFELHIVGVVAKNYNEDIPKEMTGIVNLYLTNAYSSEDEFNTNCPSEKVEY  240
*****

SP|Q9Y696|CLIC4_HUMAN  EIAYSQVAKRLTK  253
SP|Q9QYB1|CLIC4_MOUSE  EIAYSQVAKRLTK  253
*****

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FIG. 2B

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SP|Q00299|CLIC1  -----N-AEEQPCQVELEVAGSDGAGISGSGSGSLFMVYLNKGVVFESVTTVDLE  48
SP|Q15247|CLIC2  -----MSGLPQGTQVDPEIELEVAGSDGSGSIGNCPFCQRLFMILWLKGVVFESVTTVDLE  54
SP|Q95833|CLIC3  -----MAETELQLEVAGSDGSGSYGRCFPCQRLFMVLLKGVVDFLTITVDL  48
SP|Q9Y696|CLIC4  MALSMPLNGLKE-EEFLIELEVAGSDGSGSIGNCPFSQRLFMILWLKGVVFESVTTVDLE  59
*****

SP|Q00299|CLIC1  EETETVONLCPSGGLPFLLYGTETVNTDTNIEEFLEAVLCPEYFEEALNPEHTAGMD  108
SP|Q15247|CLIC2  TENPEELDLAPGTNPEFLVYNKELTDFIEIEEFLEQTLAPPYFHLSPYKESFQVGC  114
SP|Q95833|CLIC3  RESPDVLKDFAPSGGLPILLYDSDAETDTLQIEDFLEETLGPDPFSLAPSYKESHTAGN  106
SP|Q9Y696|CLIC4  EEPADLQHLAPGTHPEFTTENSEVETDVNTEEFLEEVLCPPEYLELSEKSPESHTAGMD  119
*****

SP|Q00299|CLIC1  EIAKFSAYIKNSPEANEALERGLLXTLOKLDVYLNRPIDPEIDENGMKIKFSTKFLD  168
SP|Q15247|CLIC2  NLFKFSAYIKNSPEANEALERGLLXTLOKLDVYLNRPIDPEIDENGMKIKFSTKFLD  174
SP|Q95833|CLIC3  DVFNKFSAYIKNSPEANEALERGLLXTLOKLDVYLNRPIDPEIDENGMKIKFSTKFLD  164
SP|Q9Y696|CLIC4  EIAKFSAYIKNSPEANEALERGLLXTLOKLDVYLNRPIDPEIDENGMKIKFSTKFLD  179
*****

SP|Q00299|CLIC1  DEENTLADCNILFELHIVGVVAKNYNEDIPKEMTGIVNLYLTNAYSSEDEFNTNCPSEKVEY  228
SP|Q15247|CLIC2  DGDQLTLADCSILFELHIVGVVAKNYNEDIPKEMTGIVNLYLTNAYSSEDEFNTNCPSEKVEY  234
SP|Q95833|CLIC3  DGDQLTLADCSILFELHIVGVVAKNYNEDIPKEMTGIVNLYLTNAYSSEDEFNTNCPSEKVEY  224
SP|Q9Y696|CLIC4  DEENTLADCNILFELHIVGVVAKNYNEDIPKEMTGIVNLYLTNAYSSEDEFNTNCPSEKVEY  232
*****

SP|Q00299|CLIC1  EELAYEQVAKRLTK-  241
SP|Q15247|CLIC2  IENTYANVAKQNS-  247
SP|Q95833|CLIC3  ILAAYRPAVHSP-  236
SP|Q9Y696|CLIC4  EIAYSQVAKRLTK  253
*****

```



```
SP:P09493-5|isoform 5 -----MA 2
TR:D9Y2V7|isoform 6 -----MA 2
TR:Q59GR8|variant GGRDFRTAPGRGGRRTTEREGRGGPFALGSGDSRGSEVRRAAAGLSHCSEPPASLPAGAMA 60
TR:B7Z722|isoform CRA_1 -----MA 2
TR:Q1ZYLS|variant 6 -----MA 2
TR:B7Z596|cDNA FLJ55130 -----MA 2
**

SP:P09493-5|isoform 5 GSSSLEAVERKINSLSQEQADAAEEAGTLQRELDNERELMETAEADVASLNHNIQLVEEE 62
TR:D9Y2V7|isoform 6 GSSSLEAVERKINSLSQEQADAAEEAGTLQRELDNERELMETAEADVASLNHNIQLVEEE 62
TR:Q59GR8|variant GSSSLEAVERKINSLSQEQADAAEEAGTLQRELDNERELMETAEADVASLNHNIQLVEEE 120
TR:B7Z722|isoform CRA_1 GSSSLEAVERKINSLSQEQADAAEEAGTLQRELDNERELMETAEADVASLNHNIQLVEEE 62
TR:Q1ZYLS|variant 6 GSSSLEAVERKINSLSQEQADAAEEAGTLQRELDNERELMETAEADVASLNHNIQLVEEE 62
TR:B7Z596|cDNA FLJ55130 GSSSLEAVERKINSLSQEQADAAEEAGTLQRELDNERELMETAEADVASLNHNIQLVEEE 62
*****

SP:P09493-5|isoform 5 LDNAQERLATALQNLAAEKAADSESRGMKVIESRAQKDEEKMEIQEIQLEAKNHIAEDA 122
TR:D9Y2V7|isoform 6 LDNAQERLATALQNLAAEKAADSESRGMKVIESRAQKDEEKMEIQEIQLEAKNHIAEDA 122
TR:Q59GR8|variant LDNAQERLATALQNLAAEKAADSESRGMKVIESRAQKDEEKMEIQEIQLEAKNHIAEDA 180
TR:B7Z722|isoform CRA_1 LDNAQERLATALQNLAAEKAADSESRGMKVIESRAQKDEEKMEIQEIQLEAKNHIAEDA 122
TR:Q1ZYLS|variant 6 LDNAQERLATALQNLAAEKAADSESRGMKVIESRAQKDEEKMEIQEIQLEAKNHIAEDA 122
TR:B7Z596|cDNA FLJ55130 LDNAQERLATALQNLAAEKAADSESRGMKVIESRAQKDEEKMEIQEIQLEAKNHIAEDA 122
*****

SP:P09493-5|isoform 5 DRKYEEVAKMLVIESDLENAEERAELESEGECALKEEELKTVTNNHLSLEAQAEKYSQKE 182
TR:D9Y2V7|isoform 6 DRKYEEVAKMLVIESDLENAEERAELESEGECALKEEELKTVTNNHLSLEAQAEKYSQKE 182
TR:Q59GR8|variant DRKYEEVAKMLVIESDLENAEERAELESEGECALKEEELKTVTNNHLSLEAQAEKYSQKE 240
TR:B7Z722|isoform CRA_1 DRKYEEVAKMLVIESDLENAEERAELESEGECALKEEELKTVTNNHLSLEAQAEKYSQKE 182
TR:Q1ZYLS|variant 6 DRKYEEVAKMLVIESDLENAEERAELESEGECALKEEELKTVTNNHLSLEAQAEKYSQKE 182
TR:B7Z596|cDNA FLJ55130 DRKYEEVAKMLVIESDLENAEERAELESEGECALKEEELKTVTNNHLSLEAQAEKYSQKE 182
*****

SP:P09493-5|isoform 5 DRKYEEVAKMLVIESDLENAEERAELESEGECALKEEELKTVTNNHLSLEAQAEKYSQKE 241
TR:D9Y2V7|isoform 6 DRKYEEVAKMLVIESDLENAEERAELESEGECALKEEELKTVTNNHLSLEAQAEKYSQKE 241
TR:Q59GR8|variant DRKYEEVAKMLVIESDLENAEERAELESEGECALKEEELKTVTNNHLSLEAQAEKYSQKE 299
TR:B7Z722|isoform CRA_1 DRKYEEVAKMLVIESDLENAEERAELESEGECALKEEELKTVTNNHLSLEAQAEKYSQKE 241
TR:Q1ZYLS|variant 6 DRKYEEVAKMLVIESDLENAEERAELESEGECALKEEELKTVTNNHLSLEAQAEKYSQKE 241
TR:B7Z596|cDNA FLJ55130 DRKYEEVAKMLVIESDLENAEERAELESEGECALKEEELKTVTNNHLSLEAQAEKYSQKE 242
*****

SP:P09493-5|isoform 5 LNED----- 245
TR:D9Y2V7|isoform 6 LNED----- 245
TR:Q59GR8|variant LNED----- 303
TR:B7Z722|isoform CRA_1 LLKLHNM----- 248
TR:Q1ZYLS|variant 6 LNED----- 245
TR:B7Z596|cDNA FLJ55130 IFQFGKIXIDTILCTCTFFLCVWGFSLWLLNS 275
: :
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Figure 3

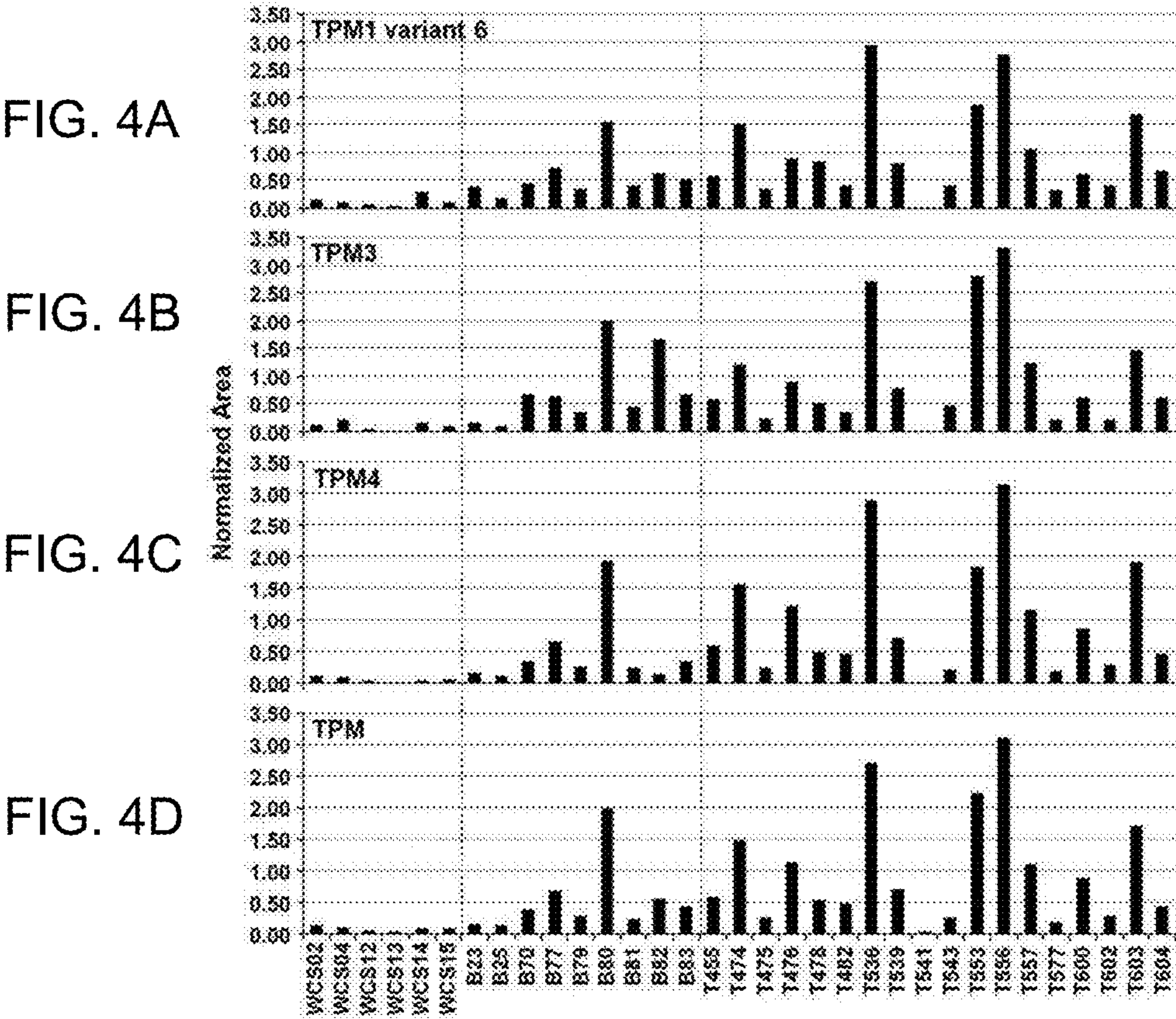


FIG. 5A

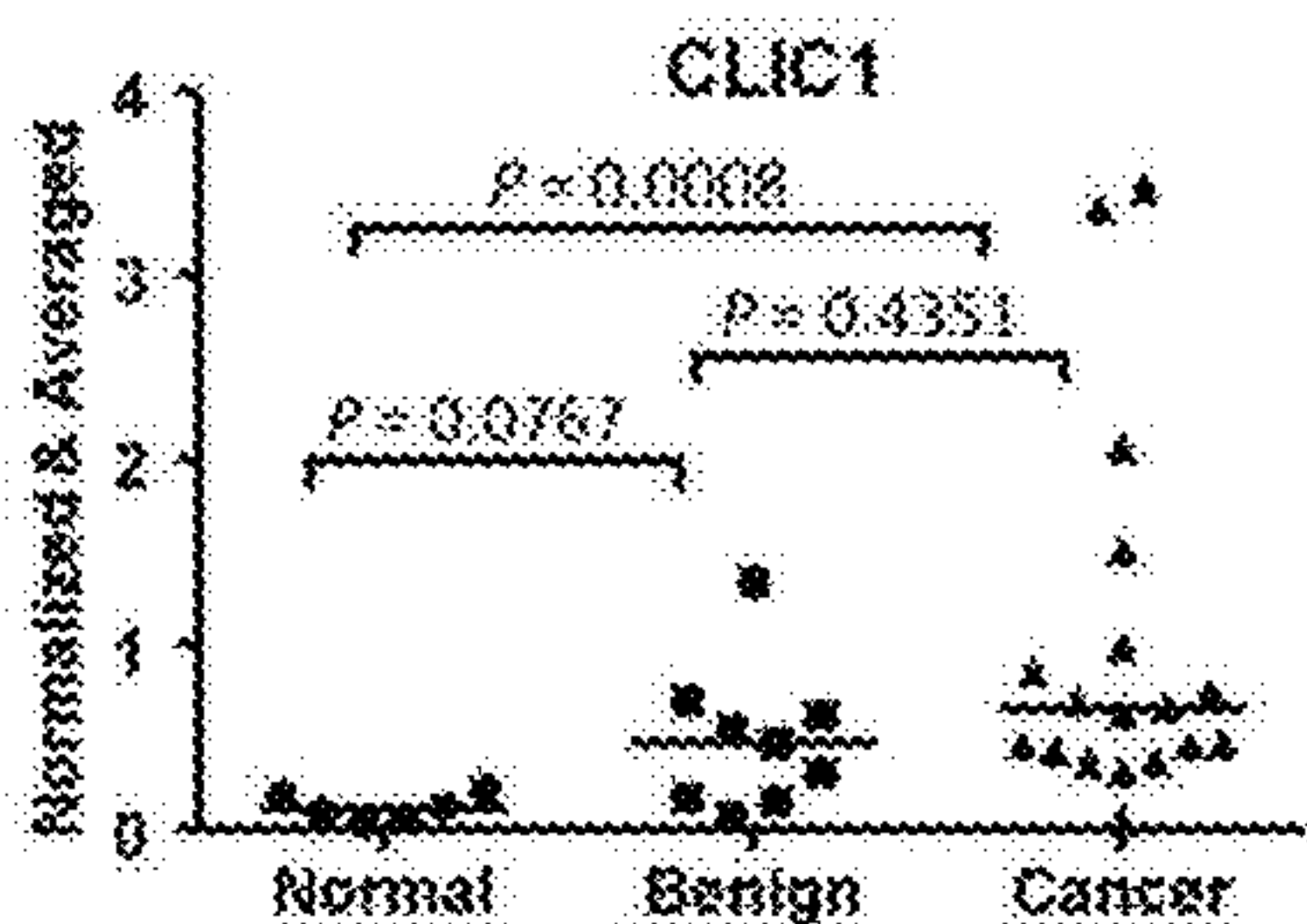


FIG. 5B

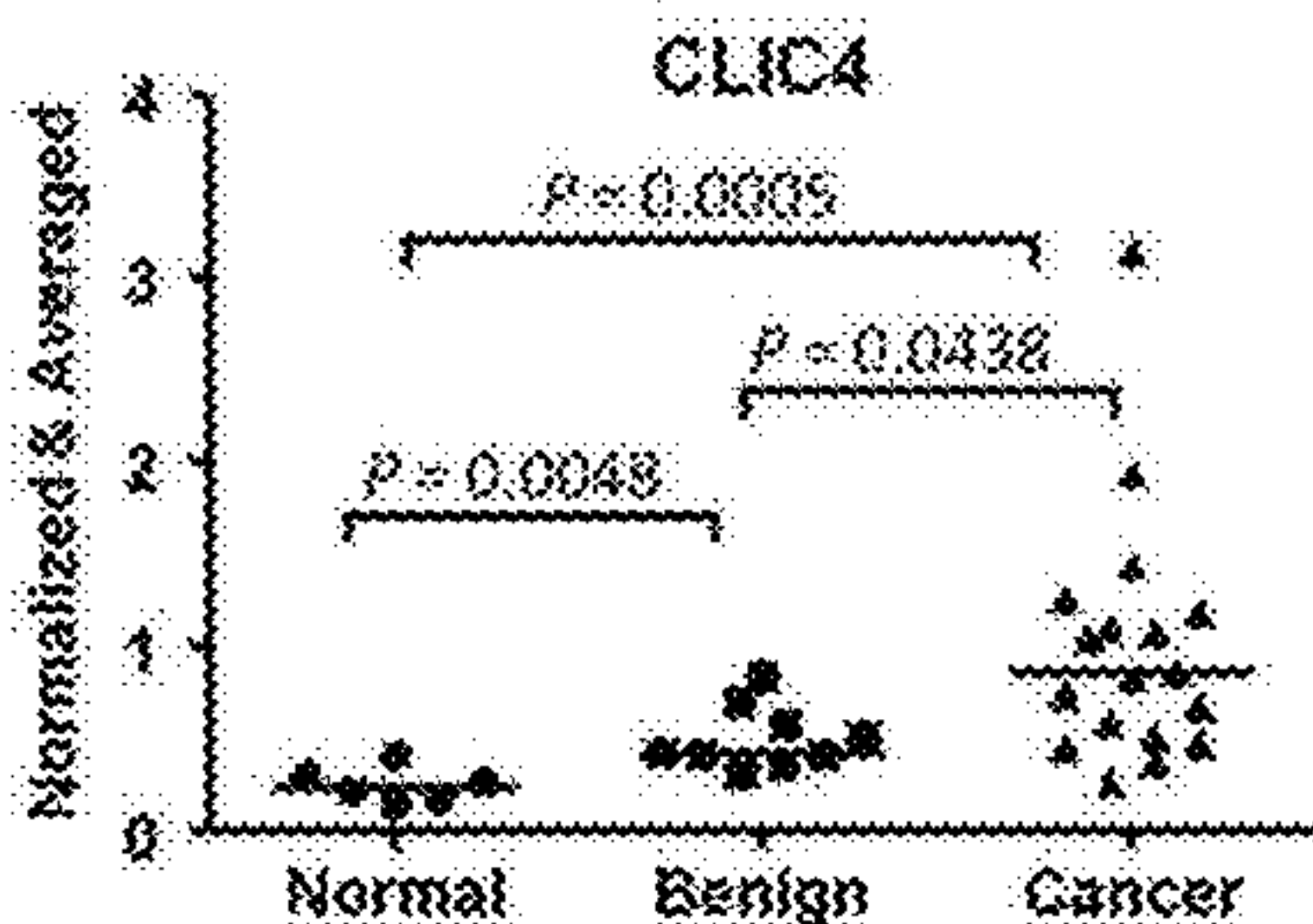


FIG. 5C

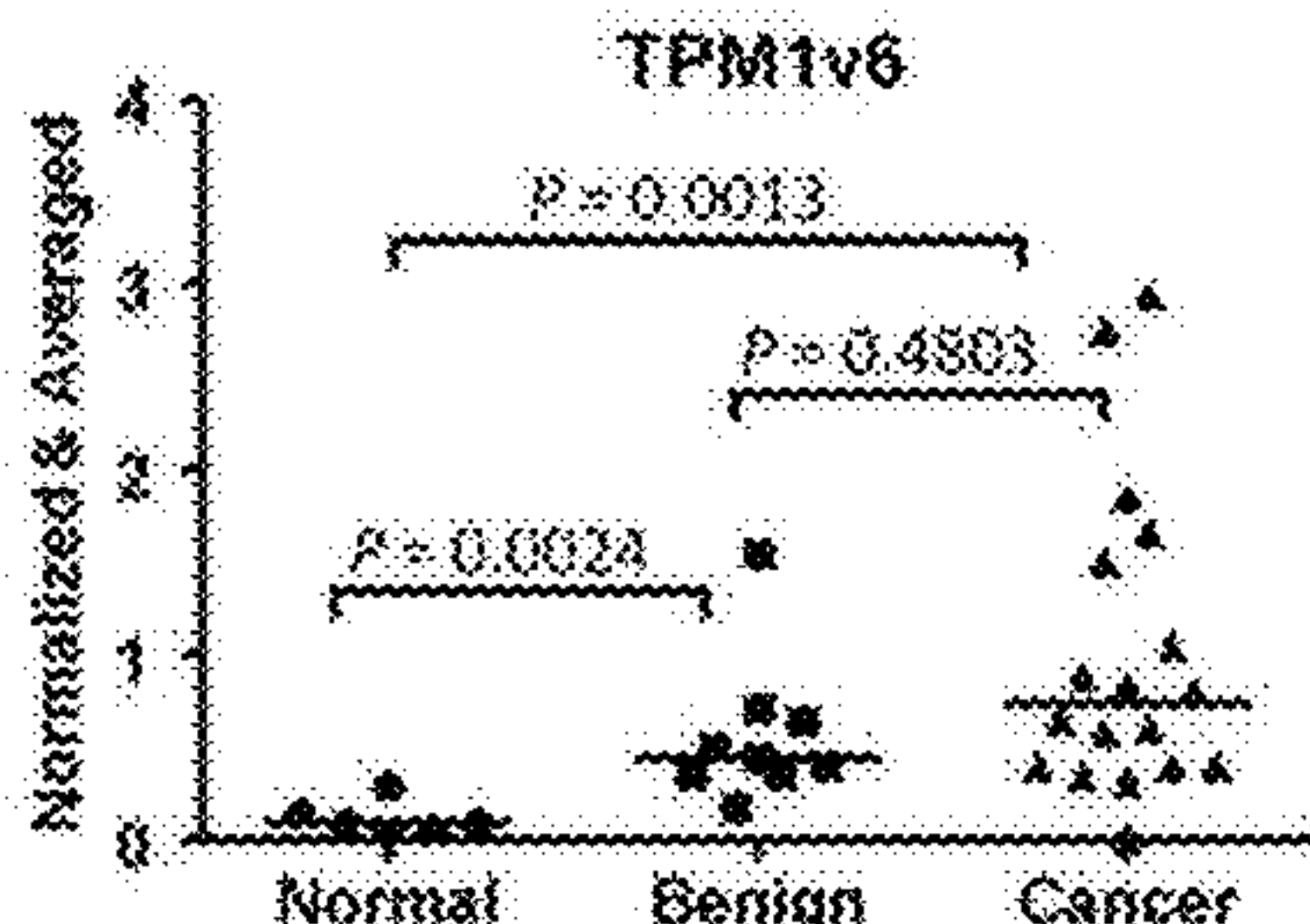


FIG. 5D

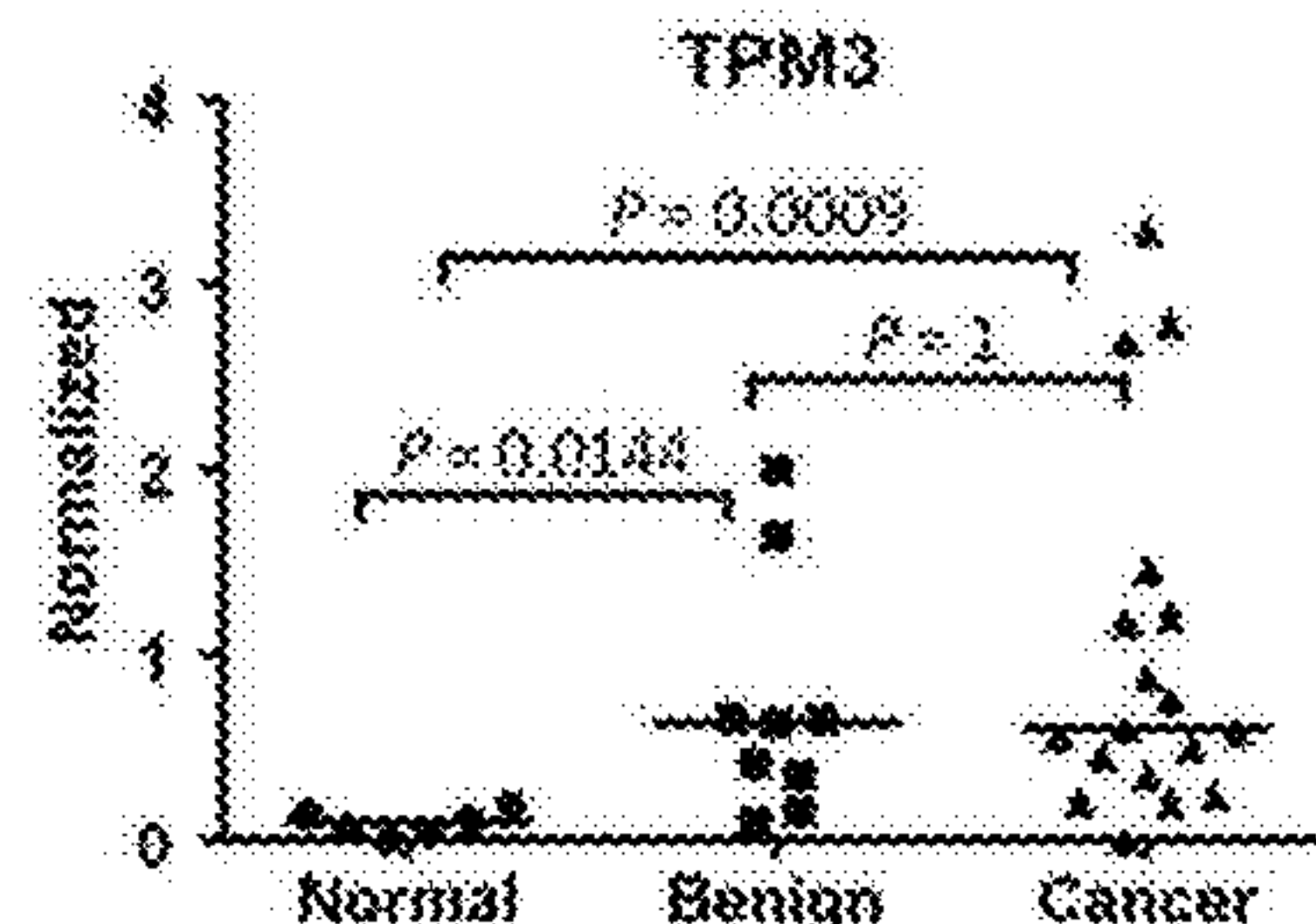


FIG. 5E

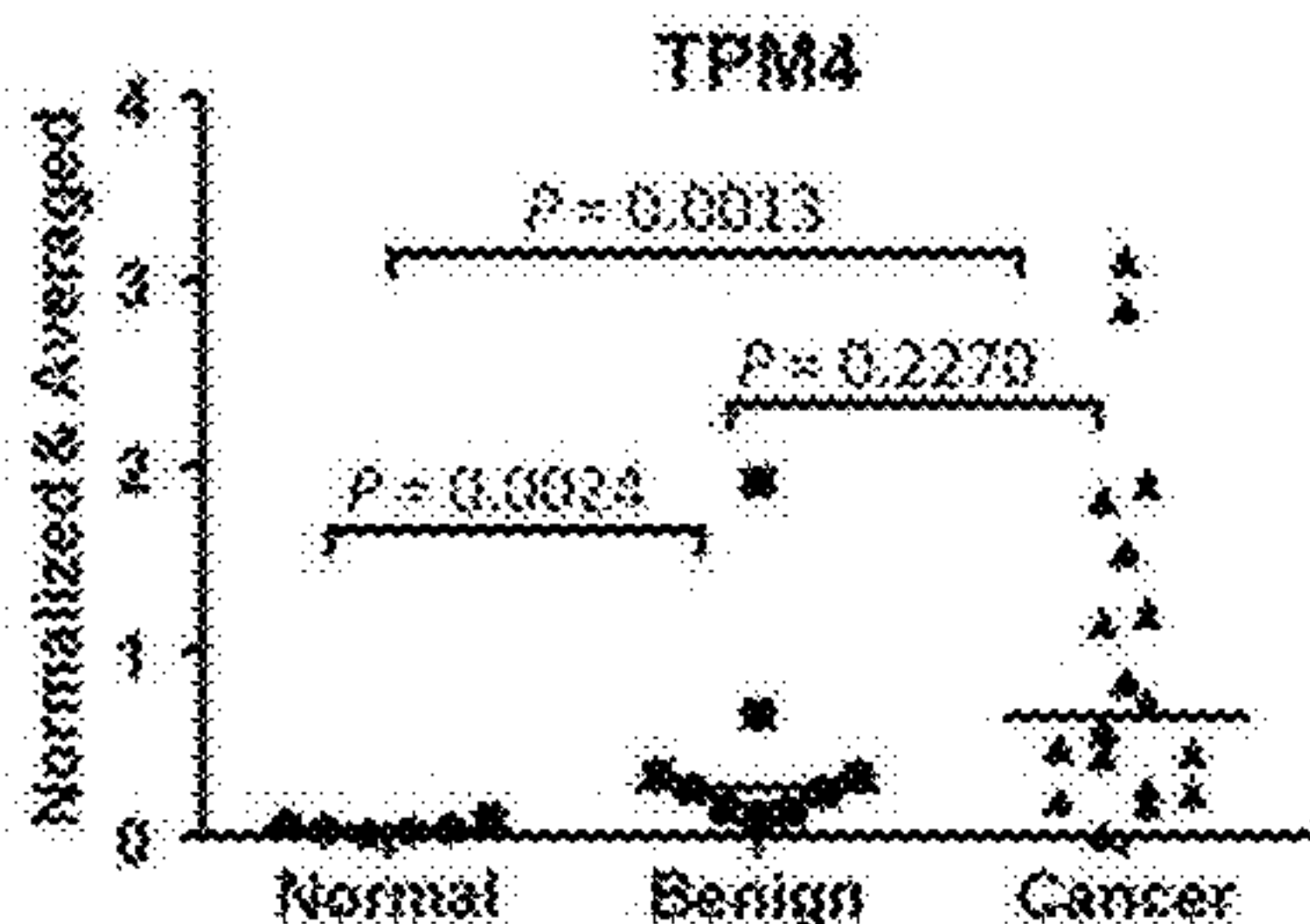


FIG. 5F

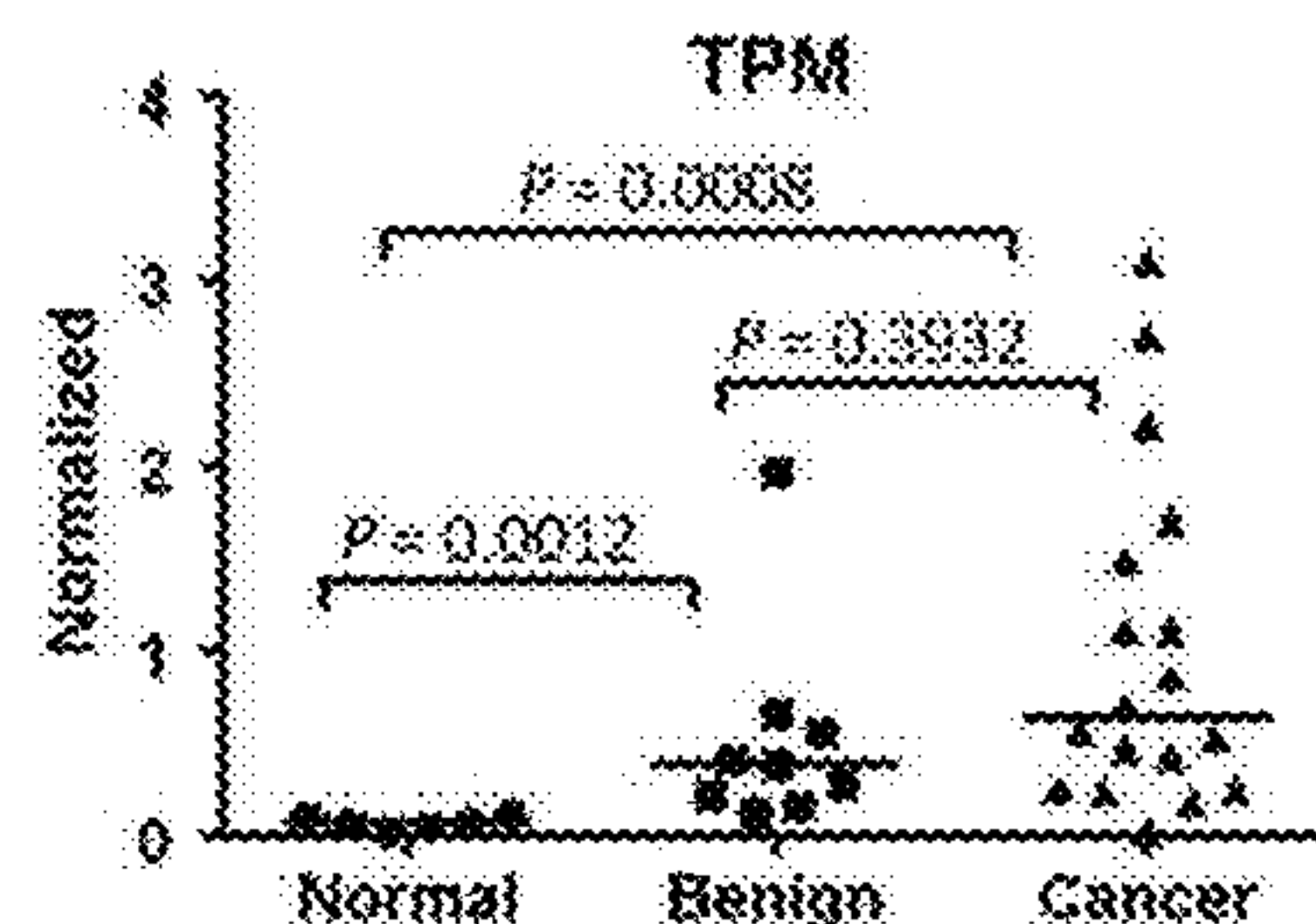


FIG. 6A

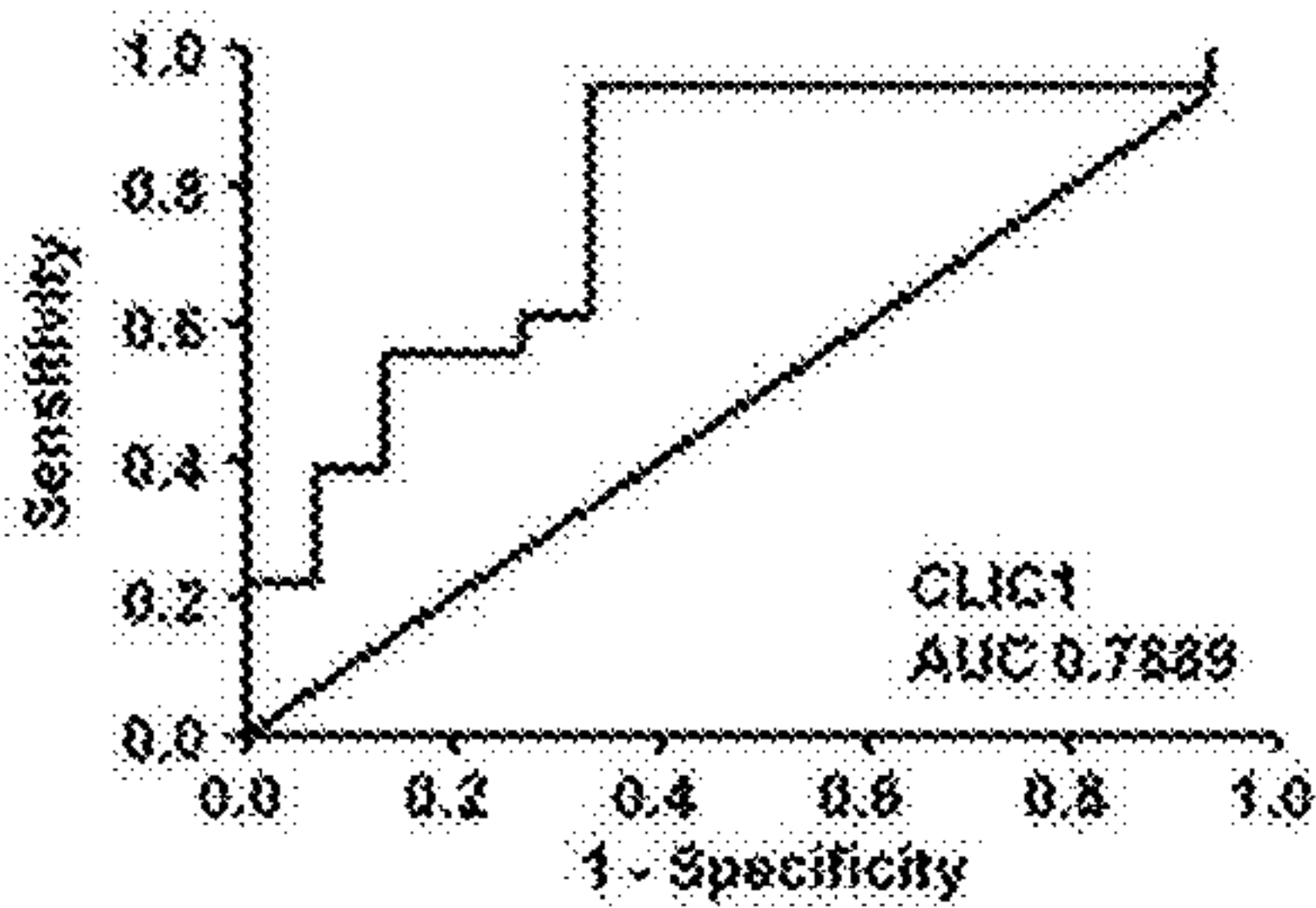


FIG. 6B

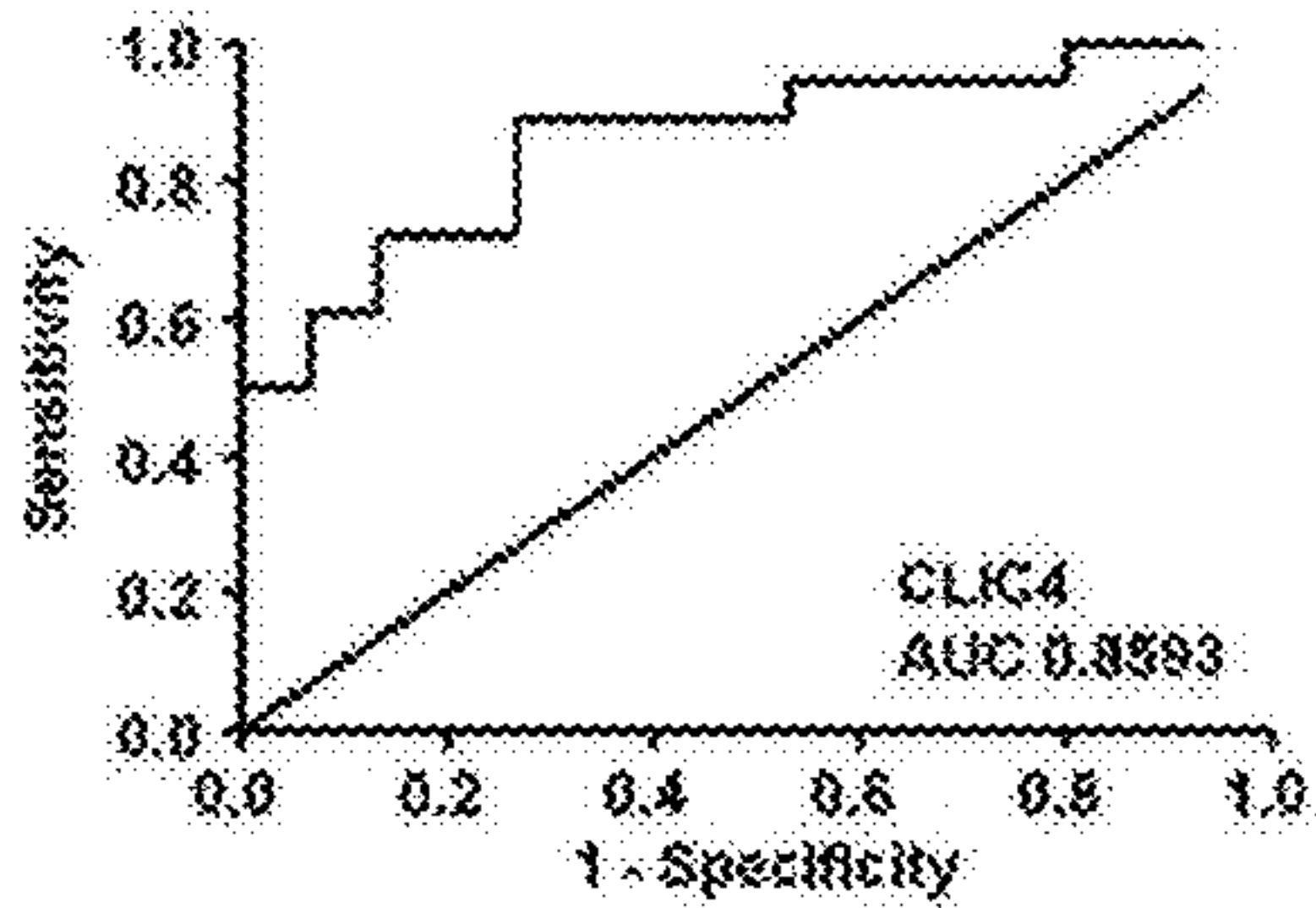


FIG. 6C

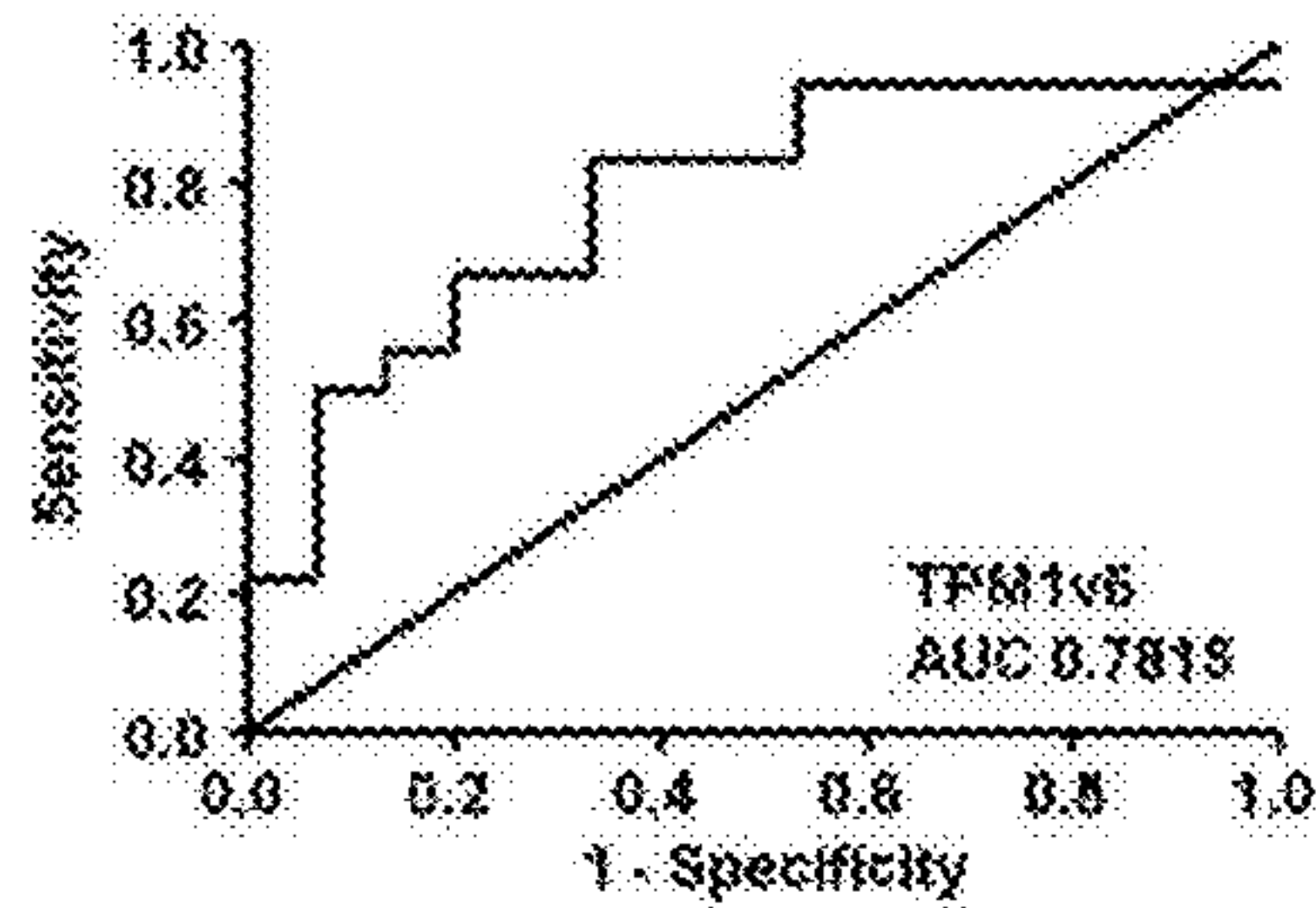


FIG. 6D

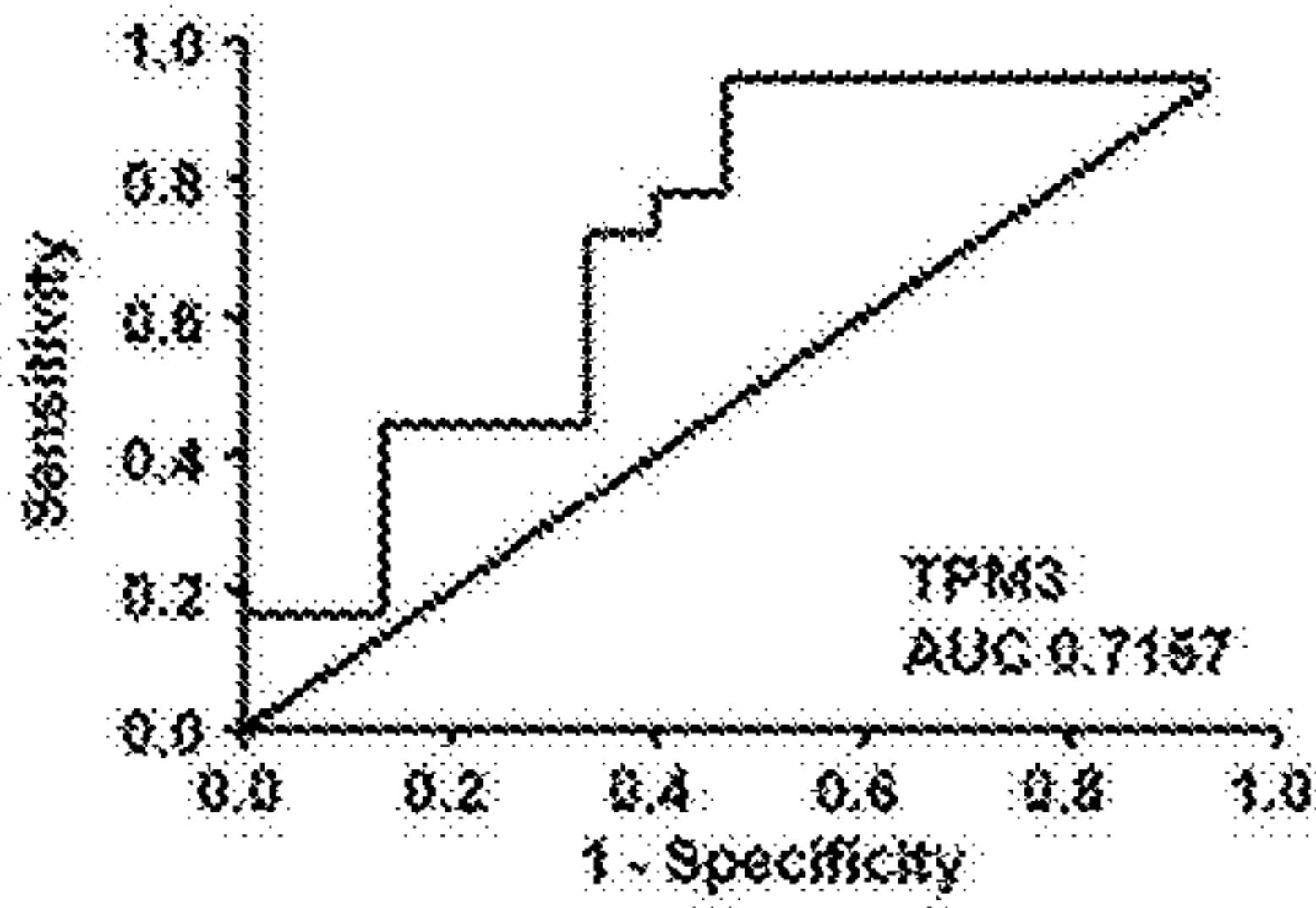


FIG. 6E

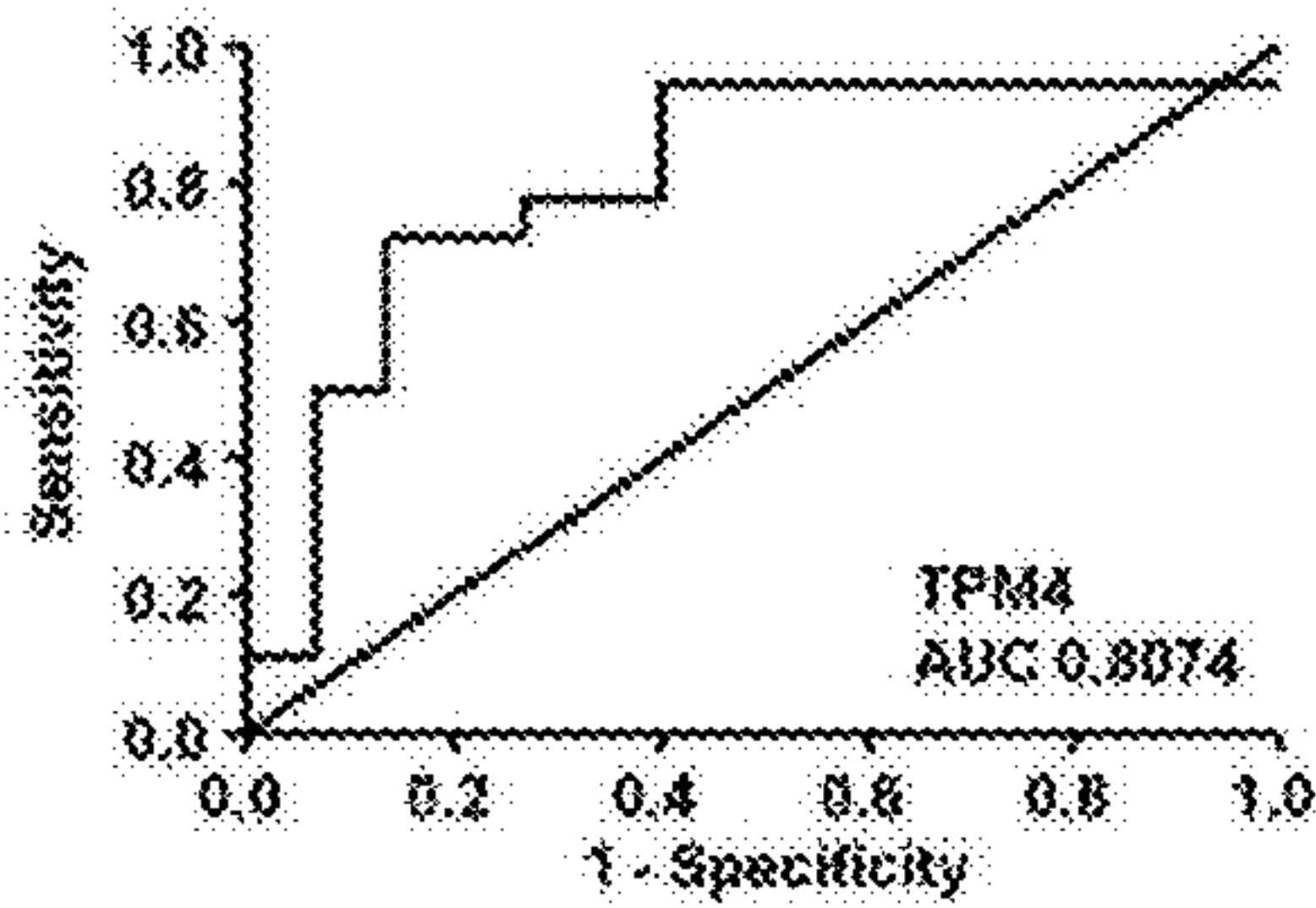
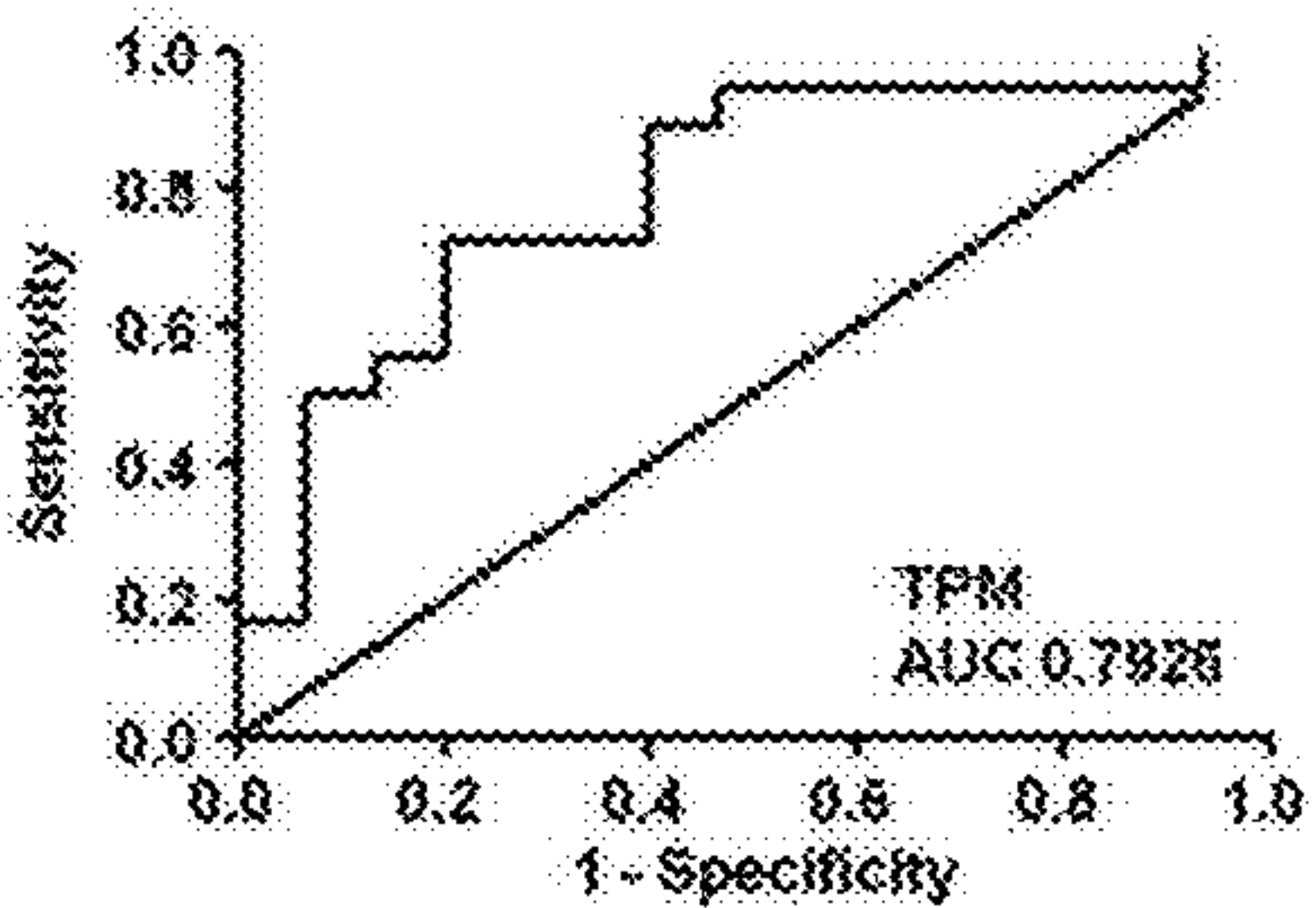


FIG. 6F



METHODS AND COMPOSITIONS FOR THE DIAGNOSIS OF OVARIAN CANCER

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation of U.S. patent application Ser. No. 16/450,602, filed Jun. 24, 2019, which is continuation of U.S. patent application Ser. No. 15/884,691, filed Jan. 31, 2018, which is a divisional of U.S. patent application Ser. No. 14/045,452, filed Oct. 3, 2013, now U.S. Pat. No. 9,903,870, issued Feb. 27, 2018, which claims the benefit of the priority of U.S. Provisional Patent Application No. 61/709,695, filed Oct. 4, 2012. These applications are incorporated herein by reference in their entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made with government support under Grant Nos. CA131582 and CA10815, awarded by the National Institutes of Health. The government has certain rights in this invention.

BACKGROUND OF THE INVENTION

[0003] Epithelial ovarian cancer (EOC) is the most lethal gynecological cancer in the United States and the fifth-leading cause of cancer-related death in women in the United States. An estimated 22,280 new cases were detected and 15,500 deaths occurred in the US in 2012. When diagnosed early (Stages I/II), treatment is generally successful, with a five-year survival rate of up to 90%. Unfortunately, most cases are not detected until after the cancer has spread, resulting in a dismal five-year survival rate for patients with advanced disease (stages III and IV) of 30% or less. The high mortality rate of ovarian cancer is due largely to the lack of effective screening tests for early detection or diagnosis of EOC.

[0004] Current screening methods for ovarian cancer typically use a combination of pelvic examination, transvaginal ultrasonography, and assays for protein biomarkers, such as serum cancer antigen 125 (CA-125). CA125 is recognized as a poor protein biomarker for early detection due to its high false positive rate and poor sensitivity and specificity. Assays for human epididymis protein-4 (HE4), or multivariate OVA1 are only approved for monitoring disease recurrence, therapeutic response, or for use in managing women with an ovarian adnexal mass.

[0005] A recently completed study comparing many of these protein biomarkers showed that none of them performed better than CA125 as a biomarker for ovarian cancer. A few groups also have used panels of biomarkers and obtained better sensitivity and specificity than CA125 alone when used in diagnostic samples. However, a recent study found that available biomarker panels did not outperform CA125 when used in pre-diagnostic samples.

[0006] Compositions and methods are urgently needed to diagnose early-stage EOC with high sensitivity and specificity and for clinical management of the disease after initial diagnosis.

SUMMARY OF THE INVENTION

[0007] In one aspect, a diagnostic reagent or device comprises the biomarker chloride intracellular channel protein 4 (CLIC4) or an isoform, pro-form, modified molecular form,

or unique peptide fragment or nucleic acid fragment thereof. In another aspect, a diagnostic reagent or device comprises a ligand capable of specifically complexing with, binding to, or quantitatively detecting or identifying the biomarker chloride intracellular channel protein 4 (CLIC4) or an isoform, pro-form, modified molecular form, or unique peptide fragment or nucleic acid fragment thereof. In certain embodiments, the biomarker sequence or ligand in the reagent or device is associated with a molecule or moiety capable alone or in combination with one or more additional molecules of generating a detectable signal. In other embodiments, the biomarker sequence or ligand in the reagent or device is associated with a substrate on which the sequence or ligand is immobilized.

[0008] In another aspect, a diagnostic reagent or device comprises one or more tropomyosin proteins, e.g., TPM1, TPM2, TPM3, TPM4, or a common or shared sequence formed of two or more TPM proteins or an isoform, pro-form, modified molecular form, unique peptide fragment or nucleic acid fragment thereof, or proteins in the same biomarker family or expressed from a related gene, having at least 20% sequence homology or sequence identity with any tropomyosin biomarker. In another aspect, a diagnostic reagent or device comprises a ligand capable of specifically complexing with, binding to, or quantitatively detecting one or more tropomyosin proteins, e.g., TPM1, TPM2, TPM3, TPM4, or a common or shared sequence in two or more TPM proteins or an isoform, pro-form, modified molecular form, or unique peptide fragment or nucleic acid fragment thereof or proteins in the same biomarker family or expressed from a related gene, having at least 20% sequence homology or sequence identity with any tropomyosin biomarker. In certain embodiments, the biomarker sequences or ligand(s) in the reagent or device are associated with a molecule or moiety capable alone or in combination with one or more additional molecules of generating a detectable signal. In other embodiments, the biomarker sequences or ligand(s) in the reagent or device are associated with a substrate on which the ligand is immobilized.

[0009] In still a further aspect, the diagnostic reagent or device comprises a set of multiple biomarkers or multiple ligands to biomarkers, each ligand individually capable of specifically complexing with, binding to, or quantitatively detecting or identifying a single biomarker. In one embodiment of such diagnostic reagents or devices, one required biomarker is CLIC4. In another embodiment of such diagnostic reagents or devices, required biomarkers are one or more tropomyosin proteins, e.g., TPM1, TPM2, TPM3, TPM4, or a common or shared sequence in two or more TPM proteins, as defined herein.

[0010] In still another aspect, the diagnostic reagent or devices described above further comprise a set of multiple biomarkers or multiple ligands, each ligand individually capable of specifically complexing with, binding to, or quantitatively detecting or identifying a single biomarker, depending upon whether the reagent is formed of biomarker sequences or ligands to biomarker sequences. The additional biomarker is one that indicates the presence of ovarian cancer in a human subject. The biomarker sequences or fragments are derived from the selected additional biomarker. Each additional ligand is capable of specifically complexing with, binding to, or quantitatively detecting, or identifying the additional biomarker. In one embodiment, the additional marker is chloride intracellular channel pro-

tein 1 (CLIC1) or an isoform, pro-form, modified molecular form, or unique peptide fragment or unique nucleic acid fragment of the additional biomarker or proteins in the same biomarker family or expressed from a related gene, having at least 20% sequence homology or sequence identity with any tropomyosin biomarker.

[0011] In one embodiment in which at least a required biomarker is CLIC4, the additional biomarker is one or more tropomyosin proteins, e.g., TPM1, TPM2, TPM3, TPM4, or a common or shared sequence within two or more TPM proteins, or an isoform, pro-form, modified molecular form, or unique peptide fragment or unique nucleic acid fragment thereof, proteins in the same biomarker family or expressed from a related gene, having at least 20% sequence homology or sequence identity with any tropomyosin biomarker. In another embodiment, the biomarkers are CLIC4 and at least one or more additional biomarkers selected from one or multiple tropomyosin biomarker proteins 1 through 4, and/or CLIC1. Still additional ovarian cancer biomarkers can form part of the set of multiple markers.

[0012] In yet another aspect, a kit, panel or microarray comprising at least one diagnostic reagent with is a biomarker CLIC4 and/or one or more tropomyosins, or ligands capable of binding thereto, and optionally one or more reagents that are different ovarian cancer biomarkers discussed herein or ligands capable of binding thereto.

[0013] In a further aspect, a method for diagnosing or detecting or monitoring the progress of ovarian cancer in a subject is provided. In one embodiment, the method comprises contacting a sample obtained from a test subject with a diagnostic reagent or device comprising a ligand capable of specifically complexing with, binding to, or quantitatively detecting or identifying the biomarker chloride intracellular channel protein 4 (CLIC4) or an isoform, pro-form, modified molecular form, or unique peptide fragment or nucleic acid fragment thereof. The protein levels of CLIC4 (or peptides of CLIC4 serving as surrogates of the CLIC4 protein) are then detected or measured in the sample or from a protein level profile generated from the sample. The protein levels of the CLIC4 biomarker in the subject's are compared with the level of the same biomarker in a reference standard. A significant change in protein level of the subject's sample from that in the reference standard indicates a diagnosis, risk, or the status of progression or remission of ovarian cancer in the subject. In one embodiment of this method, an additional step involves detecting or measuring in the sample or from a protein level profile generated from the sample, the protein levels of one or more additional ovarian cancer biomarkers; and comparing the protein levels of the CLIC4 biomarker in relation to the levels of the additional biomarkers in the subject's sample with the same biomarkers in a reference standard or profile. Analogous methods are provided for other biomarkers or biomarker sets described herein.

[0014] In another aspect, use of any of the diagnostic reagents described herein in a method for the diagnosis of ovarian cancer is provided.

[0015] Other aspects and advantages of these compositions and methods are described further in the following detailed description of the preferred embodiments thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] FIG. 1A is a quantitation of the biomarker tropomyosin 1 (TPM1) extracted from an LC-MS chromatogram

using label-free discovery mode, and GeLC-MS/MS analysis of patient serum pools. Intensities of identified peptides unique to this biomarker were summed for each of four serum pools (B-benign disease; C1, C2, C3-three different advanced ovarian cancer pools. LC-MS data from corresponding gel fractions of different serum pools were aligned and quantitatively compared using Elucidator software.

[0017] FIG. 1B is a quantitation as described in FIG. 1A for the biomarker tropomyosin 2 (TPM2), expressed from a different gene than that expressing TPM1.

[0018] FIG. 1C is a quantitation as described in FIG. 1A for the biomarker tropomyosin 3 (TPM3), expressed from a different gene than that expressing TPM1.

[0019] FIG. 1D is a quantitation as described in FIG. 1A for the biomarker tropomyosin 4 (TPM4), expressed from a different gene than that expressing TPM1.

[0020] FIG. 1E is a quantitation as described in FIG. 1A for the biomarker chloride ion channel protein 1 (CLIC1).

[0021] FIG. 1F is a quantitation as described in FIG. 1A for the biomarker chloride ion channel protein 4 (CLIC4) expressed from a different gene than that expressing CLIC1.

[0022] FIG. 2A is a sequence alignment of CLIC4 human (SwissProt Ref: Q9Y696; SEQ ID NO: 1) and 99% identical mouse (SwissProt Ref: Q9QYB1; SEQ ID NO: 2) CLIC4 sequence.

[0023] FIG. 2B is a sequence alignment of four human CLIC biomarkers expressed from different genes in the CLIC family. These biomarker gene products are of similar size and located in the 25-50 kDa region (CLIC1—SwissProt Ref: 000299; SEQ ID NO: 3; CLIC2—SwissProt Ref: O15247; SEQ ID NO: 4; CLIC3—SwissProt Ref: 095833; SEQ ID NO: 5; and CLIC4—SwissProt Ref: Q9Y696; SEQ ID NO: 1). Database identifiers are from the Swiss-Prot database. Tryptic sites (K or R) are indicated in bold—underlined sequences—peptides identified in the xenograft mouse serum. Grey highlight—peptides identified only in the patient serum pools. Lower case sequences—peptides identified in both the xenograft mouse and patient serum. Boxed sequences—peptides used for MRM quantitation.

[0024] FIG. 3 is a sequence alignment of selected isoforms of the biomarker TPM1, i.e., TPM1 isoform 5 SEQ ID NO: 6, TPM1 isoform 6 SEQ ID NO: 7, TPM1 variant SEQ ID NO: 8, TPM1 isoform CRA_i SEQ ID NO: 9, TPM1 variant 6 SEQ ID NO: 10, and TPM1 cDNA FLJ55130 SEQ ID NO: 11. TPM1 isoforms that contain all five xenograft-identified peptides (lower case sequences) were aligned using the Clustal algorithm. Two additional peptides (grey highlight) were subsequently identified in analyses of ovarian cancer patient sample pools. Inclusion of these peptides suggests the presence of TPM1 Q1ZYL5 or B7Z596. The database type (SP, Swiss-Prot entry; TR, TrEMBL entry), identifier, and brief description are indicated for each sequence. Tryptic sites (K or R) are indicated in bold. Boxed sequences—peptides used for MRM quantitation.

[0025] FIGS. 4A-4D are GeLC-MRM quantitations of tropomyosin biomarkers in individuals without ovarian cancer (WCS), individuals with benign (B), and individuals with late-stage (T) ovarian cancer. The following peptides were used to derive these values:

TABLE 1

Peptides represented in FIGS. 4A-D		
Biomarker	Peptide	SEQ ID NO:
TPM1 variant 6 (FIG. 4A)	ETAEADVASLNR	AA43-54 of SEQ ID NO: 10
	SLQEQADAAEER	AA16-27 of SEQ ID NO: 10
	LVIIIESDLER	AA133-142 of SEQ ID NO: 10
	AELSEGQVR	AA147-155 of SEQ ID NO: 10
TPM3 (FIG. 4B)	IQVLQQQADDAEER	12
TPM4 (FIG. 4C)	AEGDVAALNR	13
	IQALQQQADEAEDR	14
	[K]LVILEGELER	15
TPM (common) (FIG. 4D)	[R]IQLVEEELDR	16

[0026] FIG. 5A is a scatter plot showing GeLC-MRM quantitation of the CLIC1 biomarker in serum of normal (n=6), benign (n=9), and ovarian cancer patients (15 Stage III, 3 Stage IV). P-values were calculated using the Mann-Whitney test with Bonferroni adjustment. Horizontal bars in each group indicate the median serum level of the protein. Peptides associated with this biomarker are shown in Table 5.

[0027] FIG. 5B is a scatter plot showing GeLC-MRM quantitation of the CLIC4 biomarker as described in FIG. 5A. Peptides associated with this biomarker are shown in Table 4.

[0028] FIG. 5C is a scatter plot showing GeLC-MRM quantitation of the TPM1, variant 6 biomarker as described in FIG. 5A. Peptides associated with this biomarker are shown in Table 6.

[0029] FIG. 5D is a scatter plot showing GeLC-MRM quantitation of the TPM3 biomarker as described in FIG. 5A. Peptides associated with this biomarker are shown in Table 7.

[0030] FIG. 5E is a scatter plot showing GeLC-MRM quantitation of the TPM4 biomarker as described in FIG. 5A. Peptides associated with this biomarker are shown in Table 7.

[0031] FIG. 5F is a scatter plot showing GeLC-MRM quantitation of the TPM family of biomarkers as described in FIG. 5A. The peptide common to all four gene products and used for this quantitation is shown in Table 8.

[0032] FIG. 6A is a ROC curve of the CLIC1 biomarker generated from Control (6 normal, 9 benign) and Cancer (15 Stage III, 3 Stage IV) datasets. The area under the ROC curve is indicated for each biomarker. The peptides associated with each biomarker are shown in Tables 4-8.

[0033] FIG. 6B is a ROC curve of the CLIC4 biomarker, as described in FIG. 6A.

[0034] FIG. 6C is a ROC curve of the TPM1, variant 6 biomarker, as described in FIG. 6A.

[0035] FIG. 6D is a ROC curve of the TPM3 biomarker, as described in FIG. 6A.

[0036] FIG. 6E is a ROC curve of the TPM4 biomarker, as described in FIG. 6A.

[0037] FIG. 6F is a ROC curve of the TPM family, as described in FIG. 6A.

DETAILED DESCRIPTION OF THE INVENTION

[0038] New serological biomarkers for early detection and clinical management of ovarian cancer are urgently needed, and many candidates have been reported. A major challenge frequently encountered when validating candidates in patients is establishing quantitative assays that distinguish between highly homologous proteins. It is important to differentiate between protein isoforms as well as related protein members of a family because these sequence differences are often associated with a vital distinct role that is critical to the protein structure or function. In this regard, any assay, including sandwich ELISA assays, could give misleading results if the biomarker specificity is unknown or if multiple related biomarker proteins are quantitated as a group.

[0039] The present inventors developed a gel-based, label-free MRM quantitation approach (GeLC-MRM) as a rapid, first-level biomarker verification strategy using human plasma or serum samples. As disclosed in the examples below, the inventors used in-depth GeLC-MS/MS analysis of patient serum pools and isoform-specific MRM assays (Tang et al, 2012, cited above; Tang, H. Y et al, *J Proteome Res* 2011, 10, (9), 4005-17; and Beer, L. A et al, *J Proteome Res* 2011, 10, (3), 1126-38) to identify and quantitate additional EOC biomarkers. Related proteins in two protein families were detected that significantly distinguished between cancer and control patients to a high sensitivity.

[0040] The inventors were able to use quantitative MRM assays to distinguish between all related protein members and the isoforms of certain members that were detectable in low abundance in pools of ovarian cancer patient sera. By using a combination of unique and shared peptides together with correlation and factor analysis, the inventors determined that such unidentified related proteins either do not occur at a significant level or change in parallel with the proteins explicitly defined by unique peptides. In summary, the inventors found that the CLIC4 biomarker and at least four tropomyosins biomarker proteins (TPM1 variant 6, TPM2, TPM3, and TPM4) are significantly elevated in ovarian cancer patients compared with non-cancer controls. These proteins, as well as the previously identified biomarkers CLIC1, PRDX6, and CTSD, among others identified in PCT/US2012/54136, are promising new EOC biomarkers for distinguishing between patient cohorts and diagnosing ovarian cancer from non-cancer controls. As such, they form the basis of novel diagnostic reagents and devices, as well as methods for diagnosing or detecting the existence or absence of, or monitoring the progress of, ovarian cancer in a subject. Such compositions and method use one or more of the biomarkers, e.g., CLIC4 in optional combination with one or more other ovarian cancer-associated biomarkers, or CLIC4 in combination with multiple related TPM proteins, to diagnose and monitor the progression and treatment of EOC.

[0041] Protein abundance levels of biomarkers in blood, in some embodiments, are dependent upon expression levels in tissues of origin (e.g., ovarian tumors), as well as rate of shedding into the blood and rate of clearance from the blood. While increased expression in a tumor often will correlate

with increased abundance levels being observed in the blood, this is not necessarily always true. Therefore, the methods and compositions in one aspect refer to compositions that detect protein biomarkers and to protein assay methods. However, one of skill in the art, given the teachings contained herein, would readily understand that nucleic acid expression levels of the biomarkers and reagents and methods for their detections may be similarly practiced, without undue experimentation.

[0042] Diagnostic reagents that can detect and measure the target biomarkers and sets of biomarkers identified herein and methods for evaluating the level or ratios of these target biomarkers vs. their level(s) in a variety of reference standards or controls of different conditions or stages in ovarian cancer are valuable tools in the early detection and monitoring of ovarian cancer.

I. Definitions

[0043] “Patient” or “subject” as used herein means a female mammalian animal, including a human, a veterinary or farm animal, a domestic animal or pet, and animals normally used for clinical research. In one embodiment, the subject of these methods and compositions is a human.

[0044] By “biomarker” or “biomarker signature” as used herein is meant a single protein or a combination of proteins or peptide fragments thereof, the protein levels or relative protein levels or ratios of which significantly change (either in an increased or decreased manner) from the level or relative levels present in a subject having one physical condition or disease or disease stage from that of a reference standard representative of another physical condition or disease stage. Throughout this specification, wherever a particular biomarker is identified by name, it should be understood that the term “biomarker” includes CLIC4 and/or multiple related proteins of the tropomyosin families. These biomarkers may be combined to form certain sets of biomarkers or ligands to biomarkers in diagnostic reagents. Still other “additional” biomarkers are mentioned specifically herein in combination with CLIC4 and/or the multiple tropomyosin protein members. Biomarkers described in this specification include any physiological molecular forms, or modified physiological molecular forms, isoforms, pro-forms, and peptide fragments thereof, unless otherwise specified. It is understood that all molecular forms useful in this context are physiological, e.g., naturally occurring in the species. Preferably the peptide fragments obtained from the biomarkers are unique sequences, such as those exemplified below. However, it is understood that fragments other than those explicitly identified may be obtained readily by one of skill in the art in view of the teachings provided herein.

[0045] By “isoform” or “multiple molecular form” is meant an alternative expression product or variant of a single gene in a given species, including forms generated by alternative splicing, single nucleotide polymorphisms, alternative promoter usage, alternative translation initiation, small genetic differences between alleles of the same gene, and posttranslational modifications (PTMs) of these sequences.

[0046] By “related proteins” or “proteins of the same family” are meant expression products of different genes or related genes identified as belonging to a common family. Related proteins in the same biomarker family may or may not share related functions. Related proteins can be readily identified as having significant sequence identity either over

the entire protein or a significant part of the protein that is typically referred to as a “domain”; typically proteins with at least 20% sequence homology or sequence identity can be readily identified as belonging to the same protein family.

[0047] By “homologous protein” is meant an alternative form of a related protein produced from a related gene having a percent sequence similarity or identity of greater than 20%, greater than 30%, greater than 40%, greater than 50%, greater than 60%, greater than 70%, greater than 75%, greater than 80%, greater than 85%, greater than 90%, greater than 95%, greater than 97%, or greater than 99%.

[0048] “Reference standard” as used herein refers to the source of the reference biomarker levels. The “reference standard” is preferably provided by using the same assay technique as is used for measurement of the subject’s biomarker levels in the reference subject or population, to avoid any error in standardization. The reference standard is, alternatively, a numerical value, a predetermined cutpoint, a mean, an average, a numerical mean or range of numerical means, a numerical pattern, a ratio, a graphical pattern or a protein abundance profile or protein level profile derived from the same biomarker or biomarkers in a reference subject or reference population. In an embodiment, in which expression of nucleic acid sequences encoding the biomarkers is desired to be evaluated, the reference standard can be an expression level of one or more biomarkers or an expression profile.

[0049] “Reference subject” or “Reference Population” defines the source of the reference standard. In one embodiment, the reference is a human subject or a population of subjects having no ovarian cancer, i.e., healthy controls or negative controls. In yet another embodiment, the reference is a human subject or population of subjects with one or more clinical indicators of ovarian cancer, but who did not develop ovarian cancer. In still another embodiment, the reference is a human subject or a population of subjects having benign ovarian nodules or cysts. In still another embodiment, the reference is a human subject or a population of subjects who had ovarian cancer, following surgical removal of an ovarian tumor. In another embodiment, the reference is a human subject or a population of subjects who had ovarian cancer and were evaluated for biomarker levels prior to surgical removal of an ovarian tumor. Similarly, in another embodiment, the reference is a human subject or a population of subjects evaluated for biomarker levels following therapeutic treatment for ovarian cancer. In still another embodiment, the reference is a human subject or a population of subjects prior to therapeutic treatment for an ovarian cancer. Similarly, in another embodiment, the reference is a human subject or a population of subjects without ovarian cancer but which tests positive for a protein level of CA-125 or HE4. Similarly, in another embodiment, the reference is a human subject or a population of subjects with ovarian cancer but which tests negative for a protein level of CA125 or HE4. In still other embodiments of methods described herein, the reference is obtained from the same test subject who provided a temporally earlier biological sample. That sample can be pre- or post-therapy or pre- or post-surgery.

[0050] Other potential reference standards are obtained from a reference that is a human subject or a population of subjects having early stage ovarian cancer. In another embodiment the reference is a human subject or a population of subjects having advanced stage ovarian cancer. In still

another embodiment, the reference is a human subject or a population of subjects having a subtype of epithelial ovarian cancer. In still another embodiment, the reference is a human subject or a population of subjects having serous ovarian cancer or serous papillary adenocarcinoma. In still another embodiment, the reference is a human subject or a population of subjects having mucinous ovarian cancer. In still another embodiment, the reference is a human subject or a population of subjects having clear cell ovarian cancer. In still another embodiment, the reference is a subject or a population of subjects having endometrioid ovarian cancer. In another embodiment, the reference is a human subject or a population of subjects having Mullerian ovarian cancer. In another embodiment, the reference is a human subject or a population of subjects having undifferentiated ovarian cancer or an ovarian sarcoma. In another embodiment, the reference standard is a combination of two or more of the above reference standards.

[0051] Selection of the particular class of reference standards, reference population, biomarker levels or profiles depends upon the use to which the diagnostic/monitoring methods and compositions are to be put by the physician and the desired result, e.g., initial diagnosis of ovarian cancer or other ovarian condition, clinical management of patients with ovarian cancer after initial diagnosis, including, but not limited to, monitoring for reoccurrence of disease or monitoring remission or progression of the cancer and either before, during or after therapeutic or surgical intervention, selecting among therapeutic protocols for individual patients, monitoring for development of toxicity or other complications of therapy, predicting development of therapeutic resistance, and the like. Such reference standards or controls are the types that are commonly used in similar diagnostic assays for other biomarkers.

[0052] “Sample” as used herein means any biological fluid or tissue that contains the ovarian cancer biomarkers identified herein. The most suitable samples for use in the methods and with the compositions are samples which require minimal invasion for testing, e.g., blood samples, including serum, plasma, whole blood, and circulating tumor cells. It is also anticipated that other biological fluids, such as saliva or urine, vaginal or cervical secretions, and ascites fluids or peritoneal fluid may be similarly evaluated by the methods described herein. Also, circulating tumor cells or fluids containing them are also suitable samples for evaluation in certain embodiments of this invention. The samples may include biopsy tissue, tumor tissue, surgical tissue, circulating tumor cells, or other tissue. Such samples may further be diluted with saline, buffer or a physiologically acceptable diluent. Alternatively, such samples are concentrated by conventional means. In certain embodiments, e.g., those in which expression levels of nucleic acid sequences encoding the biomarkers are desired to be evaluated, the samples may include biopsy tissue, surgical tissue, circulating tumor cells, or other tissue. In one embodiment, the sample is a tumor secretome, i.e., any fluid or medium containing the proteins secreted from the tumor. These shed proteins may be unassociated, associated with other biological molecules, or enclosed in a lipid membrane such as an exosome. In another embodiment, the sample is plasma.

[0053] By “significant change in protein level” is meant an increased protein level of a selected biomarker in comparison to that of the selected reference standard or control or relative to a predetermined cutpoint; a decreased protein

level of a selected biomarker in comparison to that of the selected reference or control or relative to a predetermined cutpoint; or a combination of a pattern or relative pattern of certain increased and/or decreased biomarkers.

[0054] The degree of change in biomarker protein level can vary with each individual and is subject to variation with each population. For example, in one embodiment, a large change, e.g., 2-3 fold increase or decrease in protein levels of a small number of biomarkers, e.g., from 1 to 9 characteristic biomarkers, is statistically significant. In another embodiment, a smaller relative change in 10 or more (i.e., about 10, 20, 24, 29, or 30 or more biomarkers) is statistically significant. The degree of change in any biomarker(s) expression varies with the condition, such as type of ovarian cancer and with the size or spread of the cancer or solid tumor. The degree of change also varies with the immune response of the individual and is subject to variation with each individual. For example, in one embodiment of this invention, a change at or greater than a 1.2 fold increase or decrease in protein level of a biomarker or more than two such biomarkers, or even 3 or more biomarkers, is statistically significant. In another embodiment, a larger change, e.g., at or greater than a 1.5 fold, greater than 1.7 fold or greater than 2.0 fold increase or a decrease in expression of a biomarker(s) is statistically significant. This is particularly true for cancers without solid tumors. Still alternatively, if a single biomarker protein level is significantly increased in biological samples which normally do not contain measurable protein levels of the biomarker, such increase in a single biomarker level may alone be statistically significant. Conversely, if a single biomarker protein level is normally decreased or not significantly measurable in certain biological samples which normally do contain measurable protein levels of the biomarker, such decrease in protein level of a single biomarker may alone be statistically significant.

[0055] A change in protein level of a biomarker required for diagnosis or detection by the methods described herein refers to a biomarker whose protein level is increased or decreased in a subject having a condition or suffering from a disease, specifically ovarian cancer, relative to its expression in a reference subject or reference standard. Biomarkers may also be increased or decreased in protein level at different stages of the same disease or condition. The protein levels of specific biomarkers differ between normal subjects and subjects suffering from a disease, benign ovarian nodules, or cancer, or between various stages of the same disease. Protein levels of specific biomarkers differ between pre-surgery and post-surgery patients with ovarian cancer. Such differences in biomarker levels include both quantitative, as well as qualitative, differences in the temporal or relative protein level or abundance patterns among, for example, biological samples of normal and diseased subjects, or among biological samples which have undergone different disease events or disease stages. For the purpose of this invention, a significant change in biomarker protein levels when compared to a reference standard is considered to be present when there is a statistically significant ($p < 0.05$) difference in biomarker protein level between the subject and reference standard or profile, or significantly different relative to a predetermined cut-point.

[0056] For example, in one embodiment, the test subject's biomarker(s) levels are compared with a healthy reference standard. If the subject has ovarian cancer, the selected EOC biomarker(s), e.g., CLIC 4 and/or TPM1, 2, 3, and/or 4, will

typically show a change in protein level from the levels in the healthy reference standard, thus permitting diagnosis of ovarian cancer. In another example, these biomarker(s) differentially change in protein level (either by increased or decreased protein level) when the biomarker levels or relative levels from the sample of a subject having one of the following conditions is compared to a reference subject or population having another of the following physical conditions. These “conditions” include no ovarian cancer, the presence of benign ovarian nodules, the presence of an ovarian cancer or subtype, the condition following surgical removal of an ovarian tumor; the condition prior to surgical removal of an ovarian tumor; the condition following a specific therapeutic treatment for an ovarian tumor; the condition prior to a specific therapeutic treatment for an ovarian tumor. It is further anticipated that the biomarker(s) expression levels may change and the changes may be detected during treatment for ovarian cancer. In another embodiment, a condition includes that of a subject having undiagnosed clinical symptoms of abdominal pain or other abdominal condition of unknown origin. Still other embodiments of “conditions” as defined above include early stage ovarian cancer; advanced stage ovarian cancer, a subtype of epithelial ovarian cancer, serous ovarian cancer; mucinous ovarian cancer, clear cell ovarian cancer, endometrioid ovarian cancer, Mullerian ovarian cancer; undifferentiated ovarian cancer, serous papillary adenocarcinoma; and sarcoma.

[0057] The term “ligand” refers with regard to protein biomarkers to a molecule that binds or complexes, with a biomarker protein, molecular form or peptide, such as an antibody, antibody mimic or equivalent that binds to or complexes with a biomarker identified herein, a molecular form or fragment thereof. In certain embodiments, in which the biomarker expression is to be evaluated, the ligand can be a nucleotide sequence, e.g., polynucleotide or oligonucleotide, primer or probe.

[0058] As used herein, the term “antibody” refers to an intact immunoglobulin having two light and two heavy chains or fragments thereof capable of binding to a biomarker protein or a fragment of a biomarker protein. Thus a single isolated antibody or fragment may be a monoclonal antibody, a synthetic antibody, a recombinant antibody, a chimeric antibody, a humanized antibody, or a human antibody. The term “antibody fragment” refers to less than an intact antibody structure, including, without limitation, an isolated single antibody chain, an Fv construct, a Fab construct, an Fc construct, a light chain variable or complementarity determining region (CDR) sequence, etc.

[0059] As used herein, “labels” or “reporter molecules” are chemical or biochemical moieties useful for labeling a ligand, e.g., amino acid, peptide sequence, protein, or antibody. “Labels” and “reporter molecules” include fluorescent agents, chemiluminescent agents, chromogenic agents, quenching agents, radionucleotides, enzymes, substrates, cofactors, inhibitors, radioactive isotopes, magnetic particles, and other moieties known in the art. “Labels” or “reporter molecules” are capable of generating a measurable signal and may be covalently or noncovalently joined to a ligand.

[0060] As used herein the term “cancer” refers to or describes the physiological condition in mammals that is typically characterized by unregulated cell growth. More specifically, as used herein, the term “cancer” means any ovarian cancer. In one embodiment, the ovarian cancer is an

epithelial ovarian cancer or subtype as referred to in “conditions” above. In still an alternative embodiment, the cancer is an “early stage” (I or II) ovarian cancer. In still another embodiment, the cancer is a “late stage” (III or IV) ovarian cancer.

[0061] The term “tumor,” as used herein, refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues.

[0062] By “therapeutic reagent” or “regimen” is meant any type of treatment employed in the treatment of cancers with or without solid tumors, including, without limitation, chemotherapeutic pharmaceuticals, biological response modifiers, radiation, diet, vitamin therapy, hormone therapies, gene therapy, surgical resection, etc.

[0063] The term “microarray” refers to an ordered arrangement of binding/complexing array elements or ligands, e.g. antibodies, on a substrate.

[0064] In the context of the compositions and methods described herein, reference to “at least two,” “at least five,” etc. of the biomarkers listed in any particular biomarker set means any and all combinations of the biomarkers identified. Specific biomarkers for the biomarker profile do for use in this invention include CLIC4, but may also include any biomarker, fragment or molecular form, as discussed herein.

[0065] By “significant change in expression” is meant an upregulation in the expression level of a nucleic acid sequence, e.g., genes or transcript, encoding a selected biomarker, in comparison to the selected reference standard or control; a downregulation in the expression level of a nucleic acid sequence, e.g., genes or transcript, encoding a selected biomarker, in comparison to the selected reference standard or control; or a combination of a pattern or relative pattern of certain upregulated and/or down regulated biomarker genes. The degree of change in biomarker expression can vary with each individual as stated above for protein biomarkers.

[0066] The term “polynucleotide,” when used in singular or plural form, generally refers to any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. Thus, for instance, polynucleotides as defined herein include, without limitation, single- and double-stranded DNA, DNA including single- and double-stranded regions, single- and double-stranded RNA, and RNA including single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or include single- and double-stranded regions. In addition, the term “polynucleotide” as used herein refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term “polynucleotide” specifically includes cDNAs. The term includes DNAs (including cDNAs) and RNAs that contain one or more modified bases. In general, the term “polynucleotide” embraces all chemically, enzymatically and/or metabolically modified forms of unmodified polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including simple and complex cells.

[0067] The term “oligonucleotide” refers to a relatively short polynucleotide of less than 20 bases, including, without limitation, single-stranded deoxyribonucleotides, single- or double-stranded ribonucleotides, RNA:DNA hybrids and double-stranded DNAs. Oligonucleotides, such as single-stranded DNA probe oligonucleotides, are often synthesized

by chemical methods, for example using automated oligonucleotide synthesizers that are commercially available. However, oligonucleotides can be made by a variety of other methods, including in vitro recombinant DNA-mediated techniques and by expression of DNAs in cells and organisms.

[0068] One skilled in the art may readily reproduce the compositions and methods described herein by use of the amino acid sequences of the biomarkers and other molecular forms, which are publicly available from conventional sources.

[0069] It should be understood that while various embodiments in the specification are presented using “comprising” language, under various circumstances, a related embodiment is also be described using “consisting of” or “consisting essentially of” language. It is to be noted that the term “a” or “an”, refers to one or more, for example, “an immunoglobulin molecule,” is understood to represent one or more immunoglobulin molecules. As such, the terms “a” (or “an”), “one or more,” and “at least one” is used interchangeably herein.

[0070] Unless defined otherwise in this specification, 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 and by reference to published texts, which provide one skilled in the art with a general guide to many of the terms used in the present application.

II. Biomarkers and Biomarker Signatures Useful in the Methods and Compositions

[0071] The “targets” of the compositions and methods of these inventions include, in one aspect, CLIC4, optionally with other biomarkers identified herein, fragments, particularly unique fragments thereof, and molecular forms thereof. In certain embodiments, superior diagnostic tests for diagnosing the existence of ovarian cancer utilize at least one of the ligands that bind or complex with CLIC4, or one of the fragments or molecular forms thereof. In other embodiments, superior diagnostic tests for distinguishing ovarian cancer from one of the conditions recited above utilize multiple ligands, each individually detecting a different specific target biomarker identified herein, or isoform, modified form or peptide thereof. In still other methods, no ligand is necessary, e.g., MRM assays.

[0072] Thus, in one aspect, the target biomarker of the methods and compositions described herein is chloride intracellular channel protein 4 (CLIC4) or an isoform, pro-form, modified molecular form, or unique peptide fragment or nucleic acid fragment thereof. The amino acid sequences for CLIC4 and its molecular forms are publically available, such as in GENBANK. In one embodiment, the biomarker CLIC4 is Uniprot ID no. Z9Y696 or a peptide fragment thereof. Certain fragments of CLIC4 that may be useful as targets in the methods and compositions described herein include one or more peptide fragments, such as, but not limited to, those identified below. It should be understood that, depending upon the context, any reference to CLIC4 herein also refers to a peptide and the molecular form thereof, as well as the nucleotide sequences encoding CLIC4 and/or any of its unique peptides or forms. Among such fragments useful for detection of CLIC4 are

- (a)
GVVFSVTTVDLK,
aa 49-60 of SEQ ID NO: 1
- (b)
LDEYLNSPLPDEIDENSMEDIK,
aa 151-172 of SEQ ID NO: 1
- (c)
NSRPEANEALER,
aa 131-142 of SEQ ID NO: 1
- (d)
YLTNAYSRL,
aa 20-27 of SEQ ID NO: 1
- (d)
EVEIAYSDEVAK,
aa 139-249 of SEQ ID NO: 1
- (e)
LFMILWLK,
aa 41-48 of SEQ ID NO: 1
- (f)
DEFTNTCPSDK,
aa 228-238 of SEQ ID NO: 1
- (g)
IEEFLEEVLCPPK,
aa 91-103 of SEQ ID NO: 1
- (h)
FLDGNEMTLADCNLLPK,
aa 178-194 of SEQ ID NO: 1
- (i)
EEDKEPLIELFVK,
aa 12-24 of SEQ ID NO: 1
or
- (j)
HPESNTAGMDIFAK,
aa 111-124 of SEQ ID NO: 1.

[0073] In some embodiments, the methionine in each sequence is the oxidized form; in other embodiments, the methionine in each sequence is in the unoxidized form.

[0074] In another embodiment the target biomarker of the methods and compositions described herein is Tropomyosin 1 (TPM1), as described in International Patent Application No. PCT/US12/54136. The amino acid sequence for TPM1 is publically available, such as in GENBANK. In one embodiment, an isoform of TPM1 is TPM1, variant 6 (UniProt ID No. Q1ZYL5) or TPM1, variant 8 (UniProt ID No. B7Z596). Certain fragments of TPM1 may also be useful as targets in the methods and compositions described herein. It should be understood that, depending upon the context, any reference to TPM1 herein also refers to any of its peptides or molecular forms, as well as the nucleotide sequences encoding TPM1 and/or any of the peptides.

[0075] In another embodiment the target biomarker of the methods and compositions described herein is Tropomyosin 2 (TPM2). The amino acid sequence for TPM2 is publically available, such as in GENBANK. In another embodiment, an isoform of TPM2 is TPM2 beta chain (UniProt ID No. UR1H_P07951, UR1H_Q5TCU3, or UR1H_Q5TCU8) or TPM2, isoform 2 (UniProt ID No. P07951-2, A7XZE4). Certain fragments of TPM2 may also be useful as targets in the methods and compositions described herein. It should be understood that, depending upon the context, any reference to TPM2 herein also refers to any of its peptides or molecu-

lar forms, as well as the nucleotide sequences encoding TPM2 and/or any of the peptides.

[0076] In another embodiment the target biomarker of the methods and compositions described herein is Tropomyosin 3 (TPM3). The amino acid sequence for TPM3 is publically available, such as in GENBANK. In still another embodiment, an isoform of TPM3 is TPM3 alpha-3 chain (UniProt ID No. P06753, Q5VU59, Q5HYB6, B2RDE1, or E2RB38). Certain fragments of TPM3 may also be useful as targets in the methods and compositions described herein. It should be understood that, depending upon the context, any reference to TPM3 herein also refers to any of its peptides or molecular forms, as well as the nucleotide sequences encoding TPM3 and/or any of the peptides.

[0077] In another embodiment the target biomarker of the methods and compositions described herein is Tropomyosin 4 (TPM4). The amino acid sequence for TPM4 is publically available, such as in GENBANK. In a further embodiment, an isoform of TPM4 is TPM4 alpha-4 chain (UniProt ID No. P6736), or TPM4, Isoform 2 (UniProt ID No. P67936-2). Certain fragments of TPM4 may also be useful as targets in the methods and compositions described herein, e.g., Table 3. It should be understood that, depending upon the context, any reference to TPM4 herein also refers to any of its peptides or molecular forms, as well as the nucleotide sequences encoding TPM4 and/or any of the peptides.

[0078] In another embodiment the target biomarker of the methods and compositions described herein is a peptide sequence shared by two or more members of the related family of tropomyosin proteins TPM1, TPM2, TPM3 and TPM4. A common amino acid sequence for TPM or fragments thereof can be readily generated using and aligning the publically known sequences for the native TPM family members. It should be understood that, depending upon the context, any reference to common TPM herein also refers to any of its fragments or peptides derived therefrom or the common sequence generated by use of 2 or more of the TPM related proteins to generate the alignment.

[0079] In still other embodiments, the target biomarker(s) are multiple members of the related family of tropomyosin proteins, such as tropomyosin 1 (TPM1); tropomyosin 2 (TPM2); tropomyosin 3 (TPM3); tropomyosin 4 (TPM4); or a common sequence shared by two or more of these related proteins.

[0080] In still another embodiment, the target biomarker is actually a set of targets, which set contains CLIC4 (as defined above) and one or more additional EOC target biomarkers. In still another embodiment, the target biomarker is actually a set of targets, which set contains two or more related TPM family proteins or a TPM common or shared peptide or protein (as defined above) and one or more additional EOC target biomarkers. It should be understood that wherever in a following list of biomarker sets, a TPM biomarker is identified, it could be replaced or used with a common or shared TPM sequence.

[0081] Thus, among other embodiments, a set of biomarkers can be one of the following sets: CLIC4, TPM1 or CLIC4, TPM2 or CLIC4, TPM3 or CLIC4, TPM4, or CLIC4, TPM1, TPM2, or CLIC4, TPM1, TPM2, TPM3, or CLIC4, TPM1, TPM2, TPM3, TPM4, or CLIC4, TPM2, TPM3, TPM4, or CLIC4, TPM3, TPM4, or CLIC4, TPM2, TPM4, or CLIC4, TPM1, TPM3.

[0082] However, still other additional biomarkers can include any of the biomarkers identified in International

Patent Application No. PCT/US12/54136, such as CLIC 1 or an isoform, pro-form, modified molecular form, or unique peptide fragment or unique nucleic acid fragment thereof. The amino acid sequence for CLIC1 is publically available, such as in GENBANK. Certain fragments of CLIC1 that may be useful as targets in the methods and compositions described herein include one or more peptide fragments, such as, but not limited to, those identified herein. It should be understood that, depending upon the context, any reference to CLIC1 herein also refers to any of these peptides, or any molecular form of the biomarker, as well as the nucleotide sequences encoding CLIC1 and/or any of the peptides. Among useful fragments of CLIC1 include

(a)
GVTFNVTTVDTK,
aa 38-49 of SEQ ID NO: 3;

(b)
LAALNPESNTAGLDIFAK,
aa 96-113 of SEQ ID NO: 3;
or

(c)
NSNPALNDNLEK,
aa 120-131 of SEQ ID NO: 3.

[0083] Thus, in another embodiment, a desirable set of target biomarkers includes, without limitation: CLIC4 and CLIC1, or CLIC4, CLIC1, TPM1, or CLIC4, CLIC1, TPM2, or CLIC4, CLIC1, TPM3, or CLIC4, CLIC1, TPM4, or CLIC4, CLIC1, TPM1, TPM2, or CLIC4, CLIC1, TPM1, TPM2, TPM3, or CLIC4, CLIC1, TPM1, TPM2, TPM3, TPM4 or CLIC4, CLIC1, TPM2, TPM3, or CLIC4, CLIC1, TPM2, TPM3, TPM4, or CLIC4, CLIC1, TPM3, TPM4, or CLIC4, CLIC1, TPM1, TPM3, or CLIC4, CLIC1, TPM2, TPM4. In another embodiment, a desirable set of target biomarkers includes, without limitation: CLIC1, TPM2, or CLIC1, TPM3, or CLIC1, TPM4, or CLIC1, TPM1, TPM2, or CLIC1, TPM1, TPM2, TPM3, or CLIC1, TPM1, TPM2, TPM3, TPM4 or CLIC1, TPM2, TPM3, or CLIC1, TPM2, TPM3, TPM4, or CLIC1, TPM3, TPM4, or CLIC1, TPM1, TPM3, or CLIC1, TPM2, TPM4.

[0084] Still other sets of target biomarkers that include either CLIC4 and/or multiple TPM biomarkers as described above, can further include other known EOC biomarkers, which may be added to the above-identified sets either individually or in groups. In certain embodiments, the additional biomarker is cathepsin D-30 kDa (CTSD-30) or an isoform, pro-form, modified molecular form, or unique peptide fragment or unique nucleic acid fragment thereof, proteins in the same biomarker family or expressed from a related gene, having at least 20% sequence homology or sequence identity with CTSD-30. In other embodiments, the additional biomarker is peroxiredoxin-6 (PRDX6) or an isoform, pro-form, modified molecular form, or unique peptide fragment or unique nucleic acid fragment thereof, or proteins in the same biomarker family or expressed from a related gene, having at least 20% sequence homology or sequence identity with PRDX6. In still other embodiments, the additional biomarker is the known EOC biomarker CA125, or an isoform, pro-form, modified molecular form, or unique peptide fragment therefrom or proteins in the same biomarker family or expressed from a related gene, having at least 20% sequence homology or sequence identity with CA125. In still other embodiments, the additional biomarker

is the known EOC biomarker HE4, or an isoform, pro-form, modified molecular form, or unique peptide fragment therefrom or proteins in the same biomarker family or expressed from a related gene, having at least 20% sequence homology or sequence identity with HE4. In other embodiments, the additional biomarker is bisphosphoglycerate mutase (BPGM) or an isoform, pro-form, modified molecular form, or unique peptide fragment or unique nucleic acid fragment thereof, or proteins in the same biomarker family or expressed from a related gene, having at least 20% sequence homology or sequence identity with BPGM. In other embodiments, the additional biomarker is proteasome subunit alpha type-7 (PSMA7) or an isoform, pro-form, modified molecular form, or unique peptide fragment or unique nucleic acid fragment thereof, or proteins in the same biomarker family or expressed from a related gene, having at least 20% sequence homology or sequence identity with PSMA7. In other embodiments, the additional biomarker is aldose reductase (AKR1B1) or an isoform, pro-form, modified molecular form, or unique peptide fragment or unique nucleic acid fragment thereof, or proteins in the same biomarker family or expressed from a related gene, having at least 20% sequence homology or sequence identity with AKR1B1. In other embodiments, the additional biomarker is homeobox protein (HMX1) or an isoform, pro-form, modified molecular form, or unique peptide fragment or unique nucleic acid fragment thereof, or proteins in the same biomarker family or expressed from a related gene, having at least 20% sequence homology or sequence identity with HMX1. In other embodiments, the additional biomarker is melastatin 1 (TRPM1) or an isoform, pro-form, modified molecular form, or unique peptide fragment or unique nucleic acid fragment thereof, or proteins in the same biomarker family or expressed from a related gene, having at least 20% sequence homology or sequence identity with TRPM1. In other embodiments, the additional biomarker is protein CutA (CUTA) or an isoform, pro-form, modified molecular form, or unique peptide fragment or unique nucleic acid fragment thereof, or proteins in the same biomarker family or expressed from a related gene, having at least 20% sequence homology or sequence identity with CUTA. In other embodiments, the additional biomarker is SERPINB12 protein (SERPINB12), or an isoform, pro-form, modified molecular form, or unique peptide fragment or unique nucleic acid fragment thereof, or proteins in the same biomarker family or expressed from a related gene, having at least 20% sequence homology or sequence identity with SERPINB12. In other embodiments, the additional biomarker is cathepsin D-52 kDa (CTSD-52) or an isoform, pro-form, modified molecular form, or unique peptide fragment or unique nucleic acid fragment thereof, or proteins in the same biomarker family or expressed from a related gene, having at least 20% sequence homology or sequence identity with CTSD-52.

[0085] In still other multiple biomarker combinations with either CLIC4 and/or multiple TPM biomarkers or a TPM common or shared biomarker, include, without limitation, or consist of, the following exemplary combinations of biomarkers or combinations that include different molecular forms of the same biomarker, for diagnosis of ovarian cancer or for monitoring the progression of the severity of disease or remission of disease:

[0086] CLIC4 with one or more of CLIC1, PRDX6, BPGM, PSMA7, AKR1B1, HMX1, TRPM1, CUTA, SERPINB12, CTSD-30 kDa or CTSD-52 kDa; or

[0087] CLIC4 and one or multiple TPM 1-4, as above, with one or more of CLIC1, PRDX6, BPGM, PSMA7, AKR1B1, HMX1, TRPM1, CUTA, SERPINB12, CTSD-30 kDa or CTSD-52 kDa; or

[0088] CLIC4 with one or more of CLIC1, PRDX6, BPGM, PSMA7, AKR1B1, HMX1, TRPM1, CUTA, SERPINB12, CTSD-30 kDa or CTSD-52 kDa, and CA125 and/or HE4; or

[0089] CLIC4 and one or multiple TPM 1-4, as above, with CLIC1, PRDX6, BPGM, PSMA7, AKR1B1, HMX1, TRPM1, CUTA, SERPINB12, CTSD-30 kDa or CTSD-52 kDa, and CA125 and/or HE4.

[0090] In still another aspect, a biomarker combination includes, without limitation, or consists of, the following exemplary combinations of biomarkers for diagnosis of ovarian cancer or for monitoring the progression of the severity of disease or remission of disease:

[0091] CLIC4, CTSD-30 kDa, and/or CLIC1, and/or PRDX6, or

[0092] CLIC4, CTSD-30 kDa, CLIC1, and PRDX6;

[0093] CLIC4, CTSD-30 kDa, CLIC1, PRDX6 and at least two TPM proteins selected from TPM1, TPM2, TPM3, TPM4 or common or shared TPM;

[0094] CLIC4, CTSD-30 kDa, CLIC1, PRDX6 and BPGM,

[0095] CLIC4, CTSD-30 kDa, CLIC1, PRDX6 and PSMA7, CLIC4, CTSD-30 kDa, CLIC1, PRDX6, BPGM, and at least two TPM proteins selected from TPM1, TPM2, TPM3, TPM4 or common or shared TPM;

[0096] CLIC4, CTSD-30 kDa, CLIC1, PRDX6, PSMA7, and at least two TPM proteins selected from TPM1, TPM2, TPM3, TPM4 or common or shared TPM;

[0097] CLIC4, CTSD-30 kDa, CLIC1, PRDX6, BPGM and PSMA7, and at least two TPM proteins selected from TPM1, TPM2, TPM3, TPM4 or common or shared TPM.

[0098] Still other permutations of the biomarkers listed herein may form the multiple biomarker targets or an isoform, pro-form, modified molecular form, or peptide fragment thereof in the compositions and methods of this invention. One of skill in the art may readily form appropriate combinations from the biomarkers listed herein, and from any isoform, pro-form, modified molecular form, post-translational modification, or unique peptide fragment or unique nucleic acid fragment thereof, proteins in the same biomarker family or expressed from a related gene, having at least 20% sequence homology or sequence identity with a specifically named biomarker herein. It is further anticipated that CLIC4 alone, multiple tropomyosins (TPM1, TPM2, TPM3, TPM4 or common or shared TPM) alone, or CLIC4 in concert with multiple tropomyosins may be further combined with other known EOC markers to form desirable target sets.

[0099] For example, among desirable biomarker signatures are signatures that comprise, or consist of, at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more of the biomarkers described herein or any other known ovarian cancer biomarker or molecular forms or peptides thereof. It is contemplated that even higher abundance biomarkers (including some identified in PCT/US12/54136) may be useful when combined in a panel or signature with the low abundance biomarker CLIC4 and/or multiple TPMs.

[0100] As further stated above, the biomarkers/biomarker signatures described above, can in another embodiment, refer to nucleic acid sequences, genes and transcripts encoding the biomarkers and expression profiles thereof.

II. Diagnostic Reagents, Devices and Kits

[0101] A. Labeled or Immobilized Biomarkers or Peptides or Molecular Forms

[0102] Thus, in one aspect, a diagnostic reagent or device comprises the biomarker chloride intracellular channel protein 4 (CLIC4) or an isoform, pro-form, modified molecular form, or unique peptide fragment or nucleic acid fragment thereof. In one embodiment, the biomarker protein or nucleic acid is associated with a molecule or moiety capable alone or in combination with one or more additional molecules of generating a detectable signal. In another embodiment, the biomarker protein or nucleic acid is associated with a substrate and is immobilized.

[0103] In another embodiment, a diagnostic reagent or device comprises multiple members of the related family of tropomyosin proteins, such as tropomyosin 1 (TPM1); tropomyosin 2 (TPM2); tropomyosin 3 (TPM3); tropomyosin 4 (TPM4); or a common or shared sequence formed of two or more of these related proteins, or multiple isoforms, pro-forms, modified molecular forms, or unique peptide fragments or nucleic acid fragments thereof. In one embodiment, the biomarker protein or nucleic acid is associated with a molecule or moiety capable alone or in combination with one or more additional molecules of generating a detectable signal. In another embodiment, the biomarker protein or nucleic acid is associated with a substrate and is immobilized.

[0104] In another embodiment, a diagnostic reagent or device comprises a CLIC4 and one or multiple members of the related family of tropomyosin proteins, or the TPM common or shared sequence, or multiple isoforms, pro-forms, modified molecular forms, or unique peptide fragments or nucleic acid fragments thereof. In one embodiment, the biomarker proteins or nucleic acid sequences are each associated with a molecule or moiety capable alone or in combination with one or more additional molecules of generating a detectable signal. In another embodiment, the biomarker protein or nucleic acid is associated with a substrate and is immobilized. Where the reagent or device contains multiple biomarker sequences, the detectable moieties and signals can each be different, so as to identify different results.

[0105] In still other embodiments, the device or reagent designed to identify CLIC4 and/or multiple TPMs can include additional EOC biomarker proteins, fragments or nucleic acid sequences, such as the disclosed in International Patent Application No. PCT/US12/54136, incorporated by reference herein. Thus, a diagnostic reagent or device as described herein can comprise 1 to 2, 3, 4, 5, 6, 7, 8, 10, 15 or 20 or more biomarker sequences.

[0106] In still other embodiments, diagnostic reagents or devices for use in the methods of diagnosing ovarian cancer include CLIC4 and/or multiple TPMs and one or more additional target biomarkers or peptide fragments identified herein, including, but not limited to, isoforms or molecular forms thereof, pro-form, modified molecular form, or unique peptide fragment or nucleic acid fragment thereof, or proteins in the same biomarker family or expressed from a

related gene, having at least 20% sequence homology or sequence identity with the identified biomarker.

[0107] In still another embodiment of these sets can include one or more additional biomarker that indicates the presence of ovarian cancer in a human subject. In certain embodiments, the additional biomarker is cathepsin D-30 kDa (CTSD-30) or an isoform, pro-form, modified molecular form, or unique peptide fragment or unique nucleic acid fragment thereof, proteins in the same biomarker family or expressed from a related gene, having at least 20% sequence homology or sequence identity with CTSD-30. In other embodiments, the additional biomarker is peroxiredoxin-6 (PRDX6) or an isoform, pro-form, modified molecular form, or unique peptide fragment or unique nucleic acid fragment thereof, or proteins in the same biomarker family or expressed from a related gene, having at least 20% sequence homology or sequence identity with PRDX6. In still other embodiments, the additional biomarker is CA125, or an isoform, pro-form, modified molecular form, or unique peptide fragment therefrom or proteins in the same biomarker family or expressed from a related gene, having at least 20% sequence homology or sequence identity with CA125. In other embodiments, the additional biomarker is bisphosphoglycerate mutase (BPGM) or an isoform, pro-form, modified molecular form, or unique peptide fragment or unique nucleic acid fragment thereof, or proteins in the same biomarker family or expressed from a related gene, having at least 20% sequence homology or sequence identity with BPGM. In other embodiments, the additional biomarker is proteasome subunit alpha type-7 (PSMA7) or an isoform, pro-form, modified molecular form, or unique peptide fragment or unique nucleic acid fragment thereof, or proteins in the same biomarker family or expressed from a related gene, having at least 20% sequence homology or sequence identity with PSMA7. In other embodiments, the additional biomarker is aldose reductase (AKR1B1) or an isoform, pro-form, modified molecular form, or unique peptide fragment or unique nucleic acid fragment thereof, or proteins in the same biomarker family or expressed from a related gene, having at least 20% sequence homology or sequence identity with AKR1B1. In other embodiments, the additional biomarker is homeobox protein (HMX1) or an isoform, pro-form, modified molecular form, or unique peptide fragment or unique nucleic acid fragment thereof, or proteins in the same biomarker family or expressed from a related gene, having at least 20% sequence homology or sequence identity with HMX1. In other embodiments, the additional biomarker is melastatin 1 (TRPM1) or an isoform, pro-form, modified molecular form, or unique peptide fragment or unique nucleic acid fragment thereof, or proteins in the same biomarker family or expressed from a related gene, having at least 20% sequence homology or sequence identity with TRPM1. In other embodiments, the additional biomarker is protein CutA (CUTA) or an isoform, pro-form, modified molecular form, or unique peptide fragment or unique nucleic acid fragment thereof, or proteins in the same biomarker family or expressed from a related gene, having at least 20% sequence homology or sequence identity with CUTA. In other embodiments, the additional biomarker is SERPINB12 protein (SERPINB12), or an isoform, pro-form, modified molecular form, or unique peptide fragment or unique nucleic acid fragment thereof, or proteins in the same biomarker family or expressed from a related gene, having at least 20% sequence homology or sequence identity

with SERPINB12. In other embodiments, the additional biomarker is cathepsin D-52 kDa (CTSD-52) or an isoform, pro-form, modified molecular form, or unique peptide fragment or unique nucleic acid fragment thereof, or proteins in the same biomarker family or expressed from a related gene, having at least 20% sequence homology or sequence identity with CTSD-52.

[0108] In such embodiments of the diagnostic reagents and devices, at least one of the biomarkers is associated with a detectable label or portion of a detectable label system. In another embodiment, a diagnostic reagent includes one or more target biomarker or peptide fragment identified herein, immobilized on a substrate. In still another embodiment, combinations of such labeled or immobilized biomarkers are suitable reagents and components of a diagnostic kit or device. In another aspect, suitable embodiments of such labeled or immobilized reagents include at least one, 2, 3, 4, 5, 6, 7, 8, 9, 10 or all 20 or more biomarkers identified herein or their unique peptide fragments.

[0109] In one aspect the reagent, device or kit as described above further comprises or consists of ligands that individually specifically complex with, bind to, or quantitatively detect or identify multiple isoforms or multiple related proteins of any of biomarkers mentioned herein. In another aspect, the reagent, device or kit comprises or consists of ligands that individually specifically complex with, bind to, or quantitatively detect or identify two or more biomarkers identified in the groups specifically discussed above. Still other diagnostic reagents are the surrogate peptides used for the MRM assays, such as, but not limited to, the peptides disclosed herein.

[0110] Any combination of labeled or immobilized biomarkers as described above can be assembled in a diagnostic kit or device for the purposes of diagnosing ovarian cancer, such as those combinations of biomarkers discussed herein.

[0111] For these reagents, the labels may be selected from among many known diagnostic labels, including those described above. Similarly, the substrates for immobilization in a device may be any of the common substrates, glass, plastic, a microarray, a microfluidics card, a chip, a bead or a chamber.

[0112] B. Labeled or Immobilized Ligands that Bind or Complex with the Biomarkers

[0113] In another aspect, a diagnostic reagent or device comprises a ligand capable of specifically complexing with, binding to, or quantitatively detecting or identifying the biomarker chloride intracellular channel protein 4 (CLIC4) or an isoform, pro-form, modified molecular form, or unique peptide fragment or nucleic acid fragment thereof. In one embodiment, the ligand is associated with a molecule or moiety capable alone or in combination with one or more additional molecules of generating a detectable signal. In another embodiment, the ligand is associated with a substrate on which the ligand is immobilized.

[0114] In still other embodiments, the diagnostic reagent or device comprises a set of multiple ligands, each ligand individually capable of specifically complexing with, binding to, or quantitatively detecting or identifying a single biomarker or an isoform, pro-form, modified molecular form, or unique peptide fragment or nucleic acid fragment thereof. In another embodiment, a diagnostic reagent or device comprises a ligand capable of specifically complexing with, binding to, or quantitatively detecting or identifying multiple members of the related family of tropomyosin

proteins, such as tropomyosin 1 (TPM1); tropomyosin 2 (TPM2); tropomyosin 3 (TPM3); tropomyosin 4 (TPM4); or a common or shared sequence within two or more of these related proteins. In another embodiment, a diagnostic reagent or device comprises multiple ligands, each ligand capable of identifying a different TPM family member.

[0115] In another embodiment, a diagnostic reagent or device comprises a ligand capable of specifically complexing with, binding to, or quantitatively detecting or identifying CLIC4 and one or more ligands capable of specifically complexing with, binding to, or quantitatively detecting or identifying individually one or multiple members of the related family of tropomyosin proteins, or the TPM common or shared sequence. In still other embodiments, the device or reagent designed to identify CLIC4 and/or multiple TPMs can contain ligand capable of specifically complexing with, binding to, or quantitatively detecting or identifying other additional EOC biomarkers, such as the unique peptides directed to the above biomarkers and as disclosed in International Patent Application No. PCT/US12/54136, incorporated by reference herein.

[0116] Thus, a diagnostic reagent or device as described herein can comprise 1 to 2, 3, 4, 5, 6, 7, 8, 10, 15 or 20 or more such ligands to identify an equivalent number of biomarkers. In still other embodiments, diagnostic reagents or devices for use in the methods of diagnosing ovarian cancer include ligand capable of specifically complexing with, binding to, or quantitatively detecting or identifying CLIC4 and/or multiple TPMs and one or more additional target biomarkers or peptide fragments identified herein, including, but not limited to, isoforms or molecular forms thereof, pro-form, modified molecular form, or unique peptide fragment or nucleic acid fragment thereof, or proteins in the same biomarker family or expressed from a related gene, having at least 20% sequence homology or sequence identity with the identified biomarker.

[0117] In still another embodiment, one or more of said ligands in the diagnostic reagent or device is associated with a molecule or moiety capable alone or in combination with one or more additional molecules of generating a detectable signal, a detectable label or portion of a detectable label system. In another embodiment, a diagnostic reagent includes one or more ligand capable of specifically complexing with, binding to, or quantitatively detecting or identifying target biomarker or peptide fragment identified herein, immobilized on a substrate. In still another embodiment, combinations of such labeled or immobilized ligands are suitable reagents and components of a diagnostic kit or device.

[0118] In one aspect the reagent, device or kit comprises or consists of ligands that complex with, bind to, or quantitatively detect or identify multiple isoforms or multiple related proteins of any of biomarkers mentioned herein. In another aspect, the reagent, device or kit comprises or consists of ligands that individually specifically complex with, bind to, or quantitatively detect or identify two or more biomarkers in a biomarker family. Still other diagnostic reagents are the ligands to the surrogate peptides used for the MRM assays, such as, but not limited to, the peptides disclosed herein.

[0119] Any combination of labeled or immobilized ligands can be assembled in a diagnostic kit or device for the

purposes of diagnosing ovarian cancer, such as those combinations of ligands to target biomarker sets discussed herein.

[0120] For these reagents, the labels may be selected from among many known diagnostic labels, including those described above. Similarly, the substrates for immobilization in a device may be any of the common substrates, glass, plastic, a microarray, a microfluidics card, a chip, a bead or a chamber.

[0121] In another embodiment, the diagnostic reagent or device includes a ligand that binds to or complexes with a biomarker as identified above or a unique peptide thereof, or a molecular form thereof, or a related protein family member expressed by a different, related gene, or a combination of such ligands. In certain aspects, the diagnostic reagent or device includes ligands which can include an antibody or fragment of an antibody, an antibody mimic, a synthetic antibody, a single chain antibody or an equivalent that binds to or complexes with a single biomarker, said ligand optionally associated with a detectable label or with a substrate. Such antibodies may be presently extant in the art or presently used commercially, such as those available as part of commercial antibody sandwich ELISA assay kits or that may be developed by techniques now common in the field of immunology. A recombinant molecule bearing the binding portion of a biomarker antibody, e.g., carrying one or more variable chain CDR sequences that bind e.g., CLIC4, TPM1, TPM2, TPM3, TPM4, PRDX6, CTSD-30, CDSD-52, CLIC1, etc. may also be used in a diagnostic assay. As used herein, the term “antibody” may also refer, where appropriate, to a mixture of different antibodies or antibody fragments that bind to the selected biomarker. Such different antibodies may bind to different biomarkers or different portions of the same biomarker protein than the other antibodies in the mixture. Such differences in antibodies used in the assay may be reflected in the CDR sequences of the variable regions of the antibodies. Such differences may also be generated by the antibody backbone, for example, if the antibody itself is a non-human antibody containing a human CDR sequence, or a chimeric antibody or some other recombinant antibody fragment containing sequences from a non-human source. Antibodies or fragments useful in the compositions or methods described herein may be generated synthetically or recombinantly, using conventional techniques or may be isolated and purified from plasma or further manipulated to increase the binding affinity thereof. It should be understood that any antibody, antibody fragment, or mixture thereof that binds one of the biomarkers described herein or a particular sequence of the selected biomarker or peptide fragment thereof may be employed in the compositions and methods, regardless of how the antibody or mixture of antibodies was generated. Various forms of antibody, e.g., polyclonal, monoclonal, recombinant, chimeric, as well as fragments and components (e.g., CDRs, single chain variable regions, etc.) or antibody mimics or equivalents may be used in place of antibodies. The ligand itself may be labeled or immobilized.

[0122] In another embodiment, the reagent ligands are nucleotide sequences, the diagnostic reagent is a polynucleotide or oligonucleotide sequence that hybridizes to gene, gene fragment, gene transcript or nucleotide sequence encoding a biomarker discussed herein or encoding a unique peptide thereof. Such a polynucleotide/oligonucleotide can be a probe or primer, and may itself be labeled or immobi-

lized. In one embodiment, ligand-hybridizing polynucleotide or oligonucleotide reagent(s) are part of a primer-probe set, and the kit comprises both primer and probe. Each said primer-probe set amplifies a different gene, gene fragment or gene expression product that encodes a different biomarker discussed herein, optionally including one or more additional known biomarkers, also as described above. For use in the compositions the PCR primers and probes are preferably designed based upon intron sequences present in the biomarker gene(s) to be amplified selected from the gene expression profile. The design of the primer and probe sequences is within the skill of the art once the particular gene target is selected. The particular methods selected for the primer and probe design and the particular primer and probe sequences are not limiting features of these compositions. A ready explanation of primer and probe design techniques available to those of skill in the art is summarized in U.S. Pat. No. 7,081,340, with reference to publically available tools such as DNA BLAST software, the Repeat Masker program (Baylor College of Medicine), Primer Express (Applied Biosystems); MGB assay-by-design (Applied Biosystems); Primer3 (Steve Rozen and Helen J. Skaletsky (2000) Primer3 on the WWW for general users and for biologist programmers and other publications.

[0123] In general, optimal PCR primers and probes used in the compositions described herein are generally 17-30 bases in length, and contain about 20-80%, such as, for example, about 50-60% G+C bases. Melting temperatures of between 50 and 80° C., e.g. about 50 to 70° C. are typically preferred.

[0124] In one embodiment, such a ligand binding to a protein biomarker or a unique peptide contained therein, can be an antibody which specifically binds a single biomarker such as CLIC4, or a unique peptide in that single biomarker or a nucleic acid sequence which hybridizes to the nucleic acid sequence encoding CLIC4 or its unique peptide. In one embodiment, such a ligand desirably binds to a protein biomarker or a unique peptide contained therein, and can be an antibody which specifically binds to one or more of the TPM family members, e.g., TPM1, 2, 3, or 4 individually, collectively, or individually to a unique peptide in that single TPM biomarker, or to a TPM common or shared biomarker. In another embodiment, such a ligand can hybridize to the nucleic acid sequence encoding the protein biomarker or a unique peptide contained therein, where the biomarker is one or more of the TPM family members, e.g., TPM1, 2, 3, or 4 individually, collectively, or individually to a nucleic acid encoding a unique peptide in that single TPM biomarker, or to a TPM common or shared biomarker.

[0125] In another embodiment, suitable labeled or immobilized reagents include at least 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11 or more ligands or antibodies, in which each ligand binds to or complexes with a single biomarker protein/peptide, fragment, or molecular form of the biomarker(s) listed in detail above. In some of the embodiments in which the combination of biomarkers including those listed specifically above or another known additional biomarker that may be in higher abundance in serum, ligands to each of these additional biomarkers may be employed in the diagnostic reagent.

[0126] Any combination of labeled or immobilized biomarker ligands can be assembled in a diagnostic kit or device for the purposes of diagnosing ovarian cancer.

[0127] Thus, a kit or device can contain multiple reagents or one or more individual reagents. For example, one

embodiment of a composition includes a substrate upon which the biomarkers or ligands are immobilized. In another embodiment, the kit also contains optional detectable labels, immobilization substrates, optional substrates for enzymatic labels, as well as other laboratory items.

[0128] The diagnostic reagents, devices, or kits compositions based on the biomarkers or fragments described herein or including the ligands thereto, optionally associated with detectable labels, can be presented in the format of a microfluidics card, a chip or chamber, a bead or a kit adapted for use with assays formats such as sandwich ELISAs, multiple protein assays, platform multiplex ELISAs, such as the BioRad Luminex platform, mass spectrometry quantitative assays, or PCR, RT-PCR or Q PCR techniques.

[0129] The selection of the ligands, biomarker sequences, their length, suitable labels and substrates used in the reagents and kits are routine determinations made by one of skill in the art in view of the teachings herein of which biomarkers form signatures suitable for the diagnosis of ovarian cancer. Assembly of the ligands and biomarkers discussed herein, attachment to a substrate, and assembly into the form of a microarray, a microfluidics card, a chip, a bead, or a chamber employ techniques known in the art.

IV. Methods for Diagnosing or Monitoring Ovarian Cancer

[0130] In another embodiment, a method for diagnosing or detecting or monitoring the progress of ovarian cancer and treatment of ovarian cancer in a subject comprises, or consists of, a variety of steps.

[0131] A. Sample Preparation

[0132] The test sample is obtained from a human subject who is to undergo the treatment or is in the process of being treated. The subject's sample can in one embodiment be provided before initial diagnosis, so that the method is performed to diagnose the existence of an ovarian cancer.

[0133] In another embodiment, depending upon the reference standard and markers used, the method is performed to diagnosis the stage of ovarian cancer. In another embodiment, depending upon the reference standard and markers used, the method is performed to diagnosis the type or subtype of ovarian cancer from the types and subtypes identified above. In another embodiment, the subject's sample can be provided after a diagnosis, so that the method is performed to monitor progression of an ovarian cancer. In another embodiment, the sample can be provided prior to surgical removal of an ovarian tumor or prior to therapeutic treatment of a diagnosed ovarian cancer and the method used to thereafter monitor the effect of the treatment or surgery, and to check for relapse. In another embodiment, the sample can be provided following surgical removal of an ovarian tumor or following therapeutic treatment of a diagnosed ovarian cancer, and the method performed to ascertain efficacy of treatment or relapse. In yet another embodiment the sample may be obtained from the subject periodically during therapeutic treatment for an ovarian cancer, and the method employed to track efficacy of therapy or relapse. In yet another embodiment the sample may be obtained from the subject periodically during therapeutic treatment to enable the physician to change therapies or adjust dosages. In one or more of these embodiments, the subject's own prior sample can be employed in the method as the reference standard.

[0134] Preferably where the sample is a fluid, e.g., blood, serum or plasma, obtaining the sample involves simply withdrawing and preparing the sample in traditional fashion for contact with the diagnostic reagent. Where the sample is a tissue or tumor sample, it may be prepared in conventional manner for contact with the diagnostic reagent.

[0135] The method further involves contacting the sample obtained from a test subject with a diagnostic reagent as described above under conditions that permit the reagent to bind to or complex with one or more biomarker(s), e.g., CLIC4 and/or multiple TPM biomarkers, and/or additional biomarkers which may be present in the sample. This method may employ any of the suitable diagnostic reagents or kits or compositions described above.

[0136] B. Measuring Biomarker Levels

[0137] Thereafter, a suitable assay is employed to detect or measure in the sample the protein level (actual or relative) of one or more biomarker(s), including CLIC4 and/or multiple TPMs, and/or additional biomarkers discussed herein. Alternatively, a suitable assay is employed to generate a protein abundance profile (actual or relative or ratios thereof) of multiple biomarkers discussed herein from the sample or of multiple different molecular forms of the same biomarker or both. In another embodiment, the above method further includes measuring in the biological sample of the subject the protein level of one or more additional biomarkers, such as CTSD-30 kDa, CA125 or other known ovarian cancer biomarker identified herein or other known EOC biomarker known in the art.

[0138] In another embodiment, the above method further includes measuring in the biological sample of the subject the protein levels of two or more additional biomarkers which form with CLIC4 and/or multiple or common or shared TPMs, a biomarker protein abundance signature for ovarian cancer. In one embodiment, the measurement of all target biomarkers occurs in a single sample. In another embodiment, the measurement of all target biomarkers occurs in a multiple samples from a single patient. It should be understood that the measurement of all biomarkers need not occur simultaneously or in the same assay. Results from multiple assays may be combined providing that they are performed within a reasonable time for comparison with the other target biomarker levels.

[0139] The measurement of the biomarker(s) in the biological sample may employ any suitable ligand, e.g., antibody, antibody mimic or equivalent (or antibody to any second biomarker) to detect the biomarker protein, as described above. Similarly, the antibodies may be tagged or labeled with reagents capable of providing a detectable signal, depending upon the assay format employed. Such labels are capable, alone or in concert with other compositions or compounds, of providing a detectable signal. Where more than one antibody is employed in a diagnostic method for a single biomarker, e.g., such as in a sandwich ELISA, the labels are desirably interactive to produce a detectable signal. Most desirably, the label is detectable visually, e.g. colorimetrically. A variety of enzyme systems operate to reveal a colorimetric signal in an assay, e.g., glucose oxidase (which uses glucose as a substrate) releases peroxide as a product that in the presence of peroxidase and a hydrogen donor such as tetramethyl benzidine (TMB) produces an oxidized TMB that is seen as a blue color. Other examples include horseradish peroxidase (HRP) or alkaline phosphatase (AP), and hexokinase in conjunction with glucose-

6-phosphate dehydrogenase that reacts with ATP, glucose, and NAD⁺ to yield, among other products, NADH that is detected as increased absorbance at 340 nm wavelength.

[0140] Other label systems that may be utilized in the methods and devices of this invention are detectable by other means, e.g., colored latex microparticles (Bangs Laboratories, Indiana) in which a dye is embedded may be used in place of enzymes to provide a visual signal indicative of the presence of the resulting selected biomarker-antibody complex in applicable assays. Still other labels include fluorescent compounds, radioactive compounds or elements. Preferably, an anti-biomarker antibody is associated with, or conjugated to a fluorescent detectable fluorochromes, e.g., fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), coriphosphine-O (CPO) or tandem dyes, PE-cyanin-5 (PC5), and PE-Texas Red (ECD). Commonly used fluorochromes include fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), and also include the tandem dyes, PE-cyanin-5 (PC5), PE-cyanin-7 (PC7), PE-cyanin-5.5, PE-Texas Red (ECD), rhodamine, PerCP, fluorescein isothiocyanate (FITC) and Alexa dyes. Combinations of such labels, such as Texas Red and rhodamine, FITC+PE, FITC+PECy5 and PE+PECy7, among others may be used depending upon assay method.

[0141] Detectable labels for attachment to antibodies useful in diagnostic assays and devices of this invention may be easily selected from among numerous compositions known and readily available to one skilled in the art of diagnostic assays. The biomarker-antibodies or fragments useful in this invention are not limited by the particular detectable label or label system employed. Thus, selection and/or generation of suitable biomarker antibodies with optional labels for use in this invention is within the skill of the art, provided with this specification, the documents incorporated herein, and the conventional teachings of immunology.

[0142] Similarly the particular assay format used to measure the selected biomarker in a biological sample may be selected from among a wide range of protein assays, such as described in the examples below. Suitable assays include enzyme-linked immunoassays, sandwich immunoassays, homogeneous assays, immunohistochemistry formats, or other conventional assay formats. In one embodiment, a serum/plasma sandwich ELISA is employed in the method. In another embodiment, a mass spectrometry-based assay is employed. In another embodiment, a MRM assay is employed, in which antibodies are used to enrich the biomarker in a manner analogous to the capture antibody in sandwich ELISAs. One of skill in the art may readily select from any number of conventional immunoassay formats to perform this invention.

[0143] Other reagents for the detection of protein in biological samples, such as peptide mimetics, synthetic chemical compounds capable of detecting the selected biomarker may be used in other assay formats for the quantitative detection of biomarker protein in biological samples, such as high pressure liquid chromatography (HPLC), immunohistochemistry, etc.

[0144] Employing ligand binding to the biomarker proteins or multiple biomarkers forming the signature enables more precise quantitative assays, as illustrated by the multiple reaction monitoring (MRM) mass spectrometry (MS) assays. As an alternative to specific peptide-based MRM-MS assays that can distinguish specific protein isoforms and proteolytic fragments, the knowledge of specific molecular

forms of biomarkers allows more accurate antibody-based assays, such as sandwich ELISA assays or their equivalent. Frequently, the isoform specificity, protein domain specificity and affects of posttranslational modifications on binding of immune reagents used in pre-clinical (and some clinical) diagnostic tests are not well defined. MRM-MS assays were used to quantitative the levels of a number of the low abundance biomarkers in samples, as discussed in the examples.

[0145] In one embodiment, suitable assays for use in these methods include immunoassays using antibodies or ligands to the above-identified biomarkers and biomarker signatures. In another embodiment, a suitable assay includes a multiplexed MRM based assay for two more biomarkers that include one or more of the proteins/unique peptides described herein. It is anticipated that ultimately the platform most likely to be used in clinical assays will be multi-plexed or parallel sandwich ELISA assays or their equivalent, primarily because this platform is the technology most commonly used to quantify blood proteins in clinical laboratories. MRM MS assays may continue to be used productively to help evaluate the isoform/molecular form specificity of any existing immunoassays or those developed in the future. In addition, multiplexed quantitative MS assays such as MRM MS may replace ELISA assays in clinical laboratories in some situations.

[0146] C. Detection of a Change in Biomarker Abundance Level and Diagnosis

[0147] The protein level of the one or more biomarker(s) in the subject's sample or the protein abundance profile of multiple said biomarkers as detected by the use of the assays described above is then compared with the level of the same biomarker or biomarkers in a reference standard or reference profile. In one embodiment, the comparing step of the method is performed by a computer processor or computer-programmed instrument that generates numerical or graphical data useful in the appropriate diagnosis of the condition. Optionally, the comparison may be performed manually.

[0148] The detection or observation of a change in the protein level of a biomarker or biomarkers in the subject's sample from the same biomarker or biomarkers in the reference standard can indicate an appropriate diagnosis. An appropriate diagnosis can be identifying a risk of developing ovarian cancer, a diagnosis of ovarian cancer (or stage or type thereof), a diagnosis or detection of the status of progression or remission of ovarian cancer in the subject following therapy or surgery, a determination of the need for a change in therapy or dosage of therapeutic agent. The method is thus useful for early diagnosis of disease, for monitoring response or relapse after initial diagnosis and treatment or to predict clinical outcome or determine the best clinical treatment for the subject.

[0149] In one embodiment, the change in protein level of each biomarker can involve an increase of a biomarker or multiple biomarkers in comparison to the specific reference standard. In one embodiment, the biomarker CLIC4 is increased in a subject sample from a patient having ovarian cancer when compared to the levels of these biomarkers from a healthy reference standard. In another embodiment, the biomarkers are increased in a subject sample from a patient having ovarian cancer prior to therapy or surgery, when compared to the levels of these biomarkers from a post-surgery or post-therapy reference standard.

[0150] In another embodiment, the change in protein level of each biomarker can involve a decrease of a biomarker or multiple biomarkers in comparison to the specific reference standard. In one embodiment, the biomarkers are decreased in a subject sample from a patient having ovarian cancer following surgical removal of a tumor or following chemotherapy/radiation when compared to the levels of these biomarkers from a pre-surgery/pre-therapy ovarian cancer reference standard or a reference standard which is a sample obtained from the same subject pre-surgery or pre-therapy.

[0151] In still other embodiments, the changes in protein levels of the biomarkers may be altered in characteristic ways if the reference standard is a particular type of ovarian cancer, e.g., serous, epithelial, mucinous or clear cell, or if the reference standard is derived from benign ovarian cysts or nodules.

[0152] The results of the methods and use of the compositions described herein may be used in conjunction with clinical risk factors to help physicians make more accurate decisions about how to manage patients with ovarian cancers. Another advantage of these methods and compositions is that diagnosis may occur earlier than with more invasive diagnostic measures.

[0153] D. Alternative Assay Embodiments

[0154] In an alternative embodiment, the method of diagnosis or risk of diagnosis involves using the nucleic acid hybridizing reagent ligands described above to detect a significant change in expression level of the subject's sample biomarker or biomarkers from that in a reference standard or reference expression profile which indicates a diagnosis, risk, or the status of progression or remission of ovarian cancer in the subject. These methods may be performed in other biological samples, e.g., biopsy tissue samples, tissue removed by surgery, or tumor cell samples, including circulating tumor cells isolated from the blood, to detect or analyze a risk of developing an ovarian cancer, as well as a diagnosis of same. Such methods are also known in the art and include contacting a sample obtained from a test subject with a diagnostic reagent comprising a ligand which is a nucleotide sequence capable of hybridizing to a nucleic acid sequence encoding a biomarker or biomarker combination described herein, e.g., CLIC4 and/or multiple TPM proteins, said ligand associated with a detectable label or with a substrate. Thereafter one would detect or measure in the sample or from an expression profile generated from the sample, the expression levels of one or more of the biomarkers or ratios thereof. The expression level(s) of the biomarker(s) in the subject's sample or from an expression profile or ratio of multiple said biomarkers are then compared with the expression level of the same biomarker or biomarkers in a reference standard. A significant change in expression level of the subject's sample biomarker or biomarkers from that in the reference standard indicates a diagnosis, risk, or the status of progression or remission of ovarian cancer in the subject.

[0155] Suitable assay methods include methods based on hybridization analysis of polynucleotides, methods based on sequencing of polynucleotides, proteomics-based methods or immunochemistry techniques. The most commonly used methods known in the art for the quantification of mRNA expression in a sample include northern blotting and in situ hybridization; RNase protection assays; and PCR-based methods, such as reverse transcription polymerase chain reaction (RT-PCR) or qPCR. Alternatively, antibodies may

be employed that can recognize specific DNA-protein duplexes. The methods described herein are not limited by the particular techniques selected to perform them. Exemplary commercial products for generation of reagents or performance of assays include TRI-REAGENT, Qiagen RNeasy mini-columns, MASTERPURE Complete DNA and RNA Purification Kit (EPICENTRE®, Madison, Wis.), Paraffin Block RNA Isolation Kit (Ambion, Inc.) and RNA Stat-60 (Tel-Test), the MassARRAY-based method (Sequenom, Inc., San Diego, Calif.), differential display, amplified fragment length polymorphism (AFLP), and BeadArray™ technology (Illumina, San Diego, Calif.) using the commercially available Luminex 100 LabMAP system and multiple color-coded microspheres (Luminex Corp., Austin, Tex.) and high coverage expression profiling (HiCEP) analysis.

[0156] The comparison of the quantitative or relative expression levels of the biomarkers may be done analogously to that described above for the comparison of protein levels of biomarkers.

[0157] E. Non-Ligand-Based Analysis

[0158] In another aspect, a method for diagnosing or detecting or monitoring the progress of ovarian cancer in a subject involves non-ligand based methods, such as mass spectrometry. For example, proteins in a biological sample obtained from a test subject may be contacted with a chemical or enzymatic agent and the proteins, including the biomarkers contained therein fragmented in the sample. The digested sample or portions thereof are injected into a mass spectrometer and the protein levels or ratios of one or more of the biomarker or biomarker combinations described herein, optionally with other known biomarkers, modified molecular forms, peptides and unique peptides or ratios thereof, are quantitatively identified or measured by mass spectrometry. The protein levels of the biomarkers in the subject's sample are then compared with the level of the same biomarker or biomarkers in a reference standard or to a predetermined cutoff derived from the reference standard. In one embodiment, the agent is a proteolytic enzyme. In another embodiment, the agent is trypsin.

[0159] A significant change in protein level of the subject's sample biomarker or biomarkers from that in the reference standard or from a predetermined cutoff indicates a diagnosis, risk, or the status of progression or remission of ovarian cancer in the subject.

[0160] Thus, the various methods, devices and steps described above can be utilized in an initial diagnosis of ovarian cancer or other ovarian condition, as well as in clinical management of patients with ovarian cancer after initial diagnosis. Uses in clinical management of the various devices, reagents and assay methods, include without limitation, monitoring for reoccurrence of disease or monitoring remission or progression of the cancer and either before, during or after therapeutic or surgical intervention, selecting among therapeutic protocols for individual patients, monitoring for development of toxicity or other complications of therapy, and predicting development of therapeutic resistance.

[0161] In one embodiment, the method involves enriching the biomarker protein or one or more peptides produced by specific proteolysis in the sample by contacting the sample with an antibody prior to injecting into a mass spectrometer in a manner analogous to a capture antibody in a conventional sandwich ELISA. In another embodiment, the method involves depleting the sample of non-target proteins prior to

injecting sample into a mass spectrometer. The depletion may also be performed using antibodies to the non-targets. The method described herein may use liquid chromatographic mass spectrometry, such as HPLC. One such method is described in detail in the Examples below.

[0162] F. Illustrative Embodiments

[0163] In one embodiment, a method for diagnosing or detecting or monitoring the progress of ovarian cancer in a subject comprises contacting a sample obtained from a test subject with a diagnostic reagent or device comprising a ligand capable of specifically complexing with, binding to, or quantitatively detecting or identifying the biomarker chloride intracellular channel protein 4 (CLIC4) or an isoform, pro-form, modified molecular form, or unique peptide fragment or nucleic acid fragment thereof. Thereafter, the method involves detecting or measuring in the sample or from a protein level profile generated from the sample, the protein levels of the CLIC4 biomarker. Another step includes comparing the protein levels of the CLIC4 biomarker in the subject's with the level of the same biomarker in a reference standard. A significant change in protein level of the subject's sample from that in the reference standard indicates a diagnosis, risk, or the status of progression or remission of ovarian cancer in the subject.

[0164] In another aspect, the method comprises detecting or measuring in the sample or from a protein level profile generated from the sample, the protein levels of one or more additional ovarian cancer biomarkers; and comparing the protein levels of the CLIC4 biomarker in relation to the levels of the additional biomarkers in the subject's sample with the same biomarkers in a reference standard or profile.

[0165] In another aspect, the method uses as a reference standard a mean, an average, a numerical mean or range of numerical means, a numerical pattern, a ratio, a graphical pattern or a protein level profile derived from the same biomarker or biomarkers in a reference subject or reference population. In certain embodiments, the change in protein level of each biomarker comprises an increase in comparison to said reference or control or a decrease in comparison to said reference or control.

[0166] Use of such methods permits early diagnosis of disease, monitoring relapse after initial diagnosis and treatment, predicting clinical outcome, or determining the best clinical treatment.

IV. EXAMPLES

[0167] As discussed in detail in the examples below, it was discovered that a single member of the chloride intracellular channel proteins, CLIC4, which is expressed from a related but different gene than CLIC1, was readily and with great sensitivity, detectable in ovarian cancer patient sera. In additional aspects, related tropomyosin proteins expressed from a gene other than the gene expressing TPM1 and its isoforms, were also detectable in ovarian cancer patient sera. A multiplexed, label-free multiple reaction monitoring (MRM) assay was established to target peptides specific to the detected CLIC and TPM biomarkers, and were quantitated for ovarian cancer patients, patients with benign disease, and normal donors. In addition to CLIC1 and TPM1, which were biomarker proteins discovered in a xenograft mouse model (see PCT/US12/54136, cited above), CLIC4, TPM2, TPM3, and TPM4 were present in ovarian cancer patient sera at significantly elevated levels compared with controls. These additional biomarkers may be superior to the

previously identified biomarkers at discriminating between cancer and noncancer patients.

[0168] The invention is now described with reference to the following examples. These examples are provided for the purpose of illustration only and the invention should in no way be construed as being limited to these examples but rather should be construed to encompass any and all variations that become evident as a result of the teaching provided herein.

Example 1: Materials and Methods

[0169] A. Human Serum Collection and Processing

[0170] Sera from patients with benign ovarian tumors and from late-stage ovarian cancer patients were collected at the University of Turin, Turin, Italy, at the time of diagnosis and have been previously described (Tang et al, 2012, cited above). Control serum samples were collected from healthy, post-menopausal female donors at The Wistar Institute, Philadelphia, Pa. All specimens were processed in compliance with Institutional Review Board (IRB) and Health Insurance Portability and Accountability Act (HIPAA) requirements. Control and patient serum samples were processed for MS analysis either individually or as pools, as previously described. Dithiothreitol (DTT) was obtained from GE Healthcare (Piscataway, N.J.); and iodoacetamide from Sigma-Aldrich (St. Louis, Mo.).

[0171] B. LC-MS/MS of Patient Sample Pools

[0172] To identify all CLIC and TPM isoforms that could be detected in ovarian cancer patient sera, label-free comparisons of pooled sera were performed. One pool of serum from benign patients and three pools of advanced ovarian cancer patient serum samples were made. Serum pools were immunodepleted, separated on a 1D SDS gel for 4 cm, sliced into 40 fractions, and digested with trypsin (sequencing-grade, modified trypsin from Promega, Madison, Wis.) as described in Tang et al, 2012, cited above and incorporated herein by reference). Tryptic digests were analyzed using an LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Waltham, Mass.) connected to a NanoACQUITY UPLC system (Waters, Milford, Mass.). Peptides were eluted at 200 nL/min using a 229 min gradient consisting of 5-28% B over 168 min, 28-50% B over 51.5 min, 50-80% B over 5 min, 80% B for 4.5 min, before returning to 5% B over 0.5 min. A short blank gradient was run before injecting the next sample. The mass spectrometer was set to scan m/z from 400 to 2000. The full MS scan was collected at 60,000 resolution in the Orbitrap in profile mode followed by data-dependent MS/MS scans on the six-most-abundant ions exceeding a minimum threshold of 1000, collected in the linear trap. Monoisotopic precursor selection was enabled and charge-state screening was enabled to reject z=1 ions. Ions subjected to MS/MS were excluded from repeated analysis for 60 s.

[0173] C. Data Processing

[0174] MS/MS spectra were extracted and searched using the SEQUEST algorithm (v. 28, rev. 13, University of Washington, Seattle, Wash.) in Bioworks (v. 3.3.1, Thermo Scientific) against the human UniRef100 protein sequence database (v. June 2011) plus common contaminants. A decoy database was produced by reversing the protein sequence of each database entry, and the entire reversed database was appended in front of the forward database. Spectra were searched with a partial tryptic constraint with up to two missed cleavages, 100 ppm precursor mass tolerance, 1 Da

fragment ion mass tolerance, static modification of Cys (+57.0215 Da), and variable modification of Methionine (+15.9949 Da). Common or shared protein lists were created using DTASelect (v. 2.0, licensed from Scripps Research Institute, La Jolla, Calif.).

[0175] The following filters were applied: remove proteins that are subsets of others, full tryptic constraint, a minimum of two peptides, mass accuracy ≤ 10 ppm, and $\Delta Cn \geq 0.05$ (Wang, H et al., *J Proteome Res* 2011, 10, (11), 4993-5005). The peptide false discovery rate in the resulting dataset was less than 1%. Quantitative comparisons of all detected CLIC and TPM (related proteins from different genes and isoforms of a single gene) across serum pools were performed using Rosetta Elucidator software to compare peptide signal intensities in full MS scans. Retention time alignment, feature identification (discrete ion signals), feature extraction, and protein identifications were performed by the Elucidator system as previously described in Tang et al, 2012, Tang, et al, 2011; and Beer et al, 2011, all cited above.

[0176] D. Label-free MRM Analysis

[0177] MRM experiments were performed on a 5500 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer (AB SCIEX, Foster City, Calif.) interfaced with a NanoACQUITY UPLC system. Eight μ L of tryptic digests were injected using the partial loop injection mode onto a UPLC Symmetry trap column (180 μ m i.d. \times 2 cm packed with 5 μ m C18 resin; Waters) and then separated by RP-HPLC on a BEH C18 nanocapillary analytical column (75 μ m i.d. \times 25 cm, 1.7 μ m particle size; Waters) at 45° C. Chromatography was performed with Solvent A, which contained Milli-Q water with 0.1% formic acid, and Solvent B, which contained HPLC-grade acetonitrile (Thomas Scientific, Swedesboro, N.J.) and 0.1% formic acid. Peptides were eluted at 400 nL/min for 5-35% B over 24 min, 35% B for 3 min before returning to 5% B in 0.5 min.

[0178] To minimize sample carryover, a fast blank gradient was run between each sample. An identical reference sample was run at the beginning of each set of samples and was used to normalize variation in MRM signals caused by changes in performance of the HPLC, reverse phase column or mass spectrometer. MRM data were acquired with a spray voltage of 3300 V, curtain gas of 20 p.s.i., nebulizer gas of 10 p.s.i., interface heater temperature of 150° C., and a pause time of 3 ms. Multiple MRM transitions were monitored using unit resolution in both Q1 and Q3 quadrupoles to maximize specificity. Scheduled MRM was used to reduce the number of concurrent transitions and maximize the dwell time for each transition. The detection window was set at 3 min, and the target scan time was set at 1.8 s. Data analyses were performed using MultiQuant version 2.1 software (AB SCIEX). The most abundant transition for each peptide was used for quantitation unless interference from the matrix was observed. In these cases, another transition free of interference was chosen for quantitation.

[0179] E. Statistical Analyses

[0180] Serum levels of candidate biomarkers were compared across patient groups using the Mann-Whitney test,

and Bonferroni-adjusted P-values were reported in scatter plots. Results were considered statistically significant if the Bonferroni-adjusted P-value of the test was less than 0.05. Spearman's correlation coefficients were calculated to examine correlations among all tested tropomyosin peptides. For each candidate biomarker, a receiver operator characteristic (ROC) curve was generated and the area under the curve was calculated to reflect biomarker specific potential sensitivity and specificity for distinguishing non-cancer and cancer patients.

Example 2: Ambiguities in Identification of EOC Candidate Biomarkers and Isoforms from Analysis of Xenograft Mouse Serum

[0181] We previously identified 106 human proteins with at least two peptides from the serum of a xenograft mouse model of human ovarian endometrioid cancer (TOV-112D tumors) using a gel based, multidimensional protein profiling strategy.²¹ In that study, GeLC-MRM quantitation of several candidate biomarkers in the 20-55 kDa region showed that CLIC1, PRDX6, and the mature form of CTSD were significantly elevated in ovarian cancer patients compared with noncancer (normal and benign disease) individuals.

[0182] Tropomyosin 1 (TPM1) isoform 6 was initially identified as a human protein in the xenograft mouse serum based upon the detection of two human-specific peptides and four peptides common to human and mouse. Changes of species specificity due to database updates was not a widespread problem, because reexamination of the species specificity of our previously identified putative human peptides revealed that only seven peptides (approximately 1%) were identical to new mouse entries in the 2011 database. Since both human and mouse TPM1 are also highly homologous (98 to 99% identical, depending on specific isoforms used for comparison), it is extremely challenging to distinguish between the two species.

[0183] The confident identification of specific biomarkers proteins within proteins families is often ambiguous when using shotgun proteomics, because protease digestion destroys the connectivity between proteins and detected peptides. Inferring the correct peptide-protein association is often confounded by the presence of shared peptides from homologous proteins that are not isoforms from the same gene, alternative splice variants, or redundant database entries (Nesvizhskii, A. I.; Aebersold, R., *Mol Cell Proteomics* 2005, 4, (10), 1419-40). But for MRM quantitation, it is important to accurately determine the peptide-protein relationship to ensure the correct form of a protein is being quantitated.

[0184] In order to determine all potential peptide-protein associations for the observed TPM peptides, each peptide identified in the xenograft mouse was searched against the human UniProtKB database (February, 2012) using the BLAST algorithm. All database entries containing the peptide sequence were identified and redundant entries were manually removed. When available, gene names associated with each database entry were also extracted (see Table 2).

TABLE 2			
Peptides identified in TOV-112D xenograft mouse serum and human serum pools			
Sequence ^a	SEQ ID NO	# Unique Database Entries	Gene
TPM1 Peptides Identified in Xenograft Mouse Serum			
ETAEDVASLN ^b	aa 43-53 of SEQ ID NO. 6	6	TPM1
SLQEQADAAER ^b ,	aa 16-27 of SEQ ID NO. 10	7	TPM1
(K) LVIIESDLER ^b ,	aa 132-142 of SEQ ID NO: 10	18	TPM1
MEIQEIQLK	aa 105-113 of SEQ ID NO. 6	38	TPM1, TPM3
IQLVEEELDR ^b ,	aa 43-53 of SEQ ID NO. 6	43	TPM 1, TPM2, TPM3, TPM4
TPM1 Peptides Detected in Patient Serum Pools			
ETAEDVASLNR ^b	aa 43-54 of SEQ ID NO. 6	6	TPM1
SLQEQADAAER ^b ,	aa 16-27 of SEQ ID NO. 10	7	TPM1
(K) LVIIESDLER ^b ,	aa 132-142 of SEQ ID NO. 6	18	TPM1
MEIQEIQLK	aa 105-113 of SEQ ID NO. 6	38	TPM1, TPM3
(R) IQLVEEELDR ^b ,	aa 55-65 of SEQ ID NO. 6	43	TPM1, TPM2, TPM3, TPM4
AELSEGQVR ^b	aa 147-155 of SEQ ID NO: 10	10	TPM 1
YEEEEK ^c	aa 185-190 of SEQ ID NO. 6	57	TPM1, TPM2, TPM3, TPM4
ATDAEADVSLNR ^{c, d}	17	19	TPM1, TPM2
TPM2 Peptides Detected in Patient Serum Pools			
(K) LVILEGELER ^b	18	11	TPM 2, TPM4
(R) IQLVEEELDR ^b	aa 55-65 of SEQ ID NO: 10	43	TPM1, TPM2, TPM3, TPM4
ATDAEADVSLNR ^c	17	19	TPM1, TPM2
MELQEMQLK	20	8	TPM2
SLMASEEEYSTK	21	3	TPM2
YEEEEK ^c ,	aa 185-190 of SEQ ID NO. 6	57	TPM1, TPM2, TPM3, TPM4
EDKYEEEEK	22	31	TPM2, TPM3, TPM4
CGDLEEEELK ^c	23	6	TPM2, TPM4

TABLE 2-continued			
Peptides identified in TOV-112D xenograft mouse serum and human serum pools			
Sequence ^a	SEQ ID NO	# Unique Database Entries	Gene
TPM3 Peptides Detected in Patient Serum Pools			
(K) IQVLQQQADDAEER ^b ,	24	16	TPM3
(R) IQLVEEELDR ^b ,	aa 55-65 of SEQ ID NO: 10	43	TPM1, TPM2, TPM3, TPM4
MELQEIQLK	25	38	TPM1, TPM3
(K) LVIIEGDLER	26	19	TPM3
HIAEEADR	27	22	TPM3, TPM4
MLDQTLDLNEM	28	12	TPM3
YEEEIK ^c ,	aa 185-190 of SEQ ID NO. 6	57	TPM1, TPM2, TPM3, TPM4
EDKYEEEIK	22	31	TPM2, TPM3, TPM4
CLSAAEEK	29	14	TPM3
AADAAEVLNR	30	1	TPM3
TPM4 Peptides Detected in Patient Serum Pools			
(K) IQALQQQADEAEDR ^b	31	3	TPM4
(K) LVILEGELER ^b	18	11	TPM2, TPM4
EENVGLHQTLDTLNL NCI	32	4	TPM4
AEGLVAALNR ^b	13	4	TPM4
MEIQEMQLK	33	2	TPM4
YSEKEDKYEEEIK	34	4	TPM4
CGDLEELK ^c	23	6	TPM2, TPM4
(R) IQLVEEELDR ^b ,	aa 55-65 of SEQ ID NO: 10	43	TPM1, TPM2, TPM3, TPM4
HIAEEADR	27	22	TPM3, TPM4
EKAEGDVAALNR	35	3	TPM4
YEEEIK ^c ,	aa 185-190 of SEQ ID NO. 6	57	TPM1, TPM2, TPM3, TPM4
EDKYEEEIK	22	31	TPM2, TPM3, TPM4
TIDDLLEK	36	4	TPM4
ASDAEGDVAALNR	37	1	TPM4

NOTES

^aDetected peptides shared by multiple gene products are listed under all matching proteins. Underlined sequences were identified as human in the UniProtKB 2007 database, but also matched a mouse homolog in a later database

^bPeptide targeted for MRM. Successful quantitation.

^cPeptide targeted for MRM. Unsuccessful quantitation.

^dThis peptide is not present in the group of TPM 1 isoforms (FIG. 3) defined by peptides identified from the xenograft mouse serum.

[0185] These peptides, i.e., KLVIIESDLE, aa132-142 of SEQ ID NO: 10, LVIIESDLER, aa133-142 of SEQ ID NO: 10; M*EIQEIQLK, aa105-113 of SEQ ID NO. 6; ETAEAD-VASLNR, aa43-54 of SEQ ID NO. 6; IQLVEEELDR, aa 43-53 of SEQ ID NO:6; and SLQEQADAAEER, aa16-27 of SEQ ID NO: 10, show a great degree of ambiguity in peptide-protein association due to the large number of TPM family members and the number of known isoforms of several of the family members. Tropomyosin is encoded by four genes (TPM1 to TPM4), and each gene can further generate multiple isoforms by the use of alternative promoters and/or alternative RNA splicing. More than 40 distinct TPM family proteins and isoforms have been reported in vertebrates (Gunning, P. W et al, *Trends Cell Biol* 2005, 15, (6), 333-41; Choi, C et al, *J Struct Biol* 2012, 177, (1), 63-9). The TPM1 peptides identified from the xenograft model were initially assigned to TPM1 isoform 6 (Q7Z6L8) using the parsimony principle to explain all the identified peptides. While BLAST indicates TPM1 is present, the exact TPM1 isoform cannot be ascertained, and the presence of the related proteins, TPM2, TPM3, or TPM4 or the isoforms thereof, cannot be excluded and should be considered.

Example 3: Biomarker Proteins Detectable in Patient Serum Pools that Correlate with EOC

[0186] A. Tropomyosin Family Biomarkers

[0187] The tropomyosins are a family of actin filament-binding proteins that have a well-defined central role in

regulating muscle contraction and cytoskeletal organization in non-muscle cells. Decreased expression of specific tropomyosin isoforms is commonly associated with the transformed phenotype and has been reported for cancer cells and tissues, including EOC (Helfman, D. M. et al, *Adv Exp Med Biol* 2008, 644, 124-31; Raval, G. N. et al, *Oncogene* 2003, 22, (40), 6194-203 and Chow, S. N et al, *Eur J Gynaecol Oncol* 2010, 31, (1), 55-62). The serological level of tropomyosin is not well studied, although high plasma levels of TPM4 have been associated with asbestos exposure, and increased levels of tropomyosin serum antibodies were observed in colorectal cancer patients.

[0188] To determine which TPM family protein or isoform is/are detectable in ovarian cancer patient serum, we used an ovarian patient serum protein dataset from in-depth GeLC-MS/MS analysis of the 20-55 kDa region of one benign and three different late-stage ovarian cancer patient immunoaffinity depleted serum pools.

[0189] In the case of TPM1, one new TPM1-specific peptide and two shared peptides were discovered in the patient serum in addition to previously identified TPM1 isoform peptides from the xenograft mouse serum (See FIG. 3, Table 1 and Table 3). Table 3 shows high confidence biomarker proteins/peptides detected in patient sera.

TABLE 3

(Part 1)									
#	Uniprot ID	Name	Description	Seq. Count	Spectrum Count				
1	O00299	CLIC1	Chloride intracellular channel protein 1	12	67				
1a	Q53FB0	CLIC1	Chloride intracellular channel 1 variant	12	67				
2	Q9Y696	CLIC4	Chloride intracellular channel protein 4	10	24				
3a	Q1ZYL5	TPM1	Tropomyosin 1 alpha variant 6	9	94				
3b	B7Z596		cDNA FLJ55130, highly similar to <i>Rattus norvegicus</i> tropomyosin 1, alpha (Tpm1), transcript variant 8, mRNA	9	94				
4a	UR1H_P07951	TPM2	Tropomyosin beta chain	9	125				
4b	UR1H_Q5TCU3	TPM2	Tropomyosin 2 (beta)	9	125				
4c	UR1H_Q5TCU8	TPM2	Tropomyosin 2 (beta)	9	125				
5a	P07951-2	TPM2	Isoform 2 of Tropomyosin beta chain	9	107				
5b	A7XZE4	TPM2	Beta Troppomyosin isoform	9	107				
6	P06753	TPM3	Tropomyosin alpha-3 chain	9	77				
7a	Q5VU59	TPM3	Tropomyosin-3	12	112				
7b	Q5HYB6	TPM3	DKFZp686J1372	12	112				
7c	B2RDE1	TPM3	cDNA, FLJ96568	12	112				
7d	E2RB38	TPM3	Uncharacterized protein	12	112				
8	P67936	TPM4	Tropomyosin alpha-4 chain	16	243				
9	P67936-2	TPM4	Isoform 2 of Tropomyosin alpha-4 chain	13	149				
(Part 2)									
#	Seq Coverage	MolWt	pI	ObsM + H+	Ppm	z	XCorr	DeltCN	SpR
1	67.7	26923	5.2	1932.9794	3.1	2	4.4164	0.4663	1
1a	67.7	27015	5.2	1281.67	1.3	2	4.0828	0.3965	1
				1844.9752	0	2	4.3773	0.5176	1
				2992.3948	1.4	3	5.7564	0.546	1
				1328.6461	1.6	2	3.3785	0.4031	1

TABLE 3-continued

2	52.6	28772	5.6	1095.6344	0.2	2	2.4544	0.1432	1
				1065.6228	5.9	2	2.3876	0.0719	1
				2573.1345	4.3	2	3.7106	0.3711	1
				2061.072	1.7	3	3.2149	0.2172	2
				1572.8121	2	2	2.8708	0.1944	1
				1078.5076	-2.1	2	2.2701	0.16	6
				957.4788	-0.1	2	2.0529	0.062	13
				1264.7158	0.9	2	3.8998	0.3919	1
				2595.1702	1.8	3	4.4279	0.3832	1
				1385.6783	1.1	3	3.8417	0.2936	1
				1223.6191	3.1	2	3.0191	0.2578	1
				1079.6346	2.3	2	2.66	0.1121	1
				1313.5336	1.7	2	2.695	0.2585	1
				1602.8118	2.2	2	2.3922	0.2966	1
				1966.9231	-0.9	2	2.6045	0.239	1
				1588.8486	1.1	3	2.2303	0.1033	81
				1533.7018	1	2	1.9489	0.0741	34
3a	28.6	28509	4.8	1243.6528	0	2	3.6574	0.283	1
3b	25.5	31753	4.9	1346.6198	1.1	2	3.8906	0.3488	1
4a	21.8	32851	4.7	1186.6775	8.3	2	2.8985	0.1839	1
				1147.6036	0.8	2	3.3535	0.1265	1
				1275.6222	3.7	2	3.696	0.3306	1
				1314.7639	0.9	3	3.8858	0.3198	1
				988.5064	0.6	2	2.6379	0.1755	1
				1399.7528	-0.9	3	3.3619	0.2539	11
				810.3899	2.3	2	1.8363	0.0982	13
				1298.7697	1.4	3	4.5416	0.2826	1
4b	21.8	32815	4.7	1170.6774	3.9	2	3.6614	0.2594	1
4c	19.3	36747	4.8	1243.6528	0	2	3.6574	0.283	1
5a	22.9	32990	4.7	1332.6372	-1.3	2	3.5477	0.261	1
				1181.5554	1.2	2	2.9144	0.1323	3
				1092.4814	-5.8	2	3.0957	0.1527	2
				1399.7528	-0.9	3	3.3619	0.2539	11
				810.3899	2.3	2	1.8363	0.0982	13
				1182.5557	2.7	2	2.3489	0.1214	35
				1298.7697	1.4	3	4.5416	0.2826	1
				1170.6774	3.9	2	3.6614	0.2594	1
				1243.6528	0	2	3.6574	0.283	1
				1332.6372	-1.3	2	3.5477	0.261	1
				1181.5554	1.2	2	2.9144	0.1323	3
				1399.7528	-0.9	3	3.3619	0.2539	11
				1390.6013	-2.1	2	2.326	0.3071	1
				810.3899	2.3	2	1.8363	0.0982	13
				1182.5557	2.7	2	2.3489	0.1214	35
				1243.6528	0	2	3.6574	0.283	1
				1147.6036	0.8	2	3.3535	0.1265	1
				1284.7534	0.9	3	3.5549	0.2933	1
6	21.5	32819	4.7	1156.6655	7.2	2	3.2166	0.1613	2
				1399.7528	-0.9	3	3.3619	0.2539	11
				940.4491	0.8	2	2.3445	0.1573	1
				1316.6499	4.4	3	3.194	0.0726	2
				810.3899	2.3	2	1.8363	0.0982	13
				1182.5557	2.7	2	2.3489	0.1214	35
				1642.8102	4.3	2	5.1395	0.4843	1
7a	35.8	27175	4.8	1770.897	-0.6	3	4.6197	0.4128	1
7b	35.8	27176	4.7	1243.6528	0	2	3.6574	0.283	1
7c	33.5	29018	4.8	1147.6036	0.8	2	3.3535	0.1265	1
7d	33.5	29033	4.8	1284.7534	0.9	3	3.5549	0.2933	1
8	46.8	28522	4.7	1156.6655	7.2	2	3.2166	0.1613	2
				1399.7528	-0.9	3	3.3619	0.2539	11
				940.4491	0.8	2	2.3445	0.1573	1
				1467.6772	4.6	2	2.864	0.2482	2
				810.3899	2.3	2	1.8363	0.0982	13
				1182.5557	2.7	2	2.3489	0.1214	35
				907.4202	1.4	2	1.7519	0.0886	33
				1614.7726	0.5	2	4.8416	0.5205	1
				1742.8723	3.2	3	5.3142	0.4742	1
				1298.7697	1.4	3	4.5416	0.2826	1
				2340.1206	3	2	5.717	0.2539	1
				1170.6774	3.9	2	3.6614	0.2594	1
				1243.6528	0	2	3.6574	0.283	1
				1015.5159	-0.8	2	3.1643	0.3492	1
				1181.5554	1.2	2	2.9144	0.1323	3
				1689.7849	-0.3	3	3.9281	0.2333	1
				1092.4814	-5.8	2	3.0957	0.1527	2
				1399.7528	-0.9	3	3.3619	0.2539	11
				940.4491	0.8	2	2.3445	0.1573	1

TABLE 3-continued

9	35.9	32723	4.7	1272.659	3.7	3	3.0739	0.2454	11
				810.3899	2.3	2	1.8363	0.0982	13
				1182.5557	2.7	2	2.3489	0.1214	35
				962.4657	-2.1	2	1.5493	0.0793	90
				1298.7697	1.4	3	4.5416	0.2826	1
				2340.1206	3	2	5.717	0.2539	1
				1170.6774	3.9	2	3.6614	0.2594	1
				1243.6528	0	2	3.6574	0.283	1
				1181.5554	1.2	2	2.9144	0.1323	3
				1689.7849	-0.3	3	3.9281	0.2333	1
				1092.4814	-5.8	2	3.0957	0.1527	2
				1399.7528	-0.9	3	3.3619	0.2539	11
				1288.6102	-1.9	2	2.8922	0.1827	1
				940.4491	0.8	2	2.3445	0.1573	1
				810.3899	2.3	2	1.8363	0.0982	13
				1182.5557	2.7	2	2.3489	0.1214	35
				962.4657	-2.1	2	1.5493	0.0793	90

TABLE 3

(Part 3)		
No	Sequence ^b	SEQ ID NO.
1a	K.FLDGNELTLADCNLLPK.L	38
1b	K.GVTFNVTTVDTK.R	39
	K.LAALNPESNTAGLDIF AK.F	40
	K.VLDNYLTSPLPEEVDETS AEDEGVSR.K	41
	K.NSNPALNDNLEK.G	42
	K.LHIVQVVCK.K	43
	R.LFM*VLWLK.G	44
	R.EEFASTCPDDEEIELAYEQVAK.A	45
	R.KFLDGNELTLADCNLLPK.L	46
	K.IEEFLEAVLCPPR.Y	47
	K.IGNCPFSQR.L	48
	R.YLSNAYAR.E	49
2	KGVVFSVTTVDLK.R	aa 48-61 of SEQ ID NO: 1
	K.LDEYLN SPLPDEIDE NSM*EDIK.F	aa 150-173 of SEQ ID NO: 1
	K.NSRPEANEALER.G	aa 130-143 of SEQ ID NO: 1
	K.EVEIAYS DVAK.R	aa 237-250 of SEQ ID NO: 1
	R.LFM*ILWLK.G ^c	aa 40-49 of SEQ ID NO: 1
	R.DEFTNTCPSDK.E	aa 227-239 of SEQ ID NO: 1
	K.IEEFLEEVLCPPK.Y	aa 90-104 of SEQ ID NO: 1
	K.FLDGNEM*TLADCNLLPK.L	aa 177-195 of SEQ ID NO: 1

TABLE 3-continued

(Part 3)		
No	Sequence ^b	SEQ ID NO.
	K.EEDKEPLIELFVK.A	aa 11-25 of SEQ ID NO: 1
	K.HPESNTAGM*DIFAK.F	aa 1 10-125 of SEQ ID NO: 1
3a	R.IQLVEEELDR.A	50
3b	R.SLQEQADAA EER.A	aa 15-28 of SEQ ID NO: 10
	K.LVIIESDLER.A	aa 132-143 of SEQ ID NO: 10
	K.M*EIQEIQLK.E	aa 104-114 of SEQ ID NO. 6
	R. ETAEADV ASLNR.R	aa 42-55 of SEQ ID NO. 6
	R.KLVIIESDLER.A	aa 131-143 of SEQ ID NO: 10
	R.AELSEGQVR.Q	aa 147-156 of SEQ ID NO: 10
	R.RIQLVEEELDR.A	51
	R.YEEEEIK.V	aa 184-191 of SEQ ID NO. 6
4a	R.KLVILEGELER.S	19
4b	K.LVILEGELER.S	52
4c	R.IQLVEEELDR.A	50
	K.ATDAE ADV ASLNR.R	53
	K.M*ELQEM*QLK.E	54
	K.CGDLEEELK.I	55
	R.RIQLVEEELDR.A	51
	K.YEEEEIK.L	56
	K.EDKYEEEEIK.L	58

TABLE 3-continued		
(Part 3)		
No	Sequence ^b	SEQ ID NO.
5a	R.KLVILEGELER.S	19
5b	K.LVILEGELER.S	52
	R.IQLVEEELDR.A	50
	K.ATDAEADVASLNR.R	53
	K.M*ELQEM*QLK.E	54
	R.RIQLVEEELDR.A	51
	K.SLM*ASEEEYSTK.E	57
	K.YEEEEIK.L	56
	K.EDKYYYYEIK.L	58
6	R.IQLVEEELDR.A	50
	KM*ELQEIQLK.E	59
	R.KLVIIIEGDLER.T	60
	K.LVIIIEGDLER.T	61
	R.RIQLVEEELDR.A	51
	K.HIAEEADR.K	62
	K.AA DAAEAVASLNR.R	63
	K.YEEEEIK.I	64
	K.EDKYYYYEIK.I	65
7a	KIQVLQQQADDAEERA	66
7b	RKIQVLQQQADDAEER.A	67
7c	RIQLVEEELDR.A	50
7d	KM*ELQEIQLKE	68
	RKLVIIIEGDLER T	60
	K.LVIIIEGDLER.T	61
	RRIQLVEEELDRA	50
	KHIAEEADRK	62
	RM*LDQTLLDLNEM*	69
	KYEEEEIKI	64
	K.EDKYYYYEIK.I	65
	KCLSAA EEKY	70
8	K.IQALQQQADEAEDRA	71
	RKIQALQQQADEAEDR.A	72
	RKLVILEGELERA	73
	KEENVGLHQTLDTLNLNCL	74
	KLVILEGELERA	aa 2-13 of SEQ ID NO: 73

TABLE 3-continued		
(Part 3)		
No	Sequence ^b	SEQ ID NO.
	RIQLVEEELDRA	aa 2-14 of SEQ ID NO: 50
	KAEGDVAA LNR.R	aa 3-14 of SEQ ID NO: 75
	K.M*EIQEM*QLK.E	76
	K.YSEKEDKYEEEIK.L	77
	K.CGDLEEELKN	78
	R.RIQLVEEELDRA	50
	K.HIAEEADRK	62
	REKAEGDVAA LNR.R	75
	K.YEEEEIK.L	56
	K.EDKYYYYEIK.L	58
	K.TIDDLEEK.L	79
9	RKLVILEGELERA	73
	KEENVGLHQTLDTLNLNCL	74
	K.LVILEGELER.A	aa 2-13 of SEQ ID NO: 73
	R.IQLVEEELDR.A	50
	K.M*EIQEM*QLK.E	76
	K.YSEKEDKYEEEIK.L	77
	K.CGDLEEELKN	78
	R.RIQLVEEELDR.A	51
	KASDAEGDVAA LNR.R	80
	K.HIAEEADR.K	62
	K.YEEEEIK.L	56
	K.EDKYYYYEIK.L	58
	K.TIDDLEEK.L	79

*All peptides mapping to listed protein isoforms are shown, i.e., peptides shared by multiple protein isoforms are listed for each of these multiple proteins.
^b Different forms of the same peptide (charge states and methionine oxidation) were collapsed and displayed as a single peptide. M* indicates methionine oxidation.
^cThis peptide is also present in CLIC2, CLIC5, and CLIC6.

[0190] Based on the newly identified AELSEGQVR, AA147-155 of SEQ ID NO: 10, peptide, all observed peptides were contained within two TPM1 isoforms, TPM1 variant 6 (Q1ZYL5) or B7Z596. These two sequences share 80% identity and differ from each other at the C-terminus. Distinguishing between these isoforms was not feasible in this study due to the inability to detect any isoform-specific C-terminal peptides. Although no other TPM1 isoforms or TPM family members were conclusively identified in human serum, their presence cannot be ruled out. But the failure to detect any unique peptides to other TPM family members or

TPM1 isoforms suggests they are either not present or are present in much lower abundance in human serum.

[0191] The TPM family proteins and/or their isoforms identified in the patient sera were quantitated by summing MS intensities for all peptides unique to a specific gene product (see FIGS. 1A through 1D). There was evidence of protein products for all four TPM genes and the expressed related gene products showed elevated levels in EOC.

[0192] B. Chloride Ion Channel Protein Biomarkers

[0193] Two CLIC family proteins were identified in the patient sera and were quantitated by summing MS intensities for all peptides unique to a specific gene product (see FIGS. 1E and 1F). The previously identified CLIC1 was confirmed to be both detected and elevated in ovarian cancer patient serum compared to benign disease.

[0194] Another CLIC family member protein CLIC4 was newly identified in the ovarian cancer patient sera. CLIC4 is a multifunctional protein that has been shown to be highly expressed in ovarian cancer stroma and may play an important role in cancer development (Yao, Q et al., *Oncol Rep* 2009, 22, (3), 541-8; Shukla, A.; Yuspa, S. H., *Nucleus* 2010, 1, (2), 144-9). CLIC4 was detected by nine specific peptides and showed elevated levels in ovarian cancer patient sera, suggesting that it was an EOC biomarker. The observation of CLIC4 in ovarian cancer patient sera raised the question as to why human CLIC1 had been previously identified in the xenograft mouse serum, but CLIC4 had not been detected.

[0195] Examination of this data showed that CLIC4 was identified by four peptides, all peptides identical to mouse sequences, i.e., K.GVVFSVTTVDLK.R, aa48-61 of SEQ ID NO: 1; K.HPESNTAGM*DIFAK.F, aa110-125 of SEQ ID NO: 1; K.LDEYLNPLPDEIDENSM*EDIK.F, aa150-173 of SEQ ID NO: 1; and R.KPADLQNLAPGTH-PPFITFNSEVK.T, aa61-86 of SEQ ID NO: 1. This is not surprising, as the human and mouse CLIC4 sequences are 99% identical (See FIG. 2A and Table 4). While distinguishing between mouse and human CLIC4 is very difficult, this problem is limited to the xenograft mouse model. In con-

trast, distinguishing the different CLIC family proteins, e.g., gene products, in human serum is more straightforward, as the sequence homologies of the four CLIC genes with similar molecular weights are somewhat lower. Specifically, the two CLIC family proteins detected, CLIC1 and CLIC4, share 67% identity. Hence, most CLIC peptides observed in the xenograft mouse serum and in patient serum pools were unique to either CLIC1 or CLIC4 (see FIG. 2B).

Example 4: Development of MRM Assays for Quantitation of CLIC4 and TPM Isoforms

[0196] CLIC and TPM biomarker protein levels in individual serum samples that included control serum samples (six normal and nine benign) and late-stage cancer samples (15 Stage III and 3 Stage IV) were determined using GeLC-MRM, essentially as previously described in Tang et al 2012, cited above. Peptides were selected based on their isoform specificity and signal intensity in MRM analysis using a 5500 QTRAP mass spectrometer. Peptide candidates for MRM were derived from a combination of the LCMS/MS analyses reported above and all human plasma/serum LC-MS/MS proteomic analyses that had been performed in this laboratory.

[0197] In the case of CLIC4, selection of MRM peptides was relatively straightforward because no major isoform issues were encountered with the identified peptides (FIG. 2B). Inclusion of peptides identified from other serum proteome analyses allowed selection of peptides with the strongest MRM signal. For example, the CLIC4 peptide, YLTNAYSR, aa220-227 of SEQ ID NO: 1, was found to produce a stronger MRM signal than some of the peptides discovered in this analysis and was therefore used for MRM quantitation (See Tables 4 and 5). Table 4 shows MRM peak areas for CLIC4 peptides, which have been normalized and averaged in individual samples as an indication of the protein abundance level.

TABLE 4

Sample #		CLIC4	CLIC4	CLIC4	CLIC4 Norm & Avg
		NSRPEANEALER, aa 131-142 of SEQ ID NO: 1	YLTNAYSR aa220-227 of SEQ ID NO: 1	EVEIAYS DVAK, aa 139-249 of SEQ ID NO: 1	
Normal	WCS02	71938	75784	5917	0.171
Normal	WCS04	71504	94424	13193	0.214
Normal	WCS12	58303	67969	5269	0.146
Normal	WCS13	92896	92885	21819	0.268
Normal	WCS14	133409	130748	38618	0.410
Normal	WCS15	90830	100865	28662	0.298
Benign	B23	106848	144779	28506	0.355
Benign	B25	93943	128049	23848	0.308
Benign	B70	142389	164356	28363	0.412
Benign	B77	180776	242906	75215	0.699
Benign	B79	121455	162778	39150	0.427
Benign	B80	233045	287533	87063	0.841
Benign	B81	125616	150244	36499	0.411
Benign	B82	162386	269199	41618	0.578
Benign	B83	120162	217288	50882	0.518
Cancer	T455	361477	343172	106250	1.106
Cancer	T474	444226	449309	98203	1.264
Cancer	T475	205961	249565	36565	0.590
Cancer	T476	120474	170584	22532	0.371
Cancer	T478	236002	254455	93543	0.839
Cancer	T482	380003	516406	80345	1.187
Cancer	T536	443069	340463	308048	1.942
Cancer	T539	249682	306233	49728	0.739
Cancer	T541	87267	113303	17938	0.266

TABLE 4-continued

Sample	#	CLIC4 NSRPEANEALER, aa 131-142 of SEQ ID NO: 1	CLIC4 YLTNAYSR aa220-227 of SEQ ID NO: 1	CLIC4 EVEIAYSQVAK, aa 139-249 of SEQ ID NO: 1	CLIC4 Norm & Avg
Cancer	T543	107946	206527	44406	0.471
Cancer	T553	418869	618499	114770	1.446
Cancer	T556	959630	1244493	256878	3.143
Cancer	T557	266741	378642	60233	0.862
Cancer	T577	176341	183183	41724	0.516
Cancer	T600	360817	393236	87364	1.080
Cancer	T602	167445	266095	68203	0.679
Cancer	T603	257545	473664	89399	1.045
Cancer	T604	114903	174142	46015	0.455
Cancer	T455	361477	343172	106250	1.106

[0198] Table 5 shows MRM peak areas for CLIC1 peptides normalized and averaged in individual samples as an indication of the protein abundance level.

could be present (See FIG. 3). Therefore, AELSEGQVR, aa147-155 of SEQ ID NO: 10, which was specific to TPM1v6, and three other peptides shared by several TPM1

TABLE 5

Sample	#	CLIC1 GVTFNVTTVDTK, aa38-49 of SEQ ID NO: 3	CLIC1 LAALNPESNTAG LDIFAK, aa96-113 of SEQ ID NO: 3	CLIC1 NSNPALNDNLEK, aa120-131 of SEQ ID NO: 3	CLIC1 Norm & Avg
Normal	WCS02	112466	46620	67000	0.099
Normal	WCS04	201327	82090	148050	0.190
Normal	WCS12	33884	30160	51100	0.054
Normal	WCS13	28858	32810	30770	0.044
Normal	WCS14	164134	155000	173700	0.230
Normal	WCS15	104943	85700	87400	0.128
Benign	B23	143772	113800	131900	0.179
Benign	B25	105300	27596	58447	0.082
Benign	B70	210921	194300	220300	0.313
Benign	B77	680255	387400	491200	0.704
Benign	B79	452783	340500	393500	0.544
Benign	B80	754653	168561	528925	0.623
Benign	B81	168894	56262	137171	0.159
Benign	B82	1367992	623771	1039990	1.347
Benign	B83	549811	153388	395116	0.476
Cancer	T455	980711	546125	697954	0.999
Cancer	T474	819310	351580	518150	0.745
Cancer	T475	286624	205100	204300	0.318
Cancer	T476	414920	306150	290460	0.463
Cancer	T478	485804	298830	183150	0.435
Cancer	T482	2816690	2008424	2505141	3.373
Cancer	T536	1178896	964000	1143310	1.516
Cancer	T539	651564	398980	428100	0.667
Cancer	T541	49917	27680	36000	0.051
Cancer	T543	511810	232600	314500	0.469
Cancer	T553	1993369	1119800	1503310	2.076
Cancer	T556	2808408	2207100	2532200	3.472
Cancer	T557	651252	444800	440800	0.699
Cancer	T577	346698	214500	277400	0.380
Cancer	T600	567452	358500	471000	0.633
Cancer	T602	455781	280500	317400	0.476
Cancer	T603	771899	505560	620200	0.862
Cancer	T604	280053	204600	316300	0.367

[0199] Selecting appropriate peptides for MRM quantitation of TPM1 isoforms and TPM family proteins in general, and TPM1 specifically, was more complicated due to the large number of TPM family members and isoforms. While TPM1 variant 6 (or isoform B7Z596) was clearly identified in the human serum samples, other TPM1 isoforms also

isoforms and other TPM family members were used for MRM quantitation (see Table 1, and Table 6). Table 6 shows MRM peak areas for TPM1 peptides normalized and averaged in individual samples as an indication of the protein abundance level.

TABLE 6

Sample	#	TPM1v6 LVIIESDLR, aa133-142 of SEQ ID NO: 10	TPM1v6 AELSEGQVR aa147-155 of SEQ ID NO: 10	TPM1v6 ETAEADVAS LNR, aa43-54 of SEQ ID NO. 6	TPM1v6 SLQEQADA AEER, aa16- 27 of SEQ ID NO: 10	TPM1v6 Norm& Avg
Normal	WCS02	27232	74344	20979	11989	0.142
Normal	WCS04	8305	80477	13397	9571	0.089
Normal	WCS12	2975	54062	9440	6603	0.056
Normal	WCS13	4094	41958	0	4239	0.033
Normal	WCS14	44163	149293	40975	35597	0.287
Normal	WCS15	11741	63033	15414	9777	0.093
Benign	B23	47853	257616	51054	38261	0.357
Benign	B25	14674	123609	30139	23165	0.175
Benign	B70	46999	382365	66409	43960	0.440
Benign	B77	101250	492555	105122	72337	0.713
Benign	B79	50274	251060	48504	38126	0.355
Benign	B80	145616	872565	306980	196336	1.542
Benign	B81	45480	275283	60738	47356	0.395
Benign	B82	53860	368472	128792	84337	0.638
Benign	B83	56220	197314	86720	62744	0.500
Cancer	T455	67782	428888	90666	64820	0.583
Cancer	T474	181863	1122884	207117	179329	1.506
Cancer	T475	50783	255305	45158	34454	0.344
Cancer	T476	119220	523437	163132	86059	0.885
Cancer	T478	120774	500688	118103	98305	0.832
Cancer	T482	71530	313303	43307	31577	0.398
Cancer	T536	431057	1930569	410941	320850	2.941
Cancer	T539	104008	533374	123592	91636	0.806
Cancer	T541	0	31284	0	0	0.012
Cancer	T543	56020	257055	57840	44155	0.397
Cancer	T553	209330	1191620	294342	236825	1.854
Cancer	T556	279781	1873212	438823	361745	2.754
Cancer	T557	140328	662386	164727	117159	1.048
Cancer	T577	25267	233927	50457	44460	0.316
Cancer	T600	69940	417227	96263	70731	0.605
Cancer	T602	50571	210865	65650	51836	0.399
Cancer	T603	234813	1190812	242276	167008	1.667
Cancer	T604	57236	423975	125785	80801	0.652

[0200] We also attempted to target TPM2, TPM3, and TPM4, as products of these genes were also identified in the GeLC-MS/MS analysis of patient serum pools (Table 3). Of course, as is typically the case, strong, consistent, interference-free MRM signals could not be obtained for all desired peptides. The final MRM assay contained one peptide specific to TPM3, two peptides specific to TPM4, one peptide shared by TPM2 and TPM4, and one peptide shared by all four TPM genes (See Table 1 and Table 7). Table 7 shows MRM peak areas for TPM3 and TPM4 peptides normalized and averaged in individual samples as an indication of the protein abundance level.

TABLE 7

Sample	#	TPM3 IQVLQQADDAEER, SEQ ID NO: 12	TPM3 Norm& Avg	TPM4 IQALQQQADEADR, SEQ ID NO: 14	TPM4 AEGDVAALNR, SEQ ID NO: 13	TPM4 KLVILEGELER, SEQ ID NO: 18	TPM4 Norm& Avg
Normal	WCS02	37607	0.116	66530	657724	630468	0.110
Normal	WCS04	62540	0.192	57080	425479	419663	0.079
Normal	WCS12	11573	0.036	13120	179765	212806	0.030
Normal	WCS13	0	0.000	0	70176	140492	0.012
Normal	WCS14	44708	0.137	18371	173740	238804	0.034
Normal	WCS15	23374	0.072	21477	215144	266941	0.039
Benign	B23	50826	0.156	92784	769719	861540	0.144
Benign	B25	31580	0.097	64680	592048	641548	0.106
Benign	B70	215328	0.661	203602	1962777	1862029	0.331
Benign	B77	205003	0.630	455168	3475540	3751568	0.660
Benign	B79	113763	0.349	173730	1258455	1590148	0.257
Benign	B80	651126	2.000	1431956	10630968	9275897	1.913
Benign	B81	135085	0.415	167413	269426	1290576	0.237
Benign	B82	533252	1.638	85394	681075	841789	0.134
Benign	B83	210727	0.647	216592	1631897	1912271	0.320
Cancer	T455	181883	0.559	329762	3198211	3703179	0.517
Cancer	T474	385261	1.183	1033082	8868498	8154397	1.539
Cancer	T475	78718	0.242	138849	1355419	1412261	0.235
Cancer	T476	287108	0.882	740508	7091117	6861693	1.207

TABLE 7-continued

Sample	#	TPM3	TPM3 Norm& Avg	TPM4	TPM4	TPM4	TPM4 Norm& Avg
		IQVLQQQADDAEER, SEQ ID NO: 12		IQALQQQADEAEDR, SEQ ID NO: 14	AEGDVAALNR, SEQ ID NO: 13	KLVILEGELER, SEQ ID NO: 18	
Cancer	T478	163723	0.503	327030	2662922	2613253	0.480
Cancer	T482	114583	0.352	264911	2633676	2720894	0.453
Cancer	T536	876097	2.691	1796201	16550308	16225609	2.863
Cancer	T539	249410	0.766	492600	3835421	3916627	0.711
Cancer	T541	0	0.000	7022	47530	49422	0.009
Cancer	T543	147454	0.453	143235	1017944	977404	0.191
Cancer	T553	909385	2.793	1389863	8780337	10176023	1.830
Cancer	T556	1070392	3.288	2182304	16019177	17806655	3.116
Cancer	T557	394944	1.213	729285	6244945	7007301	1.157
Cancer	T577	66040	0.203	126311	872615	987295	0.174
Cancer	T600	191799	0.589	466198	4198007	6027998	0.842
Cancer	T602	69239	0.213	168912	1430173	1592082	0.265
Cancer	T603	477013	1.465	1191352	10077129	11834475	1.908
Cancer	T604	197319	0.606	346242	2351772	2126914	0.442

[0201] Table 8 shows MRM peak areas for the TPM common peptide normalized in individual samples as an indication of the protein abundance level.

TABLE 8

Sample	#	TPM	TPM Norm & Avg
		RIQLVEEELDR, SEQ ID NO: 16	
Normal	WCS02	716335	0.114
Normal	WCS04	680639	0.108
Normal	WCS12	273406	0.043
Normal	WCS13	121600	0.019
Normal	WCS14	519755	0.083
Normal	WCS15	444269	0.071
Benign	B23	1018792	0.162
Benign	B25	784378	0.125
Benign	B70	2396858	0.381
Benign	B77	4183895	0.065
Benign	B79	1122745	0.274
Benign	B80	12378679	1.967
Benign	B81	1384354	0.220
Benign	B82	3515417	0.559
Benign	B83	2601924	0.413
Cancer	T455	3561471	0.566
Cancer	T474	9345962	1.485
Cancer	T475	1536987	0.244
Cancer	T476	7037409	1.118
Cancer	T478	3371869	0.536
Cancer	T482	2992930	0.476
Cancer	T536	17007129	2.702
Cancer	T539	4446598	0.706
Cancer	T541	148179	0.024
Cancer	T543	1574956	0.250
Cancer	T553	13973974	2.220
Cancer	T556	19572892	3.110
Cancer	T557	6989658	1.111
Cancer	T577	1159454	0.184
Cancer	T600	5455926	0.867
Cancer	T602	1665550	0.265
Cancer	T603	10720865	1.703
Cancer	T604	2728471	0.434

Example 5: Quantitation of TPM and CLIC Isoforms in Patient Serum Samples

[0202] GeLC-MRM quantitation of the CLIC and TPM peptides and normalized protein values for individual patient samples are summarized in Tables 3-8. TPM peptide amounts were graphically compared across all serum samples as a first-level test of potential differences across

patients in peptides specific for certain isoforms and those shared by multiple isoforms both within the TPM1 isoform group and across related gene products (See Table 3).

[0203] Similarly, the protein levels (normalized and averaged peptides values) across patient samples were compared as shown in FIG. 4. All the peptides monitored displayed similar quantitative profiles, although some minor variations were observed in some samples (See FIG. 4 and Table 3). Spearman's rank correlation coefficient analysis showed that all tropomyosin peptides analyzed here are highly correlated within each patient group with P-values<0.001 (data not shown), indicating a lack of evidence that specific tropomyosins differ from other family members and isoforms in being able to distinguish between ovarian cancer, benign disease, and normal donors. Also, if alternative TPM family proteins share some of the quantified peptides, their contribution is either minor or they track with the TPM proteins and isoforms quantitated here. Since the distribution of all tropomyosin peptides is similar, the peptide (K)LVI-LEGELER SEQ ID NO: 18 that is shared between TPM2 and TPM4 was assigned to TPM4 for the purpose of calculating the TPM4 protein level. In addition, factor analysis shows that all the tropomyosin proteins analyzed here are measuring the same factor (data not shown), which is consistent with the similar quantitative profiles shown in FIG. 4.

[0204] These data show that multiple TPM related proteins and isoforms thereof are present in human serum. At least in the current cohort, fluctuations in abundance levels related to benign ovarian disease and ovarian cancer for TPM family members and their isoforms appear to change in concert. However, it is possible that certain TPM isoforms may be more selective for specific clinical applications such as monitoring responses to different therapies or disease reoccurrence. Tropomyosin proteins are also known to be modified by post-translational modifications such as acetylation and phosphorylation, and the influence of post-translationally modified forms in ovarian cancer diagnosis has not yet been tested.

[0205] GeLC-MRM quantitative results for CLIC1 and CLIC4 peptide and protein levels also are shown in Tables 6 and 5, respectively. As expected, since the same patient samples were used, CLIC1 results were similar, but not identical, to the previously reported results in Tang et al, 2012, cited above, for this protein. There was some moder-

ate variation because the two sets of label-free measurements were performed at different times and on two different instruments. That is, previous analyses were performed on an AB SCIEX 4000 QTRAP and the current results were from an AB SCIEX 5500 QTRAP instrument. The CLIC1 measurements were repeated here to provide a direct comparison to the newly identified biomarker CLIC4. For both CLIC proteins, all peptides from the same protein showed similar distributions among the individual patient samples (data not shown), indicating that the MRM signals used for quantitation were derived from the same protein and quantitation was not appreciably affected by interfering signals.

Example 6: CLIC and TPM Biomarkers can Distinguish EOC from Non-Cancer Cases

[0206] The capacities of the CLIC and TPM biomarkers to distinguish EOC cases were assessed in several ways using the GeLC-MRM quantitation data (See, Tables 4-8). A two-way comparison between the non-cancer (normal and benign) and cancer groups using the Mann-Whitney test showed that all isoforms could significantly distinguish ($P<0.05$) between cancer and non-cancer (see Table 9).

TABLE 9

GeLC-MRM comparison of non-cancer (normal and benign) versus cancer sera			
UniProt	Name	Descriptive Name	P-value ^a
O00299	CLIC1	Chloride intracellular channel protein 1	0.004
Q9Y696	CLIC4	Chloride intracellular channel protein 4	0.0002
Q1ZYL5	TPM1,	Tropomyosin 1 alpha variant 6	0.0052
	var. 6		
Q5VU59	TPM3	Tropomyosin 3	0.0337
P67936	TPM4	Tropomyosin alpha-4 chain	0.0021

NOTES

^aP-values are from the Mann-Whitney test with Bonferroni adjustment

[0207] Based on the P-value, CLIC4 appeared to be the best biomarker in distinguishing cancer from non-cancer and TPM3 was the weakest biomarker.

[0208] For further evaluation, the normal and benign samples were compared separately to the cancer group (FIG. 5). All protein isoforms could distinguish between normal donors and ovarian cancer patients at a highly significant level ($P<0.0015$). However, CLIC4 was the only biomarker that showed a significant difference between benign disease and EOC.

[0209] The TPM isoforms did not show a statistically significant difference between benign disease and cancer primarily because a single benign sample (B80) had a much higher abundance level for all TPM isoforms than other samples in that group. Future analysis of larger cohorts will allow us to more definitively identify which proteins can reliably distinguish benign disease from ovarian cancer.

[0210] Although benign ovarian tumors and ovarian cancer appear to be very different diseases at a genetic level, a major challenge at the initial diagnosis stage in the clinic is to distinguish benign ovarian conditions from malignant ones.

[0211] To evaluate the potential diagnostic efficacy for each of these proteins, receiver operating characteristic (ROC) curve analyses were performed on the non-cancer and cancer groups (See FIG. 6). Consistent with the Mann-Whitney test, CLIC4 showed the largest area under the curve

(AUC) and TPM3 showed the lowest area. It is anticipated that larger patient cohorts may support the use of CLIC4 and tropomyosin family proteins or isoforms, when used with other biomarkers simultaneously and in combination may outperform use of a single biomarker for detection and clinical monitoring of EOC.

[0212] To our knowledge, the plasma levels of CLIC4 and tropomyosin in ovarian cancer patients have not been reported previously.

[0213] It is anticipated that testing of the biomarkers and biomarkers sets described herein in larger cohorts of patients collected from different sites, longitudinal prediagnostic blood specimens, and specimens collected throughout therapeutic treatment will demonstrate results consistent with the above.

[0214] Each and every patent, patent application, and publication, including U.S. provisional patent application No. 61/709,695, the publications listed herein, and publicly available peptide sequences, cited throughout the disclosure, is expressly incorporated herein by reference in its entirety. While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention are devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims include such embodiments and equivalent variations.

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SEQUENCE LISTING

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MAGSSSLEAV RRKIRSLQEQ ADAAEERAGT LQRELDHERK LRETAEADVA SLNRRIQLVE	60
EELDRAQERL ATALQKLEEA EKAADSERG MKVIESRAQK DEEKMEIQEI QLKEAKHIAE	120
DADRYEEVA RKLVIIESDL ERAEERAELS EGKCAELEEE LKTVTNLKS LEAQAEKYSQ	180
KEDRYEEIK VLSDKLKEAE TRAEFAERSV TKLEKSIDDL EDQLYQQLEQ NRRLTNELKL	240
ALNED	245
SEQ ID NO: 8	moltype = AA length = 303
FEATURE	Location/Qualifiers

-continued

source	1..303	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 8		
GGRDFRTAPG RRGRRRTER	PGRGGPALGS QDSRGSRVRR AAAGLSHCSP PARLPSGAMA	60
GSSSLEAVRR KIRSLQEQAD	AAEERAGTLQ RELDHERKLR ETAEADVASL NRRIQLVEEE	120
LDRAQERLAT ALQKLEEA	EK AADGSEGMK VIESRAQKDE EKMEIQEIQL KEAKHIAEDA	180
DRKYEEVARK LVIIIESDLER	AEERAELSEG KCAELEELK TVTNNLKSLE AQA EKYSQKE	240
DRYEEEEKVL SDKLKEAETR	AEFAERSVTK LEKSIDDL QLYQQLEQNR RLTNELKLAL	300
NED		303
SEQ ID NO: 9	moltype = AA length = 248	
FEATURE	Location/Qualifiers	
source	1..248	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 9		
MAGSSSLEAV RRKIRSLQEQ	ADAAEERAGT LQRELDHERK LRETAEADVA SLNRRIQLVE	60
EELDRAQERL ATALQKLEEA	EKAADSESG MKVIESRAQK DEEKMEIQEI QLKEAKHIAE	120
DADRYEEVA RKLVIIESDL	ERAEERAELS EGKCAELEEE LKTVTNNLKS LEAQA EKYSQ	180
KEDRYEEEEK VLSDKLKEAE	TRAEFAERSV TKLEKSIDDL EEKVAHAKEE NLSMHQMLDQ	240
TLELNNM		248
SEQ ID NO: 10	moltype = AA length = 245	
FEATURE	Location/Qualifiers	
source	1..245	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 10		
MAGSSSLEAV RRKIRSLQEQ	ADAAEERAGT LQRELDHERK LRETAEADVA SLNRRIQLVE	60
EELDRAQERL ATALQKLEEA	EKAADSESG MKVIESRAQK DEEKMEIQEI QLKEAKHIAE	120
DADRYEEVA RKLVIIESDL	ERAEERAELS EGQVRQLEEQ LRIMDQTLKA LMAAEDKYSQ	180
KEDRYEEEEK VLSDKPKEAE	TRAEFAERSV TKLEKSIDDL EDQLYQQLEQ NRRLTNELKL	240
ALNED		245
SEQ ID NO: 11	moltype = AA length = 275	
FEATURE	Location/Qualifiers	
source	1..275	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 11		
MAGSSSLEAV RRKIRSLQEQ	ADAAEERAGT LQRELDHERK LRETAEADVA SLNRRIQLVE	60
EELDRAQERL ATALQKLEEA	EKAADSESG MKVIESRAQK DEEKMEIQEI QLKEAKHIAE	120
DADRYEEVA RKLVIIESDL	ERAEERAELS EGQVRQLEEQ LRIMDQTLKA LMAAEDKYSQ	180
KEDRYEEEEK VLSDKLKEAE	TRAEFAERSV TKLEKSIDDL EGKILSSVFS LILMVEYQPG	240
KTIFQFKGIH IDTLLCTCTF	FLCVLWGFSL WLLNS	275
SEQ ID NO: 12	moltype = AA length = 14	
FEATURE	Location/Qualifiers	
source	1..14	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 12		
IQVLQQQADD AEER		14
SEQ ID NO: 13	moltype = AA length = 10	
FEATURE	Location/Qualifiers	
source	1..10	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 13		
AEGDVAALNR		10
SEQ ID NO: 14	moltype = AA length = 14	
FEATURE	Location/Qualifiers	
source	1..14	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 14		
IQALQQQADE AEDR		14
SEQ ID NO: 15	moltype = AA length = 11	
FEATURE	Location/Qualifiers	
source	1..11	
	mol_type = protein	
	organism = Homo sapiens	

-continued

SEQUENCE: 15		
KLIVILEGELE R		11
SEQ ID NO: 16	moltype = AA length = 11	
FEATURE	Location/Qualifiers	
source	1..11	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 16		
RIQLVEEELD R		11
SEQ ID NO: 17	moltype = AA length = 13	
FEATURE	Location/Qualifiers	
source	1..13	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 17		
ATDAEADVAS LNR		13
SEQ ID NO: 18	moltype = AA length = 11	
FEATURE	Location/Qualifiers	
source	1..11	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 18		
KLIVILEGELE R		11
SEQ ID NO: 19	moltype = AA length = 13	
FEATURE	Location/Qualifiers	
source	1..13	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 19		
RKLVILEGEL ERS		13
SEQ ID NO: 20	moltype = AA length = 9	
FEATURE	Location/Qualifiers	
source	1..9	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 20		
MELQEMQLK		9
SEQ ID NO: 21	moltype = AA length = 12	
FEATURE	Location/Qualifiers	
source	1..12	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 21		
SLMASEEEYS TK		12
SEQ ID NO: 22	moltype = AA length = 9	
FEATURE	Location/Qualifiers	
source	1..9	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 22		
EDKYEEEIK		9
SEQ ID NO: 23	moltype = AA length = 9	
FEATURE	Location/Qualifiers	
source	1..9	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 23		
CGDLEEELK		9
SEQ ID NO: 24	moltype = AA length = 15	
FEATURE	Location/Qualifiers	
source	1..15	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 24		
KIQVLQQQAD DAEER		15
SEQ ID NO: 25	moltype = AA length = 9	

-continued

FEATURE	Location/Qualifiers	
source	1..9 mol_type = protein organism = Homo sapiens	
SEQUENCE: 25 MELQEIQLK		9
SEQ ID NO: 26 FEATURE	moltype = AA length = 11 Location/Qualifiers	
source	1..11 mol_type = protein organism = Homo sapiens	
SEQUENCE: 26 KLVIIEGDLE R		11
SEQ ID NO: 27 FEATURE	moltype = AA length = 8 Location/Qualifiers	
source	1..8 mol_type = protein organism = Homo sapiens	
SEQUENCE: 27 HIAEEADR		8
SEQ ID NO: 28 FEATURE	moltype = AA length = 12 Location/Qualifiers	
source	1..12 mol_type = protein organism = Homo sapiens	
SEQUENCE: 28 MLDQTL LDLN EM		12
SEQ ID NO: 29 FEATURE	moltype = AA length = 8 Location/Qualifiers	
source	1..8 mol_type = protein organism = Homo sapiens	
SEQUENCE: 29 CLSAAEEK		8
SEQ ID NO: 30 FEATURE	moltype = AA length = 13 Location/Qualifiers	
source	1..13 mol_type = protein organism = Homo sapiens	
SEQUENCE: 30 AADAAEVAS LNR		13
SEQ ID NO: 31 FEATURE	moltype = AA length = 15 Location/Qualifiers	
source	1..15 mol_type = protein organism = Homo sapiens	
SEQUENCE: 31 KIQALQQQAD EAEDR		15
SEQ ID NO: 32 FEATURE	moltype = AA length = 20 Location/Qualifiers	
source	1..20 mol_type = protein organism = Homo sapiens	
SEQUENCE: 32 EENVGLHQTL DQTLNELNCI		20
SEQ ID NO: 33 FEATURE	moltype = AA length = 9 Location/Qualifiers	
source	1..9 mol_type = protein organism = Homo sapiens	
SEQUENCE: 33 MEIQEMQLK		9
SEQ ID NO: 34 FEATURE	moltype = AA length = 13 Location/Qualifiers	
source	1..13 mol_type = protein organism = Homo sapiens	

-continued

SEQUENCE: 34 YSEKEDKYEE EIK		13
SEQ ID NO: 35 FEATURE source	moltype = AA length = 12 Location/Qualifiers 1..12 mol_type = protein organism = Homo sapiens	
SEQUENCE: 35 EKAEGDVAAL NR		12
SEQ ID NO: 36 FEATURE source	moltype = AA length = 8 Location/Qualifiers 1..8 mol_type = protein organism = Homo sapiens	
SEQUENCE: 36 TIDDLEEK		8
SEQ ID NO: 37 FEATURE source	moltype = AA length = 13 Location/Qualifiers 1..13 mol_type = protein organism = Homo sapiens	
SEQUENCE: 37 ASDAEGDVAA LNR		13
SEQ ID NO: 38 FEATURE source	moltype = AA length = 19 Location/Qualifiers 1..19 mol_type = protein organism = Homo sapiens	
SEQUENCE: 38 KFLDGNELTL ADCNLLPKL		19
SEQ ID NO: 39 FEATURE source	moltype = AA length = 14 Location/Qualifiers 1..14 mol_type = protein organism = Homo sapiens	
SEQUENCE: 39 KGVTFNVTTV DTKR		14
SEQ ID NO: 40 FEATURE source	moltype = AA length = 20 Location/Qualifiers 1..20 mol_type = protein organism = Homo sapiens	
SEQUENCE: 40 KLAALNPESN TAGLDIFAKF		20
SEQ ID NO: 41 FEATURE source	moltype = AA length = 29 Location/Qualifiers 1..29 mol_type = protein organism = Homo sapiens	
SEQUENCE: 41 KVLDNYLTSP LPEEVDETSA EDEGVSRK		29
SEQ ID NO: 42 FEATURE source	moltype = AA length = 14 Location/Qualifiers 1..14 mol_type = protein organism = Homo sapiens	
SEQUENCE: 42 KNSNPALNDN LEKG		14
SEQ ID NO: 43 FEATURE source	moltype = AA length = 11 Location/Qualifiers 1..11 mol_type = protein organism = Homo sapiens	
SEQUENCE: 43 KLHIVQVVCK K		11
SEQ ID NO: 44	moltype = AA length = 10	

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FEATURE	Location/Qualifiers	
source	1..10	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 44		
RLFMVLWLKG		10
SEQ ID NO: 45	moltype = AA length = 24	
FEATURE	Location/Qualifiers	
source	1..24	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 45		
REEFASTCPD DEEIELAYEQ VAKA		24
SEQ ID NO: 46	moltype = AA length = 20	
FEATURE	Location/Qualifiers	
source	1..20	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 46		
RKFLDGNELT LADCNLLPKL		20
SEQ ID NO: 47	moltype = AA length = 15	
FEATURE	Location/Qualifiers	
source	1..15	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 47		
KIEEFLEAVL CPPRY		15
SEQ ID NO: 48	moltype = AA length = 11	
FEATURE	Location/Qualifiers	
source	1..11	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 48		
KIGNCPFSQR L		11
SEQ ID NO: 49	moltype = AA length = 11	
FEATURE	Location/Qualifiers	
source	1..11	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 49		
KIGNCPFSQR L		11
SEQ ID NO: 50	moltype = AA length = 12	
FEATURE	Location/Qualifiers	
source	1..12	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 50		
RIQLVEEELD RA		12
SEQ ID NO: 51	moltype = AA length = 13	
FEATURE	Location/Qualifiers	
source	1..13	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 51		
RRIQLVEEEL DRA		13
SEQ ID NO: 52	moltype = AA length = 12	
FEATURE	Location/Qualifiers	
source	1..12	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 52		
KLVILEGELE RS		12
SEQ ID NO: 53	moltype = AA length = 15	
FEATURE	Location/Qualifiers	
source	1..15	
	mol_type = protein	
	organism = Homo sapiens	

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SEQUENCE: 53 KATDAEADVA SLNRR		15
SEQ ID NO: 54 FEATURE source	moltype = AA length = 11 Location/Qualifiers 1..11 mol_type = protein organism = Homo sapiens	
SEQUENCE: 54 KMELQEMQLK E		11
SEQ ID NO: 55 FEATURE source	moltype = AA length = 11 Location/Qualifiers 1..11 mol_type = protein organism = Homo sapiens	
SEQUENCE: 55 KCGDLEELK I		11
SEQ ID NO: 56 FEATURE source	moltype = AA length = 8 Location/Qualifiers 1..8 mol_type = protein organism = Homo sapiens	
SEQUENCE: 56 KYEEEIKL		8
SEQ ID NO: 57 FEATURE source	moltype = AA length = 14 Location/Qualifiers 1..14 mol_type = protein organism = Homo sapiens	
SEQUENCE: 57 KSLMASEEEY STKE		14
SEQ ID NO: 58 FEATURE source	moltype = AA length = 11 Location/Qualifiers 1..11 mol_type = protein organism = Homo sapiens	
SEQUENCE: 58 KEDKYEEEIK L		11
SEQ ID NO: 59 FEATURE source	moltype = AA length = 11 Location/Qualifiers 1..11 mol_type = protein organism = Homo sapiens	
SEQUENCE: 59 KMELQEIQLK E		11
SEQ ID NO: 60 FEATURE source	moltype = AA length = 13 Location/Qualifiers 1..13 mol_type = protein organism = Homo sapiens	
SEQUENCE: 60 RKLVIIEGDL ERT		13
SEQ ID NO: 61 FEATURE source	moltype = AA length = 12 Location/Qualifiers 1..12 mol_type = protein organism = Homo sapiens	
SEQUENCE: 61 KLVIIEGDLE RT		12
SEQ ID NO: 62 FEATURE source	moltype = AA length = 10 Location/Qualifiers 1..10 mol_type = protein organism = Homo sapiens	
SEQUENCE: 62 KHIAEEADRK		10
SEQ ID NO: 63	moltype = AA length = 15	

-continued

FEATURE	Location/Qualifiers	
source	1..15 mol_type = protein organism = Homo sapiens	
SEQUENCE: 63 KAADAAEVA SLNRR		15
SEQ ID NO: 64 FEATURE	moltype = AA length = 8 Location/Qualifiers	
source	1..8 mol_type = protein organism = Homo sapiens	
SEQUENCE: 64 KYEEEEIKI		8
SEQ ID NO: 65 FEATURE	moltype = AA length = 11 Location/Qualifiers	
source	1..11 mol_type = protein organism = Homo sapiens	
SEQUENCE: 65 KEDKYEEEEIK I		11
SEQ ID NO: 66 FEATURE	moltype = AA length = 16 Location/Qualifiers	
source	1..16 mol_type = protein organism = Homo sapiens	
SEQUENCE: 66 KIQVLQQQAD DAEERA		16
SEQ ID NO: 67 FEATURE	moltype = AA length = 17 Location/Qualifiers	
source	1..17 mol_type = protein organism = Homo sapiens	
SEQUENCE: 67 RKIQVLQQQA DDAEERA		17
SEQ ID NO: 68 FEATURE	moltype = AA length = 11 Location/Qualifiers	
source	1..11 mol_type = protein organism = Homo sapiens	
SEQUENCE: 68 KMELQEIQLK E		11
SEQ ID NO: 69 FEATURE	moltype = AA length = 13 Location/Qualifiers	
source	1..13 mol_type = protein organism = Homo sapiens	
SEQUENCE: 69 RMLDQTLLDL NEM		13
SEQ ID NO: 70 FEATURE	moltype = AA length = 10 Location/Qualifiers	
source	1..10 mol_type = protein organism = Homo sapiens	
SEQUENCE: 70 KCLSAAEEKY		10
SEQ ID NO: 71 FEATURE	moltype = AA length = 16 Location/Qualifiers	
source	1..16 mol_type = protein organism = Homo sapiens	
SEQUENCE: 71 KIQALQQQAD EAEDRA		16
SEQ ID NO: 72 FEATURE	moltype = AA length = 17 Location/Qualifiers	
source	1..17 mol_type = protein organism = Homo sapiens	

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SEQUENCE: 72		
RKIQALQQQA DEADRA		17
SEQ ID NO: 73	moltype = AA length = 13	
FEATURE	Location/Qualifiers	
source	1..13	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 73		
RKLVILEGEL ERA		13
SEQ ID NO: 74	moltype = AA length = 21	
FEATURE	Location/Qualifiers	
source	1..21	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 74		
KEENVGLHQT LDQTLNELNC L		21
SEQ ID NO: 75	moltype = AA length = 14	
FEATURE	Location/Qualifiers	
source	1..14	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 75		
REKAEGDVAA LNRR		14
SEQ ID NO: 76	moltype = AA length = 11	
FEATURE	Location/Qualifiers	
source	1..11	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 76		
KMEIQEMQLK E		11
SEQ ID NO: 77	moltype = AA length = 15	
FEATURE	Location/Qualifiers	
source	1..15	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 77		
KYSEKEDKYE EEIKL		15
SEQ ID NO: 78	moltype = AA length = 11	
FEATURE	Location/Qualifiers	
source	1..11	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 78		
KCGDLEEELK N		11
SEQ ID NO: 79	moltype = AA length = 10	
FEATURE	Location/Qualifiers	
source	1..10	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 79		
KTIDDLLEKL		10
SEQ ID NO: 80	moltype = AA length = 15	
FEATURE	Location/Qualifiers	
source	1..15	
	mol_type = protein	
	organism = Homo sapiens	
SEQUENCE: 80		
KASDAEGDVA ALNRR		15

1. A diagnostic reagent comprising one or more of:
 - (a) a ligand capable of specifically complexing with, binding to, or quantitatively detecting or identifying the biomarker chloride intracellular channel protein 4 (CLIC4) or an isoform, pro-form, modified molecular form, posttranslational modification, or unique peptide fragment or unique nucleic acid fragment thereof;
 - (b) a ligand capable of specifically complexing with, binding to, or quantitatively detecting or identifying the biomarker chloride intracellular channel protein 1 (CLIC1) or an isoform, pro-form, modified molecular form, posttranslational modification, or unique peptide fragment or unique nucleic acid fragment thereof;
 - (c) a ligand capable of specifically complexing with, binding to, or quantitatively detecting or identifying the biomarker cancer antigen 125 (CA125) or an isoform, pro-form, modified molecular form, posttranslational modification, or unique peptide fragment or unique nucleic acid fragment thereof; and
 - (d) a ligand capable of specifically complexing with, binding to, or quantitatively detecting or identifying the biomarker human epididymis protein-4 (HE4) or an isoform, pro-form, modified molecular form, posttranslational modification, or unique peptide fragment or unique nucleic acid fragment thereof,
 wherein at least one ligand is attached to a detectable label or immobilized on a solid substrate.
2. The diagnostic reagent according to claim 1, wherein the biomarker CLIC4 is Uniprot ID no. Z9Y696 or a peptide fragment thereof.
3. The diagnostic reagent according to claim 1, wherein the CLIC4 peptide fragment comprises:
 - (a) GVVFSVTTVDLK, aa 49-60 of SEQ ID NO: 1;
 - (b) LDEYLNPLPDEIDENSMEDIK, aa151-172 of SEQ ID NO: 1;
 - (c) NSRPEANEALER, aa 131-142 of SEQ ID NO: 1;
 - (d) YLTNAYSER, aa 20-27 of SEQ ID NO: 1;
 - (d) EVEIAYSDDVAK, aa 139-249 of SEQ ID NO: 1;
 - (e) LFMILWLK, aa 41-48 of SEQ ID NO: 1;
 - (f) DEFTNTCPSDK, aa 228-238 of SEQ ID NO: 1;
 - (g) IEEFLEEVLCPPK, aa 91-103 of SEQ ID NO: 1;
 - (h) FLDGNEMTLADCNLLPK, aa 178-194 of SEQ ID NO: 1;
 - (i) EEDKEPLIELFVK, aa 12-24 of SEQ ID NO: 1; or
 - (j) HPESNTAGMDIFAK, aa 111-124 of SEQ ID NO: 1;
 wherein in each sequence where methionine occurs, it is in the oxidized or unoxidized form.
4. A diagnostic reagent according to claim 1, further comprising one or more additional ligands, each additional ligand capable of specifically complexing with, binding to, or quantitatively detecting, or identifying an additional biomarker that indicates the presence of ovarian cancer in a human subject, wherein said additional biomarker is selected from:
 - i. tropomyosin 1 (TPM1);
 - ii. tropomyosin 2 (TPM2);
 - iii. tropomyosin 3 (TPM3);
 - iv. tropomyosin 4 (TPM4);
 - v. cathepsin D-30 (CTSD-30); and
 - vi. proteasome subunit alpha type-7 (PSMA7)
5. The diagnostic reagent according to claim 1, wherein the CLIC1 peptide fragment comprises:
 - (a) GVTFNVTTVDTK, aa38-49 of SEQ ID NO: 3;
 - (b) LAALNPESNTAGLDIFAK, aa96-113 of SEQ ID NO: 3; or
 - (c) NSNPALNDNLEK, aa120-131 of SEQ ID NO: 3.
6. The diagnostic reagent according to claim 4, wherein an isoform of TPM1 is TPM1, variant 6 (UniProt ID No. Q1ZYL5) or TPM1, variant 8 (UniProt ID No. B7Z596); wherein an isoform of TPM2 is TPM2 beta chain (UniProt ID No. UR1H_P07951, UR1H_Q5TCU3, or UR1H_Q5TCU8) or TPM2, isoform 2 (UniProt ID No. P07951-2, A7XZE4), wherein an isoform of TPM3 is TPM3 alpha-3 chain (UniProt ID No. P06753, Q5VU59, Q5HYB6, B2RDE1, or E2RB38); or wherein an isoform of TPM4 is TPM4 alpha-4 chain (UniProt ID No. P6736), or TPM4, Isoform 2 (UniProt ID No. P67936-2).
7. The diagnostic reagent according to claim 1, wherein any of said ligands comprises an antibody or fragment of an antibody, an antibody mimic, a synthetic antibody, a single chain antibody or an equivalent that binds to or complexes with a single biomarker.
8. The diagnostic reagent according to claim 1, wherein any of said ligands comprises a nucleotide sequence capable of hybridizing to a nucleic acid sequence encoding a single biomarker.
9. The diagnostic reagent according to claim 1, wherein said substrate is a microarray, a microfluidics card, a chip, a bead, or a chamber.
10. A kit, panel, or microarray comprising one or more multiple diagnostic reagents according to claim 1, each reagent identifying a different biomarker.
11. A method for diagnosing or detecting or monitoring the progress or treatment of ovarian cancer in a subject comprising:
 - (i) contacting a sample obtained from a test subject with a diagnostic reagent of claim 1;
 - (ii) detecting or measuring in the sample or from a protein level profile generated from the sample, the protein levels of the selected biomarkers; and
 - (iii) comparing the protein level of the selected biomarkers in the subject's sample with the level of the same biomarkers in a reference standard;
 wherein a significant change in the protein level of one or more of the selected biomarkers in the subject's sample from that in the reference standard indicates a diagnosis, risk, or the status of progression or remission of ovarian cancer in the subject.
12. The method of claim 11, wherein the diagnostic reagent comprises one or more additional ligands, each additional ligand capable of specifically complexing with, binding to, or quantitatively detecting, or identifying an additional biomarker that indicates the presence of ovarian cancer in a human subject, wherein said additional biomarker is selected from:
 - i. tropomyosin 1 (TPM1);
 - ii. tropomyosin 2 (TPM2);
 - iii. tropomyosin 3 (TPM3);
 - iv. tropomyosin 4 (TPM4);
 - v. cathepsin D-30 (CTSD-30); and
 - vi. proteasome subunit alpha type-7 (PSMA7).

- 13.** The method according to claim **12**, wherein an isoform of TPM1 is TPM1, variant 6 (UniProt ID No. Q1ZYL5) or TPM1, variant 8 (UniProt ID No. B7Z596);
- wherein an isoform of TPM2 is TPM2 beta chain (UniProt ID No. UR1H_P07951, UR1H_Q5TCU3, or UR1H_Q5TCU8) or TPM2, isoform 2 (UniProt ID No. P07951-2, A7XZE4),
- wherein an isoform of TPM3 is TPM3 alpha-3 chain (UniProt ID No. P06753, Q5VU59, Q5HYB6, B2RDE1, or E2RB38); or
- wherein an isoform of TPM4 is TPM4 alpha-4 chain (UniProt ID No. P6736), or TPM4, Isoform 2 (UniProt ID No. P67936-2).
- 14.** The method according to claim **12**, further comprising:
- detecting or measuring in the sample or from a protein level profile generated from the sample, the protein levels of one or more additional ovarian cancer biomarkers; and comparing the protein levels of the additional biomarkers in relation to the levels of the additional biomarkers in the subject's sample with the same biomarkers in a reference standard or profile.
- 15.** The method according to claim **14**, wherein the reference standard is a mean, an average, a numerical mean or range of numerical means, a numerical pattern, a ratio, a

graphical pattern or a protein level profile derived from the same biomarker or biomarkers in a reference subject or reference population.

16. The method according to claim **12**, wherein said change in the protein level of each said biomarker comprises an increase in comparison to said reference or control or a decrease in comparison to said reference or control.

17. The method according to claim **12**, wherein said diagnosis comprises early diagnosis of disease, monitoring relapse after initial diagnosis and treatment, predicting clinical outcome, or determining the best clinical treatment.

18. The method according to claim **12**, wherein the biological sample is selected from group consisting of whole blood, plasma, serum, circulating tumor cells, ascites fluid, peritoneal fluid and tumor tissue or tissue or fluid from a biopsy sample, surgical sample, or tumor cell sample.

19. The method according to claim **12**, wherein the biomarker CLIC4 is Uniprot ID no. Z9Y696 or a peptide fragment thereof.

20. The method according to claim **12**, wherein any of said ligands comprises an antibody or fragment of an antibody, an antibody mimic, a synthetic antibody, a single chain antibody or an equivalent that binds to or complexes with a single biomarker.

* * * * *