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- (72) **Inventors; and**
- (71) **Applicants :** WALFISH, Paul G. [CA/CA]; 68 Yorkville Avenue, Toronto, Ontario M5R 3V7 (CA). RALHAN, Ranju [CA/CA]; 385 Conley Street, Thornhill, Ontario L4J 6T2 (CA).
- (74) **Agents:** CHARI, Santosh K. et al.; Blake, Cassels & Graydon LLP, 199 Bay Street, Suite 4000, Commerce Court West, Toronto, Ontario M5L 1A9 (CA).
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(54) **Title:** METHODS AND COMPOSITIONS FOR THE DIAGNOSIS OF A THYROID CONDITION

(57) **Abstract:** Methods for detecting thyroid cancer or for differentiating malignant thyroid tissue from non-malignant thyroid tissue comprises measuring the amount of one or more of PGK1, PKM2, PTEN, Profilin-1, S100A6, Galectin-3 and Cyclin D1 and measuring under-expression or over-expression of these markers in comparison to normal tissue. The methods can be used with a fine needle aspiration biopsy (FNAB), a core biopsy or a cytospin.



1 **METHODS AND COMPOSITIONS FOR THE DIAGNOSIS**
2 **OF A THYROID CONDITION**
3

4 **CROSS REFERENCE TO RELATED APPLICATIONS**

5 **[0001]** This application claims priority under the Paris Convention from US Patent Application Serial
6 Number 14/738,867, filed June 13, 2015, which is incorporated herein by reference. This application
7 is also a Continuation of US Application Number 14/738,867, filed June 13, 2015, which is a
8 Continuation in Part of US Application Number 14/476,222, filed on September 3, 2014, which is a
9 Continuation of US Application Number 13/102,638, filed on May 6, 2011, which claims the benefit
10 under 35 USC §119(e) of US Provisional Patent Application No. 61/332,381, filed May 7, 2010. Each
11 of the aforementioned applications is incorporated by reference herein as set forth in its entirety.

12 **FIELD OF THE DESCRIPTION**

13 **[0002]** The present description relates to methods for diagnosing cancer using biomarkers. In
14 particular, the description relates to the use of one or more protein biomarkers in diagnosing thyroid
15 cancer.

16 **STATEMENT REGARDING SEQUENCE LISTING**

17 **[0003]** A Sequence Listing associated with this application is provided in ASCII format and is
18 submitted electronically herewith. The Sequence Listing is hereby incorporated by reference into the
19 present specification. The text file containing the Sequence listing is titled "SequenceListing.txt", was
20 created on June 10, 2016, and is approximately 560 kilobytes in size.

21 **BACKGROUND**

22 **[0004]** Thyroid cancers are the most common malignancy of the endocrine system [1]. There is
23 currently a lack of methods for accurately diagnosing thyroid cancers (TC), and in particular for
24 differentiating benign thyroid nodules from TC. Clinically detectable thyroid nodules occur in up to
25 10% of the population. In such cases, ultrasound-guided fine needle aspiration biopsy (FNA biopsy or
26 FNAB) is the most specific preoperative technique that is used for diagnosis of thyroid cancer (Baynes
27 et al., 2014). If a patient's FNAB indicates a malignant or suspicious diagnosis, clinicians will often
28 recommend removal of the thyroid gland, or thyroidectomy (Cooper et al., 2009), which is a surgically
29 invasive procedure. Generally, 15-30% of thyroid FNAB cytologic findings are indeterminate and
30 post-operative examination of the thyroid indicates benign histopathology in 75-85% of the cases,
31 suggesting that the thyroidectomy was unnecessary. Such unnecessary invasive procedures could
32 be avoided if a definitive diagnosis was available prior to surgery [25].

33 **[0005]** Patients with inconclusive results and malignant tumors are also at risk for not undergoing
34 adequate treatment as many of them undergo an initial thyroid lobectomy that must be followed up
35 with another surgery to complete the thyroidectomy following diagnosis [3]. Additionally, an issue also
36 arises due to the fact that while most papillary thyroid cancers are non-aggressive with limited to no

1 metastasis, a small percentage are in-fact aggressive and may produce distant metastasis leading to
2 higher mortality [1].

3 **[0006]** Accurate distinction between benign and malignant thyroid nodules therefore has critical
4 therapeutic implications. One of the most challenging areas of thyroid pathology include follicular
5 patterned nodules where the differential diagnosis ranges from benign entities such as adenomatous
6 hyperplastic nodule and follicular adenoma to malignant tumors, most commonly the follicular variant
7 of papillary carcinoma and less commonly, follicular carcinoma [37].

8 **[0007]** Patient genotyping has been explored as a pre-surgical diagnostic test. Currently, two
9 molecular tests based on patient tumor genotyping have become available as pre-surgical diagnostic
10 aids [30, 38, 39]. One is a gene mutation test based on genetic alterations associated with TC
11 identified in PI3K-Akt and MAPK pathways that include rat sarcoma viral oncogene (RAS) point
12 mutations [40, 41], virus-induced rapidly accelerated fibrosarcoma murine sarcoma viral oncogene
13 homolog B (BRAF V600E) [40, 41], rearranged during transfection proto-oncogene/papillary thyroid
14 carcinoma (RET-PTC) [40, 43] and paired box gene 8/peroxisome proliferator-activated receptor
15 gamma (PAX8/PPAR γ) rearrangements [40, 44]. Recently, these mutations have been tested in
16 FNAB to define a clinical algorithm for guiding the appropriate extent of initial thyroidectomy [39].
17 Another test is a gene expression classifier (GEC) test, which is based on the expression profile of
18 142 gene mRNAs [45], and has been independently assessed [46]. One such method is discussed in
19 US Patent Number 8,669,057. Although these new genomic diagnostic tests have been proposed to
20 improve the management of indeterminate nodules, several important issues remain to be resolved
21 including their cost and accuracy before being recommended for widespread clinical use. Thus,
22 patient genotyping for thyroid cancer remains to be tested further for adoption in clinical use.

23 **[0008]** Protein biomarker analyses by immunohistochemical (IHC) subcellular localization have been
24 explored as an adjunct to FNAB cytology to facilitate the pre-surgical diagnosis of TC. In comparison
25 to genomic tests, protein based IHC analysis could offer an alternative approach that would be more
26 efficacious for routine clinical use.

27 **[0009]** Recently, our group analyzed the secretomes of three TC cell lines using proteomics and
28 reported preliminary data based on a small patient cohort (6 benign tissues and 12 TC patients) to
29 demonstrate that some of these identified proteins could be detected in patients' sera and tissues
30 [47]. Furthermore, we observed differential subcellular expression of a subset of proteins in benign
31 and malignant thyroid nodules [47].

32 **[0010]** There exists a need for a biomarker or biomarker combination that would, for example,
33 accurately distinguish benign thyroid nodules from nodules which are at risk for TC among those
34 classified as indeterminate by FNAB cytology.

35 **SUMMARY OF THE DESCRIPTION**

36 **[0011]** The present description relates, in one aspect, to markers of thyroid cancer to methods of
37 diagnosing thyroid cancer and to methods of differentiating cancerous thyroid tissue from benign

thyroid tissue. For example, the methods disclosed herein would aid in distinguishing between benign and premalignant neoplastic lesions, as well as between aggressive and non-aggressive carcinomas. In a particular aspect, the description allows for such diagnoses using a tissue sample obtained from a patient prior to surgery. Such sample may be a fine needle aspirate biopsy (FNAB or FNA biopsy), a core biopsy, and/or any other preferably non-surgical sampling technique. The sample may be in the form of a cytospin. As will be understood, one advantage offered by the methods described herein is the ability for accurate pre-surgical selection of malignant thyroid nodules from benign nodules, thereby avoiding unnecessary surgery, and the associated complications, for benign cases. However, it will be also understood that the described methods are not limited to above mentioned tissue samples and that the methods can be performed on any tissue sample, whether obtained pre- or post-surgery.

[0012] In particular, polypeptides and domains thereof disclosed in Table 1 (collectively referred to herein as "Polypeptide Thyroid Cancer Markers"), and polynucleotides encoding such polypeptides and domains thereof (collectively referred to herein as "Polynucleotide Thyroid Cancer Markers") constitute biomarkers for thyroid cancer. Polypeptide Thyroid Cancer Markers and/or Polynucleotide Thyroid Cancer Markers, and portions or fragments thereof, are sometimes referred to herein as "Thyroid Cancer Markers".

[0013] Thus, Thyroid Cancer Markers and agents that interact with the Thyroid Cancer Markers, may be used in detecting, screening for, diagnosing, characterizing, and monitoring thyroid cancer (i.e., monitoring progression of the cancer or the effectiveness of a therapeutic treatment) or for assisting same, in the identification of subjects with a predisposition to thyroid cancer, and in determining patient survival. In aspects of the description, the Thyroid Cancer Markers are used in characterizing the aggressiveness of a thyroid cancer. In some aspects of the description, the Thyroid Cancer Markers are used to determine metastatic potential or patient survival.

[0014] A method of the description wherein Thyroid Cancer Marker(s) are assayed can have enhanced sensitivity and/or specificity relative to a method assaying other markers. The enhanced clinical sensitivity may be about a 5-10% increase, in particular 6-9% increase, more particularly 8% increase in sensitivity. In an embodiment, a method of the description where one or more Thyroid Cancer Marker(s) detected in tumor samples provides a thyroid cancer clinical sensitivity of at least about 80 to 99%, in particular 90 to 95%, more particularly 91%, 92%, 93%, or 94% thyroid cancer clinical sensitivity. In embodiments of the description the clinical sensitivity of a method of the description can be greater than about 80 to 90%, more particularly greater than about 80 to 85%, most particularly greater than about 83%, 84%, or 85%. Clinical sensitivity and specificity may be determined using methods known to persons skilled in the art.

[0015] In accordance with methods of the description, a Thyroid Cancer Marker in a sample can be assessed by detecting the presence in the sample of (a) a polypeptide or polypeptide fragment corresponding to the marker; (b) a transcribed nucleic acid or fragment thereof having at least a portion with which the marker is substantially identical; and/or (c) a transcribed nucleic acid or

1 fragment thereof, wherein the nucleic acid hybridizes with the marker.

2 **[0016]** One aspect of the description provides a method for detecting or characterizing a thyroid
3 cancer in a patient comprising determining the status of Thyroid Cancer Markers in a sample obtained
4 from the patient, wherein an abnormal status in the sample indicates the presence of the condition.

5 Another aspect of the description provides a method of screening for thyroid cancer in a
6 patient comprising identifying a patient at risk of having thyroid cancer or in need of screening and
7 determining the status of Thyroid Cancer Markers in a sample obtained from the patient, wherein an
8 abnormal status of the markers indicates the presence of thyroid cancer. In some embodiments, the
9 patient is at risk of developing a specific type of thyroid cancer and the abnormal status indicates the
10 presence of the specific type of thyroid cancer.

11 **[0017]** Another aspect provides a diagnostic method comprising identifying a patient who is a
12 candidate for treatment for thyroid cancer and determining the status of Thyroid Cancer Markers in a
13 sample obtained from the patient, wherein an abnormal status of the markers in the sample indicates
14 that treatment is desirable or necessary.

15 **[0018]** In aspects of the description, the abnormal status can be an elevated status, low status or
16 negative status. In an embodiment of the description for detecting or diagnosing thyroid cancer or a
17 type of thyroid cancer the abnormal status is an elevated status.

18 **[0019]** In an aspect of the description, a method is provided for detecting Thyroid Cancer Markers
19 associated with thyroid cancer in a patient comprising or consisting essentially of: (a) obtaining a
20 sample from a patient; b) detecting or identifying in the sample one or more Thyroid Cancer Markers,
21 in particular a Thyroid Cancer Marker set out in Table 1; and (c) comparing the detected amount with
22 an amount detected for a standard.

23 **[0020]** In an aspect, the description provides a method for diagnosing or screening for thyroid cancer
24 in a subject, the method comprising: (a) contacting a sample from a subject with reagents capable of
25 measuring levels of target Thyroid Cancer Markers, in particular Thyroid Cancer Markers set out in
26 Table 1; and (b) providing a diagnosis of thyroid cancer in said subject based on a significant
27 difference in the level of the Thyroid Cancer Markers in the sample from the subject over a control
28 level obtained from similar samples taken from subjects who do not have thyroid cancer or from the
29 subject at a different time.

30 **[0021]** In an embodiment of the description, a method is provided for detecting one or more of the
31 Thyroid Cancer Markers in a patient comprising or consisting essentially of: (a) obtaining a sample
32 from a patient; (b) detecting or identifying in the sample one or more of the Thyroid Cancer Markers,
33 in particular Thyroid Cancer Markers set out in Table 1; and (c) comparing the detected amounts with
34 amounts detected for a standard.

35 **[0022]** In embodiments of the description, the Thyroid Cancer Markers are chosen from tyrosine-
36 protein kinase receptor UFO (AXL) [SEQ ID NO. 1], activated leukocyte cell adhesion molecule
37 (ALCAM)/ CD166 [SEQ ID NO. 2], and prothymosin alpha (PTMA) [SEQ ID NO. 3], and optionally

Galectin-3 [SEQ ID NO. 4]. In embodiments of the description, the Thyroid Cancer Markers comprise or are selected from the group consisting of or consisting essentially of tyrosine-protein kinase receptor UFO (AXL) and activated leukocyte cell adhesion molecule (ALCAM) / CD166.

[0023] In embodiments of the description, the Thyroid Cancer Markers comprise or are selected from the group consisting of or consisting essentially of tyrosine-protein kinase receptor UFO (AXL), activated leukocyte cell adhesion molecule (ALCAM) / CD166, and prothymosin alpha (PTMA).

[0024] In embodiments of the description, the Thyroid Cancer Marker is biotinidase [SEQ ID NO. 5]. In embodiments of the description, the Thyroid Cancer Markers comprises clusterin [SEQ ID NO. 6], activated leukocyte cell adhesion molecule (ALCAM) / CD166, amyloid precursor protein like protein 2 (APLP2) [SEQ ID NO. 7], tyrosine-protein kinase receptor UFO (AXL), nucleolin [SEQ ID NO. 8], PTMA, amyloid precursor protein (APP) [SEQ ID NO. 9], 14-3-3 zeta [SEQ ID NO. 10], SET [SEQ ID NO. 11], PKM2 [SEQ ID NO. 12] or hnRNPK [SEQ ID NO. 13].

[0025] In embodiments of the description, the Thyroid Cancer Markers comprise or are selected from the group consisting of or consisting essentially of tyrosine-protein kinase receptor UFO (AXL), activated leukocyte cell adhesion molecule (ALCAM) / CD166, prothymosin-alpha (PTMA), nucleolin, biotinidase, amyloid precursor protein (APP), APLP2 and calyntenin-1 [SEQ ID NO. 14].

[0026] In embodiments of the description, the Thyroid Cancer Markers measured additionally comprise or are selected from the group consisting of or consisting essentially of clusterin, Dickkopf-related protein 3 (DKK-3) [SEQ ID NO. 15], nidogen-1 [SEQ ID NO. 16], gelsolin [SEQ ID NO. 17], and nucleobindin [SEQ ID NO. 18].

[0027] In embodiments of the description, the Thyroid Cancer Markers measured additionally comprise or are selected from the group consisting essentially of CYR61 [SEQ ID NO. 19], E-cadherin [SEQ ID NO. 20] and prothymosin-alpha.

[0028] In embodiments of the description, the Thyroid Cancer Markers measured additionally comprise or are selected from the group consisting essentially of α (alpha)-Enolase [SEQ ID NO. 21], and dystroglycan 1 [SEQ ID NO. 22].

[0029] In embodiments of the description, the Thyroid Cancer Markers measured additionally comprised or selected from the group consisting essentially of clusterin, Dickkopf-related protein 3 (DKK-3), nidogen-1, gelsolin, nucleobindin, melanoma-associated antigen [SEQ ID NO. 23], osteopontin [SEQ ID NO. 24] and plasminogen activator urokinase [SEQ ID NO. 25].

[0030] In embodiments of the description, the Thyroid Cancer Markers measured additionally comprise or are selected from the group consisting essentially of clusterin, Dickkopf-related protein 3 (DKK-3), nidogen-1, gelsolin, nucleobindin, melanoma-associated antigen, osteopontin, plasminogen activator urokinase, nucleotin, CYR61, E-cadherin and prothymosin-alpha.

[0031] In embodiments of this aspect of the description, the Thyroid Cancer Marker(s) measured comprise(s) one, two, three, four, five, six, seven, eight, nine, ten or more markers set out in Table 1.

1 **[0032]** The description further provides a non-invasive non-surgical method for detection or
2 diagnosis of thyroid cancer in a subject comprising: obtaining a sample (e.g., fluid sample) from the
3 subject; subjecting the sample to a procedure to detect Thyroid Cancer Marker(s); detecting or
4 diagnosing thyroid cancer by comparing the levels of Thyroid Cancer Marker(s) to the levels of
5 Thyroid Cancer Marker(s) obtained from a control subject with no thyroid cancer or a lower grade of
6 thyroid cancer. In embodiments of this method of the description, the Thyroid Cancer Marker(s) are
7 one or more of the markers set out in Table 1.

8 **[0033]** The description contemplates a method for determining the aggressiveness or stage of
9 thyroid cancer comprising producing a profile of levels of Thyroid Cancer Markers, and other markers
10 associated with thyroid cancer, in cells from a patient, and comparing the profile with a reference to
11 identify a profile for the test cells indicative of aggressiveness or stage of disease. In an aspect, the
12 markers are Polypeptide Thyroid Cancer Markers and the profile is generated using a mass
13 spectrometer.

14 **[0034]** In particular aspects, methods of the description are used to diagnose the stage of thyroid
15 cancer in a subject or characterizing thyroid cancer in a subject. In an embodiment, the method
16 comprises comparing: (a) levels of one or more Thyroid Cancer Markers set out in Table 1, in
17 particular the follicular thyroid cancer markers, papillary thyroid cancer markers or
18 aggressive/metastatic thyroid cancer markers set out in Table 1, from a sample from the patient; and
19 (b) levels of the Thyroid Cancer Markers in control samples of the same type obtained from patients
20 without thyroid cancer or control patients with a different stage of thyroid cancer (e.g., low grade
21 thyroid cancer) or from another sample from the subject, wherein altered levels of Thyroid Cancer
22 Markers, relative to the corresponding levels in the control samples is an indication that the patient is
23 afflicted with a more aggressive or metastatic thyroid cancer.

24 **[0035]** In embodiments, follicular thyroid cancer is diagnosed and the Thyroid Cancer Markers are
25 one or more of the Thyroid Cancer Markers for follicular thyroid cancer set out in Table 1.

26 **[0036]** In aspects of the description, a method is provided for diagnosing follicular thyroid cancer in a
27 patient comprising or consisting essentially of: (a) detecting or identifying in the sample one, two or
28 three of the Thyroid Cancer Markers for follicular thyroid cancer set out in Table 1, and optionally one
29 or more additional Thyroid Cancer Marker set out in Table 1 or 2; and (b) comparing the detected
30 amount with an amount detected for a standard, wherein a significant difference in the Thyroid Cancer
31 Markers is indicative of follicular thyroid cancer. In embodiments of the description relating to follicular
32 thyroid cancer, the Thyroid Cancer Markers comprise or are selected from the group consisting
33 essentially of calmodulin [SEQ ID NO. 26], CD44 antigen [SEQ ID NO. 27], fibronectin [SEQ ID NO.
34 28], ubiquitin A-52 residue ribosomal protein fusion product [SEQ ID NO. 29], and basement
35 membrane specific heparin sulfate core protein [SEQ ID NO. 30].

36 **[0037]** In embodiments, papillary thyroid cancer is diagnosed and the Thyroid Cancer Markers are
37 one or more of the Thyroid Cancer Markers for papillary thyroid cancer set out in Table 1. In a

particular embodiment of the description, a method is provided for diagnosing or detecting papillary thyroid cancer in a patient comprising or consisting essentially of: (a) detecting or identifying in the sample one or more Thyroid Cancer Markers for papillary thyroid cancer set out in Table 1, and optionally one or more additional Thyroid Cancer Marker set out in Table 1 and 2; and (b) comparing the detected amount with an amount detected for a standard, wherein significant difference in the amount of the Thyroid Cancer Markers is indicative of papillary thyroid cancer. In embodiments of the description relating to papillary thyroid cancer, the Thyroid Cancer Markers comprise versican [SEQ ID NO. 31], nucleolin and/or prothymosin-alpha. In embodiments of the description relating to papillary thyroid cancer, the Thyroid Cancer Markers comprise versican, nucleolin and/or prothymosin-alpha, and optionally CYR61 and/or E-cadherin.

[0038] In embodiments of the description relating to papillary thyroid cancer, the Thyroid Cancer Markers comprise one or more of, or are selected from the group consisting of or consisting essentially of gamma-glutamyl hydrolase [SEQ ID NO. 32], lysyl oxidase-like 2 [SEQ ID NO. 33], biotinidase, and nidogen-1. The Thyroid Cancer Markers for detecting papillary thyroid cancer may additionally comprise cysteine-rich angiogenic inducer, 61 (CYR61) and/or E-cadherin. In embodiments of the description relating to papillary thyroid cancer, the Thyroid Cancer Markers comprise two, three, four, five, six, seven, eight, nine, ten or all the for papillary thyroid cancer markers set out in Table 1.

[0039] In embodiments, aggressive thyroid cancer, in particular ATC, is diagnosed and the Thyroid Cancer Markers are one or more of the Thyroid Cancer Markers for aggressive/metastatic thyroid cancer set out in Table 1. In a particular aspect of the description, a method is provided for detecting Thyroid Cancer Markers associated with aggressive or metastatic thyroid cancer, in a patient comprising or consisting essentially of: (a) obtaining a sample from a patient; (b) detecting in the sample one or more Thyroid Cancer Markers for aggressive/metastatic thyroid cancer set out in Table 1 and optionally one or more additional Thyroid Cancer Marker set out in Tables 1 and 2; and (c) comparing the detected amount with an amount detected for a standard or cut-off value. In embodiments of the description relating to aggressive or metastatic thyroid cancer, the Thyroid Cancer Markers comprise two, three, four or all the aggressive/metastatic thyroid cancer markers set out in Table 1. In embodiments of the description relating to aggressive or metastatic thyroid cancer, the Thyroid Cancer Markers comprise prothymosin-alpha. In embodiments of the description relating to aggressive or metastatic thyroid cancer, the Thyroid Cancer Markers comprise one or more or all of, or are selected from the group consisting essentially of activated leukocyte cell adhesion molecule (ALCAM) / CD166, tyrosine-protein kinase receptor UFO (AXL), amyloid precursor protein like protein 2 (APLP2), cadherin-2, prothymosin-alpha, clusterin, syndecan-4 [SEQ ID NO. 34], E-cadherin, gelsolin, hnRNP A2/B1 [SEQ ID NO. 35], nucleolin, α -MCFD2 [SEQ ID NO. 36], α -NPC2 [SEQ ID NO. 37] and SET protein.

[0040] The description provides a method of assessing whether a patient is afflicted with thyroid cancer, the method comprising comparing: (a) levels of one or more Thyroid Cancer Markers set out

1 in Table 1 from the patient; and (b) standard levels of Thyroid Cancer Markers in samples of the same
2 type obtained from control patients not afflicted with thyroid cancer or with a lower grade of thyroid
3 cancer, wherein altered levels of Thyroid Cancer Markers relative to the corresponding standard
4 levels of Thyroid Cancer Markers is an indication that the patient is afflicted with thyroid cancer. In an
5 embodiment of a method of the description for assessing whether a patient is afflicted with follicular
6 thyroid cancer (FTC), levels of one or more Thyroid Cancer Markers for follicular thyroid cancer set
7 out in Table 1, in particular basement membrane specific heparin sulfate core protein, in a sample
8 from the patient are compared to a standard. In an embodiment of a method of the description for
9 assessing whether a patient is afflicted with follicular thyroid cancer (FTC), levels of one or more
10 Thyroid Cancer Markers for follicular thyroid cancer set out in Table 1 and optionally one or more
11 additional Thyroid Cancer Marker set out in Tables 1 and 2, in particular calmodulin, CD44 antigen,
12 fibronectin, ubiquitin A-52 residue ribosomal protein fusion product, and basement membrane specific
13 heparin sulfate core protein, in a sample from the patient are compared to a standard.

14 **[0041]** In an embodiment of a method of the description for assessing whether a patient is afflicted
15 with follicular thyroid cancer higher levels of one or more Thyroid Cancer Markers for follicular thyroid
16 cancer set out in Table 1 and optionally one or more additional Thyroid Cancer Marker set out in
17 Tables 1 and 2, in particular calmodulin, CD44 antigen, fibronectin, ubiquitin A-52 residue ribosomal
18 protein fusion product, or basement membrane specific heparin sulfate core protein, in a sample
19 relative to a standard or corresponding normal levels, is an indication that the patient is afflicted with
20 follicular thyroid cancer.

21 **[0042]** In an embodiment of a method of the description for assessing whether a patient is afflicted
22 with papillary thyroid cancers (PTC), levels of one or more Thyroid Cancer Markers for papillary
23 thyroid cancer set out in Table 1 in a sample from the patient are compared to a standard. In an
24 embodiment of a method of the description for assessing whether a patient is afflicted with papillary
25 thyroid cancers (PTC), levels of nucleolin and/or prothymosin-alpha, and optionally CYR61 and/or E-
26 cadherin, in a sample from the patient are compared to a standard.

27 **[0043]** In an aspect of a method of the description for assessing whether a patient is afflicted with
28 papillary thyroid cancer higher levels of one or more Thyroid Cancer Markers for papillary thyroid
29 cancer set out in Table 1 in a sample relative to a standard or corresponding normal levels, is an
30 indication that the patient is afflicted with follicular thyroid cancer. In an aspect of a method of the
31 description for assessing whether a patient is afflicted with papillary thyroid cancer higher levels of
32 one or both of nucleolin and/or prothymosin-alpha, and optionally CYR61 and/or E-cadherin, in a
33 sample relative to a standard or corresponding normal levels, is an indication that the patient is
34 afflicted with follicular thyroid cancer.

35 **[0044]** In an embodiment of a method of the description for assessing whether a patient is afflicted
36 with aggressive or metastatic thyroid cancer, levels of one or more Thyroid Cancer Markers for
37 aggressive or metastatic thyroid cancer set out in Table 1 in a sample from the patient are compared
38 to a standard. In an aspect of a method of the description for assessing whether a patient is afflicted

1 with aggressive or metastatic thyroid cancer higher levels of one or more Thyroid Cancer Markers
2 aggressive or metastatic thyroid cancer set out in Table 1 in a sample relative to a standard or
3 corresponding normal levels or levels from a patient with a lower grade of thyroid cancer, is an
4 indication that the patient is afflicted with aggressive or metastatic thyroid cancer.

5 **[0045]** In an embodiment of a method of the description for assessing whether a patient is afflicted
6 with anaplastic thyroid cancer, levels of one or more Thyroid Cancer Markers for aggressive or
7 metastatic thyroid cancer set out in Table 1 and particularly marked *** in a sample from the patient
8 are compared to a standard, and significantly different levels of the Thyroid Cancer Markers
9 compared to a standard are indicative of anaplastic thyroid cancer.

10 **[0046]** In an aspect of a method of the description for assessing whether a patient is afflicted with
11 aggressive or metastatic thyroid cancer or anaplastic thyroid cancer levels of prothymosin-alpha in a
12 sample from the patient are compared to a standard.

13 **[0047]** In aspects of the description, aggressive thyroid cancer, in particular ATC, is detected,
14 diagnosed or characterized by determination of increased levels of one or more Thyroid Cancer
15 Marker(s) aggressive or metastatic thyroid cancer set out in Table 1 and Marked *** when compared
16 to such levels obtained from a control.

17 **[0048]** In an aspect, the description provides a method for monitoring the progression of thyroid
18 cancer in a patient the method comprising: (a) detecting one or more Thyroid Cancer Marker(s) set
19 out in Table 1 and optionally Table 2 in a patient sample (e.g. biopsy sample) at a first time point; (b)
20 repeating step (a) at a subsequent point in time; and (c) comparing the levels detected in (a) and (b),
21 and thereby monitoring the progression of thyroid cancer in the patient.

22 **[0049]** The description provides a method for classifying a patient having thyroid cancer, the method
23 comprising measuring one or more Thyroid Cancer Marker(s) set out in Table 1 and optionally Table 2
24 in a fluid sample, in particular serum sample, from the patient and correlating the values measured to
25 values measured for the Thyroid Cancer Markers from thyroid cancer patients stratified in
26 classification groups. The method can be used to predict patient survival, wherein the Thyroid Cancer
27 Marker(s) are predictive of survival and wherein the classification groups comprise groups of known
28 overall survival. In aspects of this method of the description, the Thyroid Cancer Marker(s) are
29 selected from the follicular thyroid cancer markers, papillary thyroid cancer markers or
30 aggressive/metastatic thyroid cancer markers in Table 1. In various embodiments the values
31 measured can be normalized to provide more accurate quantification and to correct for experimental
32 variations.

33 **[0050]** In aspects of the description, Polynucleotide Thyroid Cancer Markers are detected and levels
34 of Polynucleotide Thyroid Cancer Markers in a sample from a patient are compared with
35 Polynucleotide Thyroid Cancer Marker levels from samples of patients without thyroid cancer, with a
36 lower grade of thyroid cancer, or from levels from samples of the same patient. A method of the
37 description may employ one or more polynucleotides, oligonucleotides, or nucleic acids capable of

1 hybridizing to Polynucleotide Thyroid Cancer Markers. The present description relates to a method for
2 diagnosing and characterizing thyroid cancer, more particularly the stage of thyroid cancer, in a
3 sample from a subject comprising isolating nucleic acids, preferably mRNA, from the sample, and
4 detecting Polynucleotide Thyroid Cancer Markers in the sample.

5 **[0051]** The description also provides methods for determining the presence or absence of thyroid
6 cancer or the aggressiveness or metastatic potential of a thyroid cancer in a subject in the subject
7 comprising detecting in the sample a level of nucleic acids that hybridize to one or more
8 Polynucleotide Thyroid Cancer Marker(s) encoding polypeptides set out in Table 1, and optionally
9 Table 2, and comparing the level(s) with a predetermined standard or cut-off value, and therefrom
10 determining the presence or absence of thyroid cancer or the aggressiveness or metastatic potential
11 of a thyroid cancer in the subject in the subject. In an embodiment a method is provided for
12 determining the aggressiveness or metastatic potential of thyroid cancer in a subject comprising (a)
13 contacting a sample taken from the subject with oligonucleotides that hybridize to one or more
14 polynucleotides encoding the Thyroid Cancer Markers for follicular thyroid cancer markers, papillary
15 thyroid cancer markers and/or aggressive/metastatic thyroid cancer markers set out in Table 1; and
16 (b) detecting in the sample a level of nucleic acids that hybridize to the oligonucleotides relative to a
17 predetermined standard or cut-off value, and therefrom determining the aggressiveness or metastatic
18 potential of the cancer in the subject.

19 **[0052]** In an aspect, the description provides a method of assessing the aggressiveness or
20 metastatic potential of a thyroid cancer in a patient, the method comprising comparing: (a) levels of
21 one or more Polynucleotide Thyroid Cancer Marker(s) set out in Table 1, in particular follicular thyroid
22 cancer markers, papillary thyroid cancer markers or aggressive/metastatic thyroid cancer markers set
23 out in Table 1, in a sample from the patient; and (b) control levels of the Polynucleotide Thyroid
24 Cancer Marker(s) in samples of the same type obtained from control patients not afflicted with thyroid
25 cancer or a lower grade of thyroid cancer, wherein altered levels of Polynucleotide Thyroid Cancer
26 Marker(s) relative to the corresponding control levels of the Polynucleotide Thyroid Cancer Marker(s)
27 is an indication of the aggressiveness or metastatic potential of the thyroid cancer.

28 **[0053]** In a particular method of the description for assessing whether a patient is afflicted with an
29 aggressive or metastatic thyroid cancer higher levels of prothymosin-alpha or nucleolin, in a sample
30 relative to the corresponding control levels is an indication that the patient is afflicted with an
31 aggressive or metastatic thyroid cancer.

32 **[0054]** Within certain embodiments, the amount of nucleic acid that is mRNA is detected via
33 amplification reactions such as polymerase chain reaction (PCR) using, for example, at least one
34 oligonucleotide primer that hybridizes to a Polynucleotide Thyroid Cancer Marker(s) or a complement
35 of such polynucleotide. Within other embodiments, the amount of mRNA is detected using a
36 hybridization technique, employing an oligonucleotide probe that hybridizes to a Polynucleotide
37 Thyroid Cancer Marker(s), or a complement thereof.

1 **[0055]** When using mRNA detection, the method may be carried out by combining isolated mRNA
2 with reagents to convert to cDNA according to standard methods; treating the converted cDNA with
3 amplification reaction reagents along with an appropriate mixture of primers to produce amplification
4 products; and analyzing the amplification products to detect the presence of Polynucleotide Thyroid
5 Cancer Marker(s) in the sample. For mRNA the analyzing step may be accomplished using RT-PCR
6 analysis to detect the presence of Polynucleotide Thyroid Cancer Marker(s). The analysis step may
7 be accomplished by quantitatively detecting the presence of Polynucleotide Thyroid Cancer Marker(s)
8 in the amplification product, and comparing the quantity of Polynucleotide Thyroid Cancer Marker(s),
9 detected against a panel of expected values for known presence or absence in normal and malignant
10 samples derived using similar primers.

11 **[0056]** Therefore, the description provides a method wherein mRNA is detected by (a) isolating
12 mRNA from a sample and combining the mRNA with reagents to convert it to cDNA; (b) treating the
13 converted cDNA with amplification reaction reagents and nucleic acid primers that hybridize to a
14 Polynucleotide Thyroid Cancer Marker(s) to produce amplification products; (d) analyzing the
15 amplification products to detect an amount of mRNA Polynucleotide Thyroid Cancer Marker(s); and
16 (e) comparing the amount of mRNA to an amount detected against a panel of expected values for
17 normal and malignant samples (e.g., samples from patients with a different stage of thyroid cancer)
18 derived using similar nucleic acid primers.

19 **[0057]** In particular embodiments of the description, the methods described herein utilize the
20 Polynucleotide Thyroid Cancer Markers placed on a microarray so that the expression status of each
21 of the markers is assessed simultaneously. In a particular aspect, the description provides a
22 microarray comprising a defined set of genes whose expression is significantly altered by a thyroid
23 cancer or procedure. The description further relates to the use of the microarray as a prognostic tool
24 to predict thyroid cancer or status of a thyroid cancer. In an embodiment, the description provides for
25 oligonucleotide arrays comprising marker sets described herein. The microarrays provided by the
26 present description may comprise probes to markers able to distinguish thyroid cancer. In particular,
27 the description provides oligonucleotide arrays comprising probes to a subset or subsets of at least 5
28 or 10 gene markers up to a full set of markers which distinguish thyroid cancer.

29 **[0058]** Protein based methods can also be used for determining the presence or absence of thyroid
30 cancer or the aggressiveness or metastatic potential of a thyroid cancer in a sample from a subject.
31 Thyroid Cancer Markers may be detected using a binding agent for Thyroid Cancer Markers,
32 preferably antibodies specifically reactive with Thyroid Cancer Markers, or parts thereof.

33 **[0059]** The description provides a method of assessing whether a patient is afflicted with thyroid
34 cancer which comprises comparing: (a) levels of one or more Polypeptide Thyroid Cancer Markers set
35 out in Table 1, and optionally Table 2 in a sample from the patient; and (b) control levels of the
36 Polypeptide Thyroid Cancer Markers in a non-cancer sample or sample from a patient with a lower
37 grade of thyroid cancer or from a sample from the patient taken at another time, wherein significantly
38 different levels of Polypeptide Thyroid Cancer Markers in the sample from the patient compared with

1 the control levels is an indication that the patient is afflicted with thyroid cancer.

2 **[0060]** In another aspect the description provides methods for determining the presence or absence
3 of thyroid cancer or the aggressiveness or metastatic potential of a thyroid cancer or classifying
4 thyroid cancer in a patient comprising the steps of (a) contacting a biological sample obtained from a
5 patient with a binding agent that specifically binds to one or more Polypeptide Thyroid Cancer
6 Marker(s) set out in Table 1 and optionally Table 2; and (b) detecting in the sample an amount of the
7 Polypeptide Thyroid Cancer Marker(s) that binds to the binding agent(s), relative to a predetermined
8 standard or cut-off value, and therefrom determining the presence or absence of thyroid cancer, the
9 aggressiveness or metastatic potential of thyroid cancer or the stage of thyroid cancer in the patient.

10 **[0061]** In an embodiment, the description relates to a method for detecting, diagnosing, staging and
11 monitoring thyroid cancer in a subject by quantitating one or more Polypeptide Thyroid Cancer
12 Marker(s) in a biological sample from the subject comprising (a) reacting the biological sample with an
13 antibody specific for one or more Polypeptide Thyroid Cancer Marker(s) set out in Table 1, and
14 optionally Table 2, which is directly or indirectly labeled with a detectable substance; and (b) detecting
15 the detectable substance.

16 **[0062]** In another embodiment the description provides a method of using antibodies to detect
17 expression of Polypeptide Thyroid Cancer Marker(s) in a sample, the method comprising: (a)
18 combining antibodies specific for Polypeptide Thyroid Cancer Marker(s) with a sample under
19 conditions which allow the formation of antibody:protein complexes; and (b) detecting complex
20 formation, wherein complex formation indicates expression of Polypeptide Thyroid Cancer Marker(s) in
21 the sample. Expression may be compared with standards and is diagnostic of thyroid cancer, stage of
22 thyroid cancer, or the aggressiveness or metastatic potential of the thyroid cancer.

23 **[0063]** Polypeptide Thyroid Cancer Markers can be determined by constructing an antibody
24 microarray in which binding sites comprise immobilized, preferably monoclonal, antibodies specific to
25 a substantial fraction of marker-derived thyroid cancer proteins of interest.

26 **[0064]** In an aspect, the description provides a method for monitoring the progression of thyroid
27 cancer in a patient, the method comprising: (a) detecting one or more Polypeptide Thyroid Cancer
28 Marker(s) set out in Table 1, and optionally Table 2, in a patient sample at a first time point; and (b)
29 repeating step (a) at a subsequent point in time; and (c) comparing the levels detected in (a) and (b),
30 and thereby monitoring the progression of thyroid cancer in the patient.

31 **[0065]** The description further relates to a method of assessing the efficacy of a therapy for thyroid
32 cancer in a patient. This method comprises comparing: (a) levels of one or more Thyroid Cancer
33 Markers set out in Table 1, and optionally Table 2, in a first sample obtained from the patient prior to
34 providing at least a portion of the therapy to the patient; and (b) levels of the Thyroid Cancer Markers
35 in a second sample obtained from the patient following therapy. Significantly different levels of Thyroid
36 Cancer Markers in the second sample, relative to the first sample, can be an indication that the
37 therapy is efficacious for inhibiting thyroid cancer. In an embodiment, the method is used to assess

the efficacy of a therapy for inhibiting thyroid cancer and significantly different levels of one or more Thyroid Cancer Markers for follicular thyroid cancer, papillary thyroid cancer and/or aggressive/metastatic thyroid cancer in Table 1, in the second sample relative to the first sample, is an indication that the therapy is efficacious for inhibiting the cancer or metastasis. The therapy may be any therapy for treating thyroid cancer including but not limited to chemotherapy, immunotherapy, gene therapy, radiation therapy, and surgical removal of tissue. Therefore, the method can be used to evaluate a patient before, during, and after therapy, for example, to evaluate the reduction in tumor burden, aggressiveness or metastatic potential of the tumor.

[0066] The description provides marker sets for diagnosing or characterizing thyroid cancer and uses thereof. A marker set may comprise a plurality of polypeptides and/or polynucleotides encoding such polypeptides comprising or consisting of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 of the markers of Table 1. In specific aspects, the markers consist of at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 polypeptides of Table 1. In an aspect the protein marker sets comprise or consist of protein clusters, or proteins in pathways comprising markers of Table 1. In embodiments of the description, a marker is provided which is selected from the group consisting of the polypeptides set forth in Table 1 which polypeptides are up-regulated biomarkers in thyroid cancer, in particular follicular thyroid cancer, papillary cancer or metastatic/aggressive thyroid cancer.

[0067] The description also provides a diagnostic composition comprising Thyroid Cancer Markers or agents that interact with Thyroid Cancer Markers. In particular, the description provides a diagnostic composition comprising Polypeptide Thyroid Cancer Markers, or agents that bind to such markers, or hybridize to or amplify Polynucleotide Thyroid Cancer Markers. In an embodiment, the composition comprises a probe that specifically hybridizes to a Polynucleotide Thyroid Cancer Marker or a fragment thereof, and a probe that specifically hybridizes to a Polynucleotide Thyroid Cancer Marker or a fragment thereof. In another embodiment a composition is provided comprising a specific primer(s) pair capable of amplifying a Polynucleotide Thyroid Cancer Marker using polymerase chain reaction methodologies. In a still further embodiment, the composition comprises a binding agent(s) (e.g. antibody) that binds to a Polypeptide Thyroid Cancer Marker or a fragment thereof. Probes, primers, and binding agents can be labeled with a detectable substance. In an embodiment, a diagnostic composition of the description comprises one or more antibodies specific for a Thyroid Cancer Marker in Table 1. In an embodiment, a diagnostic composition of the description comprises one or more primers that amplify polynucleotides encoding a Thyroid Cancer Marker in Table 1.

[0068] In another aspect, the description relates to use of an agent that interacts with a Thyroid Cancer Marker in the manufacture of a composition for diagnosing thyroid cancer, in particular the aggressiveness or metastatic potential of a thyroid cancer.

[0069] The methods of the description may also comprise detecting additional markers associated with thyroid cancer, for example the markers listed in Table 2, more particularly Galectin-3, thyroglobulin, and E-cadherin. Further, the amount of Thyroid Cancer Markers may be mathematically combined with other markers of thyroid cancer. In an embodiment the description provides a method

1 for detecting or diagnosing thyroid cancer in a subject comprising: (a) determining the amount of one
2 or more Thyroid Cancer Markers in Table 1 in a sample from the subject; (b) determining the amount
3 of other markers associated with thyroid cancer in particular markers comprising or selected from the
4 markers listed in Table 2 or from the group consisting of or consisting essentially of Galectin-3,
5 thyroglobulin and E-cadherin, in the sample; (c) mathematically combining the results of step (a) and
6 step (b) to provide a mathematical combination; and (d) comparing or correlating the mathematical
7 combination to the presence of thyroid cancer, stage of thyroid cancer or aggressiveness or
8 metastatic potential of thyroid cancer.

9 **[0070]** In a particular embodiment the description provides a method for diagnosing the
10 aggressiveness of thyroid cancer in a subject comprising: (a) determining the amount of one or more
11 Thyroid Cancer Marker(s) for aggressive or metastatic thyroid cancer set out in Table 1 from the
12 subject; (b) determining the amount of Thyroid Cancer Marker(s) in the sample; (c) determining the
13 amount of one or more of E-cadherin, CYR61, melanoma-associated antigen, osteopontin, and
14 plasminogen activator urokinase in the sample; (d) mathematically combining the results of step (a)
15 and step (b), and optionally step (c) to provide a mathematical combination; and (e) comparing or
16 correlating the mathematical combination to the aggressiveness of the thyroid cancer. The
17 combination is preferably compared to a mathematical combination for a predetermined standard. In
18 particular aspects, the description provides a method for detecting, characterizing or diagnosing
19 thyroid cancer by determining the combination of Thyroid Cancer Markers and one or more of the
20 markers listed in Table 2, or one or both of Galectin-3 and thyroglobulin in a sample from a subject.

21 **[0071]** The description also includes kits for carrying out methods of the description. In an aspect the
22 description provides a kit for detecting, diagnosing, screening for, monitoring, predicting or
23 characterizing thyroid cancer comprising Thyroid Cancer Markers. In a particular aspect, the
24 description provides a test kit for diagnosing screening for, monitoring, predicting or characterizing
25 thyroid cancer in a subject which comprises an agent that interacts with a Thyroid Cancer Marker(s).
26 In an embodiment, the kit is for assessing whether a patient is afflicted with follicular thyroid cancer
27 and it comprises reagents for identifying and/or assessing levels of the Thyroid Cancer Markers for
28 follicular thyroid cancer in Table 1. In an embodiment, the kit is for assessing whether a patient is
29 afflicted with papillary thyroid cancer and it comprises reagents for identifying and/or assessing levels
30 of the Thyroid Cancer Markers for papillary thyroid cancer in Table 1. In an embodiment, the kit is for
31 assessing whether a patient is afflicted with aggressive or metastatic thyroid cancer and it comprises
32 reagents for identifying and/or assessing levels of the Thyroid Cancer Markers for aggressive or
33 metastatic thyroid cancer in Table 1.

34 **[0072]** The description contemplates an *in vivo* method comprising administering to a mammal one
35 or more agent that carries a label for imaging and binds to a Thyroid Cancer Marker, and then
36 imaging the mammal. According to a preferred aspect of the description, an *in vivo* method for
37 imaging thyroid cancer is provided comprising: (a) injecting a patient with an agent that binds to a
38 Thyroid Cancer Marker(s), the agent carrying a label for imaging the thyroid cancer; (b) allowing the

agent to incubate *in vivo* and bind to the Thyroid Cancer Marker(s); and (c) detecting the presence of the label localized to the thyroid cancer. In an embodiment of the description the agent is an antibody which recognizes the Thyroid Cancer Marker(s). In another embodiment of the description the agent is a chemical entity which recognizes the Thyroid Cancer Marker(s). The agent carries a label to image the Thyroid Cancer Marker(s). Examples of labels useful for imaging are radiolabels, fluorescent labels (e.g. fluorescein and rhodamine), nuclear magnetic resonance active labels, positron emitting isotopes detectable by a positron emission tomography ("PET") scanner, chemiluminescers such as luciferin, and enzymatic markers such as peroxidase or phosphatase. Short-range radiation emitters, such as isotopes detectable by short-range detector probes can also be employed. The description also contemplates the localization or imaging methods described herein using multiple markers for thyroid cancer.

[0073] In an aspect, the description provides antagonists (e.g. antibodies) specific for a Thyroid Cancer Marker set out in Table 1 that can be used therapeutically to destroy or inhibit the growth of thyroid cancer cells.

[0074] In one aspect there is provided a method for diagnosing thyroid cancer, or for differentiating malignant or pre-malignant thyroid tissue from non-malignant thyroid tissue in a subject, the method comprising:

- comparing a detected amount of PGK1 in a thyroid tissue sample from the subject with a control amount of PGK1 in non-cancerous tissue;
- and diagnosing thyroid cancer or identifying malignant thyroid tissue in the subject when the detected amount of PGK1 is less than the control amount.

[0075] In another aspect, there is provided a method for diagnosing thyroid cancer, or for differentiating malignant or pre-malignant thyroid tissue from non-malignant thyroid tissue in a subject, the method comprising:

- comparing detected amounts of PGK1 and Galectin-3 in a thyroid tissue sample from the subject with control amounts of PGK1 and Galectin-3 in non-cancerous tissue;
- and diagnosing thyroid cancer or identifying malignant thyroid tissue in the subject when the detected amount of PGK1 is less than the control amount of PGK1 and the detected amount of Galectin-3 is greater than the control amount of Galectin-3.

[0076] In another aspect there is provided a method for diagnosing thyroid cancer, or for differentiating malignant or pre-malignant thyroid tissue from non-malignant thyroid tissue in a subject, the method comprising:

- comparing detected amounts of two or more biomarkers in a thyroid tissue sample from the subject with control amounts of the biomarkers in non-cancerous tissue, the biomarkers comprising PGK1, PKM2, PTEN, Profilin-1, Galectin-3 and Cyclin D1;
- and diagnosing thyroid cancer or identifying malignant thyroid tissue in the subject when (i) the detected amounts of PGK1, PKM2, PTEN, Profilin-1 or S100A6 are less than the respective control amounts and/or (ii) the detected amounts of Cyclin D1 or Galectin-3 are greater than the

1 respective control amounts.

2 **[0077]** In another aspect, there is provided a method for diagnosing thyroid cancer, or for
3 differentiating malignant or pre-malignant thyroid tissue from non-malignant thyroid tissue in a subject,
4 the method comprising:

5 - comparing an amount of Profilin-1 detected in a thyroid tissue sample from the subject with
6 a control amount of Profilin-1 in non-cancerous tissue;

7 - and diagnosing thyroid cancer or identifying malignant thyroid tissue in the subject when the
8 detected amount of Profilin-1 is less than the control amount.

9 **[0078]** In the methods described herein, the thyroid tissue sample is preferably obtained by a
10 minimally invasive procedure, such as a fine needle aspirate biopsy, a core biopsy or the like. The
11 sample may be in the form of a cytospin. As discussed above one advantage offered by the
12 methods described herein is the ability to differentiate malignant or pre-malignant tissues from
13 malignant tissues prior to undergoing an unnecessary surgical intervention.

14 **[0079]** Other objects, features and advantages of the present description will become apparent from
15 the following detailed description. It should be understood, however, that the detailed description and
16 the specific examples while indicating preferred embodiments of the description are given by way of
17 illustration only, since various changes and modifications within the spirit and scope of the description
18 will become apparent to those skilled in the art from this detailed description.

19 **DESCRIPTION OF THE DRAWINGS**

20 **[0080]** The description will now be described in relation to the drawings in which:

21 **[0081]** Figure 1 shows the verification of secreted proteins in thyroid cancer patients' sera by
22 immunoblotting in tissue culture media and sera of thyroid cancer patients. Protein samples were
23 prepared from cultured cells (50-75 µg), their respective serum-free media (SFM; 5µg), and the sera
24 (50 µg) of five thyroid cancer patients (2 non-aggressive PTC, FTC, aggressive PTC (metastatic
25 thyroid cancer) and ATC and one normal sample ('normal', S-2145, Sigma). As protein loading
26 controls, lysates were examined for actin expression, while SFM and patients' sera were stained with
27 Ponceau S (Sigma). Western blot analysis confirmed the detection of five proteins, namely clusterin,
28 CYR 61, α-enolase, nucleolin and prothymosin-alpha in the thyroid carcinoma patients' sera and in
29 the whole-cell lysate and conditioned media (CM) of the thyroid carcinoma cell lines as shown in
30 panel A. The panel B shows increase in α-MCFD2 [SEQ ID NO. 36], α-NPC2 [SEQ ID NO. 37], E
31 cadherin and caldesmon, in normal and thyroid cancer patients' sera. The panels C and D show
32 decrease in α-Enolase probed with rabbit polyclonal antibody (H300; Santa Cruz Biotech) and a
33 mouse monoclonal antibody (ab54979; Abcam). Levels of the 48-kDa α-Enolase protein expressed
34 relative to the level of this protein in benign thyroid nodule taken as 1.0 were observed to decrease in
35 ATC and metastatic PTC suggesting a reduction that correlated with disease aggressiveness. Figure
36 1 panels E, F and G show immunohistochemical analysis of α-Enolase expression in non-aggressive
37 PTC (E), metastatic PTC (F), and normal thyroid tissue (G) (Original magnification X400). Reduced

cytoplasmic and nuclear staining was observed in PTC and metastatic PTC as compared to the normal thyroid tissues; reduction was more pronounced in the aggressive PTC tissue.

[0082] Figure 2, panels B and C show increased nuclear and cytoplasmic expression of prothymosin alpha (PTMA) by immunohistochemical analysis in human thyroid carcinoma tissues - (B) PTC and (C) ATC as compared to the normal tissue (A) (Original magnification X400). Panel D shows increased nuclear nucleolin expression in normal thyroid tissue, while the PTC (E) and ATC (F) showed reduced nuclear and increased cytoplasmic expression of nucleolin (Original magnification X400). Panels G and H depict scatter plot analysis of immunohistochemical scoring of PTMA and nucleolin in different subtypes of thyroid carcinomas. Tissues were scored based on percentage positivity and immunostaining intensity. Sections were scored as positive if epithelial cells showed immunoreactivity in the plasma membrane, cytoplasm, and/or nucleus when observed by evaluators blinded to the clinical history and outcome. The percentage positive scores were assigned according to the following scale: 0, < 10% cells; 1, 10-30% cells; 2, 30-50% cells; 3, 50-70% cells; and 4, >70%. The intensity of the staining was also scored semi-quantitatively as follows: 0, no staining; 1, mild; 2, moderate; and 3, intense. The total score (0-7) was obtained by adding the percentage positivity scores and intensity scores for each section. (G) ATC displayed elevated nuclear expression of PTMA compared to normal thyroid adjacent to benign thyroid disease, insular and papillary thyroid carcinomas. ATC cases displayed strikingly elevated cytoplasmic expression of PTMA compared to PTC and insular cells. PTC staining of cytoplasmic PTMA was low. Insular (poorly differentiated) thyroid carcinomas also demonstrated an increased expression of cytoplasmic PTMA compared to PTC and normal thyroid adjacent to benign thyroid disease. (H) Nuclear expression of nucleolin was seen in all thyroid carcinoma subtypes and normal thyroid adjacent to benign thyroid disease examined. Faint cytoplasmic expression was also observed in ATC cases only. These findings suggest increase in nuclear and cytoplasmic expression of PTMA with disease aggressiveness, while increased cytoplasmic nucleolin was observed in ATC.

[0083] Figure 3, panels A, B and C show detection and verification of serum biotinidase in patients with thyroid cancer. Serum proteins from patients with benign (n=8), non-aggressive PTC (n=6) and metastatic PTC (n=6) were immunodepleted, resolved on SDS-PAGE and probed with α -biotinidase rabbit polyclonal antibody K-17 (Santa Cruz Biotech; 1:1000 dilution) or mouse monoclonal (ab54886; Abcam) antibody. The 75 kDa biotinidase protein band levels were quantified using ImageQuant software (GE Biosciences) and are expressed as a proportion of the highest benign band intensity per blot. Biotinidase levels decreased from benign levels for nearly all of the PTC cases that correlated with disease aggressiveness. Panels D-G show immunohistochemical analysis of biotinidase expression in thyroid tissues. Stronger cytoplasmic staining was observed in the non-aggressive (E) and metastatic PTC (F), whereas nuclear levels of biotinidase decreased with increasing thyroid cancer aggressiveness. Original magnification X 400. Scatter plot analysis of biotinidase cytoplasmic and nuclear expression is shown in panels H and I, benign (n=3), non-aggressive (n=21) and aggressive (n=6) PTC cases with respective mean and standard error bars indicated. Stronger

cytoplasmic staining above the benign levels was observed for all non-aggressive and aggressive thyroid cancer cases. In the nucleus, biotinidase expression was found to decrease with increased tumor aggressiveness.

[0084] Figure 4, panels A and B show verification of serum clusterin levels in thyroid cancer patients. Serum proteins from patients with benign (n=8), non-aggressive (n=6) and aggressive (n=6) PTC were immunodepleted treatment prior to electrophoresis. To detect clusterin protein, blots were prepared and probed with α -clusterin mouse monoclonal antibody (78E; Santa Cruz Biotech) at a 1:2000 dilution. The expected 40-kDa protein was detected by 78E and found to decrease in abundance from benign levels in both non-aggressive and metastatic PTC cases. This reduction was also more pronounced in patients with more aggressive disease. Panel B shows verification of serum clusterin with mouse and rabbit α -clusterin antibodies. Immunoblots containing serum proteins from benign, non-aggressive and aggressive PTC cases were probed with α -clusterin 78E mouse monoclonal or rabbit polyclonal (ab92548; Abcam) antibody. Both antibodies recognized the expected 40-kDa clusterin protein and showed reduction in serum clusterin in thyroid cancer cases, particularly for aggressive PTC. Band intensities are presented as a proportion of the benign level for the respective blot. Panels C- F show verification of clusterin expression in thyroid tissues by immunohistochemical analysis. Sections from non-aggressive PTC (C), metastatic PTC (D), and normal (E) cases were probed with α -clusterin mouse monoclonal antibody. Increased cytoplasmic staining was observed from normal levels for both thyroid cancers. Furthermore, this increase was more prominent in the aggressive PTC tissue. In all cases, no detectable membrane or nuclear expression of clusterin was observed. Minimal non-specific staining was observed when sections were probed with the 2° antibody alone (F). Original magnification X400. Panels G and H show secreted serum clusterin levels in thyroid cancer patients using ELISA. Serum samples from 39 cases (10 benign, 10 non-aggressive PTC, 19 aggressive PTC) were examined using the Human Clusterin ELISA kit (BioVendor), as recommended. Bar (G) and scatter (H) plots were generated, depicting mean and standard error values. t-test analyses indicated that a significant difference was observed in serum clusterin levels in benign and aggressive thyroid cancers ($p=0.011$).

[0085] Figure 5, panels A-D show (ALCAM)/CD166 expression in human thyroid cancer tissue using immunohistochemical analysis. (A) Moderate membrane staining is observed in benign tissue. (B) Intense membrane and cytoplasmic staining is shown for stage I PTC, compared to moderate cytoplasmic staining in stage IV PTC (D). Very weak cytoplasmic staining is shown in ATC (C). Original magnification X400. Panel E shows scatter plot analysis of (ALCAM)/CD166 cytoplasmic and membrane expression. Tissue sections from benign (n=9), non-aggressive (n=16), aggressive (n=19) PTC and ATC (n=5) cases were probed with (ALCAM)/CD166 antibody. The distribution of all immunostaining scores for cytoplasmic and membrane (ALCAM)/CD166 expression is shown, with respective mean and standard error bars indicated. Decreased membrane staining was observed for all non-aggressive and aggressive PTC and ATC cases as compared to the benign cases. Panel F shows secreted (ALCAM)/CD166 in thyroid cancer patients' sera using ELISA. Pre- and post-surgery

1 blood samples were assessed. Non-Agg refers to non-aggressive stage I or II PTC cases with no
2 extrathyroidal spread. Agg PTC refers to aggressive PTC cases that have lymph node metastasis,
3 extrathyroidal invasion, and multifocality. Very aggressive refers to the more aggressive PTC cases,
4 which are poorly differentiated and/or have distant metastasis. Pre and post-surgery non-aggressive
5 PTC and the very aggressive post-surgery PTC (ALCAM)/CD166 levels were lower than all the other
6 groups. The non-aggressive PTC (ALCAM)/CD166 levels were lower as compared to the other
7 groups.

8 **[0086]** Figure 6 shows verification of secreted proteins in thyroid cancer patients' sera. Blots
9 containing immunodepleted serum samples (50µg/lane) were probed with respective antibodies. AXL
10 receptor tyrosine kinase, pyruvate kinase 3 isoform 1 variant (PKM2), APLP2 and amyloid precursor
11 protein (APP) levels were increased in thyroid cancer patients' sera and correlated with
12 aggressiveness. 14-3-3 zeta and protein phosphatase 2A inhibitor, SET, were detected in the sera of
13 thyroid cancer patients as well.

14 **[0087]** Figure 7, panels A-F show APLP2 expression in human thyroid cancer tissues using
15 immunohistochemical analysis. (A) Benign tissues showed moderate nuclear and mild cytoplasmic
16 staining. (B) Stage II PTC showed moderate nuclear and cytoplasmic staining. (C) Stage IV tall cell
17 variant PTC showed stronger cytoplasmic staining compared to the other thyroid cancer subtypes,
18 with some mild membrane staining. (D) Stage IV PTC revealed mild membrane and cytoplasmic
19 staining compared to moderate cytoplasmic staining in multifocal stage IV PTC (E). (F) ATC tissue
20 showed moderate cytoplasmic APLP2 staining. Original magnification x 400. Panel G shows scatter
21 plot analysis of APLP2 nuclear and cytoplasmic expression. Tissue sections from benign (n=3), PTC
22 (n=7) and ATC (n=1) cases were probed with APLP2 antibody. The distribution of all immunostaining
23 scores for nuclear and cytoplasmic APLP2 expression is shown, with respective mean and standard
24 error bars indicated.

25 **[0088]** Figure 8 shows secreted AXL protein in thyroid cancer patient sera using an enzyme-linked
26 immunosorbent assay. Pre- and post-surgery blood samples were assessed. Non-Agg refers to non-
27 aggressive stage I or II PTC cases with no extrathyroidal spread. Agg PTC refers to aggressive PTC
28 cases that have lymph node metastasis, extrathyroidal invasion, and multifocality. Very-Agg refers to
29 the more aggressive PTC cases, which are poorly differentiated and/or have distant metastasis. Lt
30 Post-Non-Agg refers to long-term post-surgery non-aggressive PTC blood samples taken 4-30 years
31 after surgery with patients having no residual cancer. In long-term post-surgery non-aggressive PTC
32 and post-surgery very aggressive PTC, AXL sera levels were lower compared to their respective pre-
33 surgery levels.

34 **[0089]** Figure 9 show immunohistochemical analysis of APP, 14-3-3 zeta, AXL, SET, PKM2 and
35 hnRNPK in benign tissues and PTC. Cytoplasmic expressions of APP and 14-3-3 zeta proteins were
36 observed in PTCs, in contrast to the absent cytoplasmic and nuclear staining in benign thyroid
37 tissues. Minimal nuclear expression and no cytoplasmic expression of AXL were observed in benign
38 thyroid tissues. Cytoplasmic accumulation of AXL protein was found in the PTC cases examined.

Strong nuclear expression and no cytoplasmic expression were observed for SET in benign thyroid tissues. In PTC, SET was found to show reduced nuclear expression. PKM2 displayed nuclear and cytoplasmic expression in benign thyroid tissues. In PTC, cytoplasmic accumulation of the PKM2 protein was found to increase, often with a loss of nuclear expression. hnRNPK was found in the nucleus of benign thyroid tissues. This nuclear expression was found to decrease, with a concomitant increase in cytoplasmic hnRNPK in the PTC cases observed. Original magnification X400.

[0090] Figure 10 shows scatter plot analysis of APP, SET, AXL, 14-3-3 zeta, PKM2 and hnRNPK proteins expression. Tissue sections from benign (n=13) and papillary thyroid carcinomas (PTC, n=13) cases were probed with respective antibodies. The distribution of all immunostaining scores for these proteins expression is shown, with respective mean and standard error bars indicated.

Increased cytoplasmic staining of APP (A) and 14-3-3 zeta (D) was observed in PTC cases as compared to benign. Nuclear staining of SET protein was observed in PTC and benign cases (B). Minimal nuclear expression and no cytoplasmic expression of AXL were observed in benign thyroid tissues (C). Cytoplasmic accumulation of AXL protein was found in the PTC cases examined (C). PKM2 displayed nuclear and cytoplasmic expression in benign thyroid tissues (E). In PTC, cytoplasmic accumulation of PKM2 protein was found to increase, often with a loss of nuclear expression (E). hnRNPK was found in the nucleus of benign thyroid tissues (F). This nuclear expression was found to decrease, with a concomitant increase in cytoplasmic hnRNPK in the PTC cases observed (F).

[0091] Figure 11 shows immunohistochemical analysis of APP, AXL, PKM2 and SET proteins in mouse xenografts of human PTC-derived (BCPAP) and ATC-derived (C643) thyroid carcinoma cell lines. In agreement with the observations in human PTC, APP was observed to stain in the cytoplasm of PTC xenografts. Increased cytoplasmic expression of APP was found in the ATC xenografts.

Weak cytoplasmic expression of AXL was observed in ATC xenografts. In anaplastic mouse xenografts, decreased nuclear expression of PKM2 in a majority of cells was observed, along with cytoplasmic immunostaining compared to papillary mouse xenografts. Consistent with the observations in human tissues, strong SET nuclear expression was observed in papillary mouse xenografts. Decreased nuclear staining of SET was observed in ATC xenografts, along with cytoplasmic expression of the protein. Original magnification X400.

[0092] Figure 12 shows photomicrographs (at x400 magnification) of tissue sections showing the immunostaining expression patterns of a panel of seven markers in benign thyroid nodules, adenomas and TC. These photomicrographs illustrate differences in subcellular localization (cytoplasm and nucleus) of: (i) PGK1; (ii) PKM2; (iii) Cyclin D1; (iv) Galectin-3; (v) PTEN; (vi) S100A6 and (vii) Profilin-1. B. FNAB showing immunostaining for (i) PGK1; (ii) Cyclin D1.

[0093] Figure 13 illustrates immunostaining of proteins in benign non-neoplastic nodules and thyroid cancers in FNAB FFPE cell blocks. Representative photomicrographs given in the panel A depict nuclear localization of proteins in FNAB FFPE cell blocks from thyroid benign non-neoplastic nodules and TC respectively. (i) PGK1; (ii) Galectin-3. Original magnification x 400.

[0094] Figure 14 illustrates immunostaining of proteins in benign non-neoplastic nodules and thyroid cancers in cytosmears. Representative photomicrographs given in the panel B depict nuclear localization of proteins in cytosmears from thyroid benign non-neoplastic nodules and TC. (i) PGK1; (ii) Galectin-3. Original magnification x 400.

[0095] Figures 15A to 15D show immunofluorescence photomicrographs (original magnification x 600) of Galectin-3 staining in thyroid FNAB from benign (Figures 15A and 15B) and papillary thyroid carcinoma (PTC) (Figures 15C and 15D) patients. ThinPreps™ were prepared from FNAB, immunostained with anti-Galectin-3 (Santa Cruz Biotechnology, B2C10, 1/100) and detected with AF488-conjugated anti-mouse antibodies (Life Technologies). Samples were mounted with ProLong™ Antifade Reagents containing DAPI (Life Technologies). Figures 15C and 15D (panel B) show increased nuclear and cytoplasmic Galectin-3 in PTC as compared to that in the benign nodule shown in Figures 15A and 15B (panel A). In these figures, green depicts Galectin-3 and blue depicts DAPI.

[0096] Figures 16A to 16D show immunofluorescence photomicrographs (original magnification x 600) showing PGK1 staining of thyroid FNAB from benign (Figures 16A and 16B) and papillary thyroid carcinoma (PTC) (Figures 16C and 16D) patients. Thyroid FNAB on ThinPreps™ were prepared and were immunostained with anti-PGK1 (Santa Cruz Biotechnology, 14, 1/50) and detected with AF488-conjugated anti-mouse antibodies (Life Technologies). Samples were mounted with ProLong™ Antifade Reagents containing DAPI (Life Technologies). Figures 16C and 16D (panel B) show decreased nuclear PGK1 in PTC as compared to that in the benign nodule shown in Figures 16A and 16B (panel A). In these figures, green depicts PGK1 and blue depicts DAPI.

[0097] Figure 17A and 17B show immunofluorescence photomicrographs showing PGK1 and Galectin 3 staining in Thyroid FNAB. ThinPreps™ were immunostained with anti-PGK1 or anti-Galectin 3 antibody, and detected using AF647-conjugated anti-mouse antibodies. Slides were mounted with medium containing DAPI. In Figure 17A, red = PGK1 and blue = DAPI. In Figure 17B red = Galectin 3 and blue = DAPI. Figure 17A shows loss of PGK1 expression and Figure 17B shows cytoplasmic galectin 3 expression in FNAB.

[0098] Figures 18A to 18D show immunofluorescence photomicrographs showing cytoplasmic and nuclear PGK1 staining in Thyroid FNAB. ThinPreps™ were immunostained with PGK1 antibody, Clone 14™ (SantaCruz Biotechnologies, Santa Cruz, CA, USA). Slides were mounted with medium containing DAPI. PGK1 nuclear and cytoplasmic staining = green and Dapi = blue-nuclei.

DETAILED DESCRIPTION

[0099] The present description relates to correlations between the expression of Thyroid Cancer Markers and thyroid cancer, in particular, the stage, aggressiveness and/or metastatic potential of a thyroid cancer. The Thyroid Cancer Markers described herein provide methods for diagnosing, detecting, predicting, monitoring or characterizing thyroid cancer, in particular stage, aggressiveness or metastatic potential of a thyroid cancer. Methods are provided for screening for, diagnosing or

detecting the presence or absence of thyroid cancer, papillary, follicular or aggressive or metastatic thyroid cancer in a sample, and for monitoring the progression of thyroid cancer, as well as providing information about characteristics of a thyroid carcinoma that are relevant to the diagnosis and characterization of thyroid carcinoma in a patient.

[0100] Glossary

[0101] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this description belongs. The following definitions supplement those in the art and are directed to the present application and are not to be imputed to any related or unrelated case. Although any methods and materials similar or equivalent to those described herein can be used in the practice of the description, particular materials and methods are described herein.

[0102] Numerical ranges recited herein by endpoints include all numbers and fractions subsumed within that range (e.g. 1 to 5 includes 1, 1.5, 2, 2.75, 3, 3.90, 4, and 5). It is also to be understood that all numbers and fractions thereof are presumed to be modified by the term "about." The term "about" means plus or minus 0.1 to 50%, 5-50%, or 10-40%, preferably 10-20%, more preferably 10% or 15%, of the number to which reference is being made. As used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise.

[0103] The term "thyroid cancer" refers to any malignant process of the thyroid gland. Examples of thyroid cancers include, but are not limited to, papillary thyroid carcinoma, follicular variant of papillary thyroid carcinoma, follicular carcinoma, Hurthle cell tumor, anaplastic thyroid carcinoma, medullary thyroid cancer, thyroid lymphoma, poorly differentiated thyroid cancer and thyroid angiosarcoma. In aspects of the description, the thyroid cancer is papillary thyroid carcinoma. In aspects of the description, the thyroid cancer is a follicular variant of papillary thyroid carcinoma or follicular carcinoma ('also referred to herein as "follicular thyroid cancer"). In aspects of the description, the thyroid cancer is medullary thyroid cancer. In aspects of the description, the thyroid cancer is an aggressive cancer or has metastatic potential, in particular an aggressive medullary or follicular thyroid cancer or a medullary or follicular thyroid cancer with metastatic potential. In aspects of the description, the thyroid cancer is anaplastic thyroid carcinoma (ATC).

[0104] "Metastatic potential" refers to the ability or possibility of a cancer cell moving from the initial site (i.e. thyroid) to other sites in the body.

[0105] The term "detect" or "detecting" includes assaying, or otherwise screening or establishing the presence or absence of the target marker(s), subunits, or combinations of reagent bound targets, and the like, or assaying for ascertaining, establishing, classifying monitoring, predicting or otherwise determining one or more factual characteristics of a thyroid cancer such as aggressiveness, metastatic potential or patient survival, or assisting in same. A standard may correspond to levels quantitated for samples from control subjects with no disease or early stage disease (e.g., low grade

thyroid cancer such as papillary thyroid cancer) or from other samples of the subject.

[0106] The term “sample” and the like mean a material known or suspected of expressing or containing Thyroid Cancer Markers, or binding agents such as antibodies specific for Polypeptide Thyroid Cancer Markers. The sample may be derived from a biological source (“biological sample”), such as tissues, extracts, or cell cultures, including cells (e.g. tumor cells), cell lysates, and biological or physiological fluids, such as, for example, whole blood, plasma, serum, saliva, cerebral spinal fluid, sweat, urine, milk, peritoneal fluid and the like. A sample may be used directly as obtained from the source or following a pretreatment to modify the character of the sample, such as preparing plasma from blood, diluting viscous fluids, and the like. In certain aspects of the description, the sample is a fluid sample. In certain aspects of the description the sample is serum, plasma, whole blood, urine or saliva. In certain particular aspects of the description the sample is serum. In certain aspects of the description, the sample is a human physiological fluid, such as human serum. In aspects of the description, the sample comprises cells (or nuclei obtained from the cells) from different sites of a tumor.

[0107] The samples that may be analyzed in accordance with the description include polynucleotides from clinically relevant sources, preferably expressed RNA or a nucleic acid derived therefrom (cDNA or amplified RNA derived from cDNA that incorporates an RNA polymerase promoter). As will be appreciated by those skilled in the art, the target polynucleotides can comprise RNA, including, without limitation total cellular RNA, poly(A)⁺ messenger RNA (mRNA) or fraction thereof, cytoplasmic mRNA, or RNA transcribed from cDNA (i.e., cRNA). (i.e., cRNA; see, for example., Linsley & Schelter, U.S. patent application Ser. No. 09/411,074, or U.S. Pat. Nos. 5,545,522, 5,891,636 or 5,716,785). Methods for preparing total and poly(A)⁺ RNA are well known in the art, and are described generally, for example, in Sambrook et al., (1989, *Molecular Cloning – A Laboratory Manual* (2nd Ed.), Vols. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) and Ausubel et al, eds. (1994, *Current Protocols in Molecular Biology*, vol. 2, Current Protocols Publishing, New York). RNA may be isolated from eukaryotic cells by procedures involving lysis of the cells and denaturation of the proteins contained in the cells. Additional steps may be utilized to remove DNA. Cell lysis may be achieved with a nonionic detergent, followed by microcentrifugation to remove the nuclei and hence the bulk of the cellular DNA. (See Chirgwin et al., 1979, *Biochemistry* 18:5294-5299). Poly(A)⁺ RNA can be selected using oligo-dT cellulose (see Sambrook et al., 1989, *Molecular Cloning—A Laboratory Manual* (2nd Ed.), Vols. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). In the alternative, RNA can be separated from DNA by organic extraction, for example, with hot phenol or phenol/chloroform/isoamyl alcohol.

[0108] Target polynucleotides can be detectably labeled at one or more nucleotides using methods known in the art. The label is preferably uniformly incorporated along the length of the RNA, and more preferably, is carried out at a high degree of efficiency. The detectable label can be a luminescent label, fluorescent label, bio-luminescent label, chemiluminescent label, radiolabel, and colorimetric label.

[0109] Target polynucleotides from a patient sample can be labeled differentially from polynucleotides of a standard. The standard can comprise target polynucleotides from normal individuals (e.g. those not afflicted with or pre-disposed to thyroid cancer, in particular pooled from samples from normal individuals or patients with a different disease stage). The target polynucleotides can be derived from the same individual, but taken at different time points, and thus indicate the efficacy of a treatment by a change in expression of the markers, or lack thereof, during and after the course of treatment.

[0110] The terms "subject", "patient" and "individual" are used interchangeably herein and refer to a warm-blooded animal such as a mammal that is afflicted with thyroid cancer, or suspected of having thyroid cancer, being pre-disposed to thyroid cancer, being screened for thyroid cancer or at risk for thyroid cancer. The term includes but is not limited to domestic animals, sports animals, primates and humans. Preferably, the terms refer to a human.

[0111] As used herein, the term subject "suspected of having thyroid cancer" refers to a subject that presents one or more symptoms indicative of a thyroid cancer (e.g., a noticeable lump or mass) or is being screened for a cancer (e.g., during a routine physical). A subject suspected of having thyroid carcinoma may also have one or more risk factors. A subject suspected of having thyroid cancer has generally not been tested for cancer. However, a "subject suspected of having thyroid cancer" encompasses an individual who has received an initial diagnosis but for whom the stage of cancer is not known. The term further includes people who once had cancer (e.g., an individual in remission). As used herein, the term subject "at risk for thyroid cancer" refers to a subject with one or more risk factors for developing thyroid carcinoma. Risk factors include, but are not limited to, gender, age, genetic predisposition, environmental exposure, previous incidents of cancer, preexisting non-cancer diseases, and lifestyle. As used herein, the term "characterizing thyroid cancer in a subject" refers to the identification of one or more properties of a cancer sample in a subject, including but not limited to the subject's prognosis or survival. Cancers may be characterized by the identification of the expression of one or more markers, including but not limited to, the Thyroid Cancer Markers disclosed herein.

[0112] "Thyroid Cancer Markers for papillary thyroid cancer set out in Table 1" include the markers identified by "*" in Table 1, namely markers chosen from the markers numbered 4, 12, 25 and 26, and polynucleotides encoding same.

[0113] "Thyroid Cancer Markers for follicular thyroid cancer set out in Table 1" include the markers identified by "***" in Table 1, namely markers chosen from the markers numbered 21, 22 and 28, and polynucleotides encoding same.

[0114] "Thyroid Cancer Markers for aggressive/metastatic thyroid cancer set out in Table 1" include the markers identified by "****" in Table 1, namely markers chosen from the markers numbered 15, 17, 29, 30, 31, 32, 33, 35, 36, 38, 39, 40, 42, 43, 45, 46, 47, 49-58, 60, 65-75, 77, 78, 80, 81 and 83, 88 and polynucleotides encoding same.

1 **[0115]** Thyroid Cancer Markers include polypeptide or protein markers including without limitation a
2 native-sequence polypeptide, a polypeptide variant, a chimeric protein or fusion protein, isoforms,
3 complexes, all homologs, fragments, precursors, and modified forms and derivatives of the markers
4 (i.e., Polypeptide Thyroid Cancer Markers).

5 **[0116]** "Polypeptide" and "protein" are used interchangeably herein and indicate at least one
6 molecular chain of amino acids linked through covalent and/or non-covalent bonds. The terms include
7 peptides, oligopeptides, and proteins, and post-translational modifications of the polypeptides, e.g.
8 glycosylations, acetylations, phosphorylations, and the like. Protein fragments, analogues, mutated or
9 variant proteins, fusion proteins, and the like, are also included within the meaning of the terms.

10 **[0117]** A "native-sequence polypeptide" comprises a polypeptide having the same amino acid
11 sequence of a polypeptide derived from nature. Such native-sequence polypeptides can be isolated
12 from nature or can be produced by recombinant or synthetic means. The term specifically
13 encompasses naturally occurring truncated or secreted forms of a polypeptide, polypeptide variants
14 including naturally occurring variant forms (e.g. alternatively spliced forms or splice variants), and
15 naturally occurring allelic variants.

16 **[0118]** The term "polypeptide variant" means a polypeptide having at least about 10%, 20%, 30%,
17 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% amino acid
18 sequence identity, particularly at least about 70-80%, more particularly at least about 85%, still more
19 particularly at least about 90%, most particularly at least about 95%, 97%, or 99% amino acid
20 sequence identity with a native-sequence polypeptide. Particular polypeptide variants have at least
21 70-80%, 85%, 90%, 95%, 97% or 99% amino acid sequence identity to the sequences identified in
22 Table 1 or 2. Such variants include, for instance, polypeptides wherein one or more amino acid
23 residues are added to, or deleted from, the N- or C-terminus of the full-length or mature sequences of
24 the polypeptide, including variants from other species, but exclude a native-sequence polypeptide. In
25 aspects of the description variants retain the immunogenic activity of the corresponding native-
26 sequence polypeptide.

27 **[0119]** Sequence identity of two amino acid sequences or of two nucleic acid sequences is defined
28 as the percentage of amino acid residues or nucleotides in a candidate sequence that are identical
29 with the amino acid residues in a polypeptide or nucleic acid sequence, after aligning the sequences
30 and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not
31 considering any conservative substitutions as part of the sequence identity. Alignment for purposes of
32 determining percent amino acid or nucleic acid sequence identity can be achieved in various
33 conventional ways, for instance, using publicly available computer software including the GCG
34 program package (Devereux J. et al., Nucleic Acids Research 12(1): 387, 1984); BLASTP, BLASTN,
35 and FASTA (Atschul, S.F. et al. J. Molec. Biol. 215: 403-410, 1990). The BLAST X program is publicly
36 available from NCBI and other sources (BLAST Manual, Altschul, S. et al. NCBI NLM NIH Bethesda,
37 Md. 20894; Altschul, S. et al. J. Mol. Biol. 215: 403-410, 1990). Skilled artisans can determine
38 appropriate parameters for measuring alignment, including any algorithms needed to achieve

1 maximal alignment over the full length of the sequences being compared. Methods to determine
2 identity and similarity are codified in publicly available computer programs.

3 **[0120]** Polypeptide variants include polypeptides comprising amino acid sequences sufficiently
4 identical to or derived from the amino acid sequence of a native polypeptide which includes fewer
5 amino acids than the full-length polypeptides. A portion or fragment of a polypeptide can be a
6 polypeptide which is for example, 3-5, 8-10, 10, 15, 15-20, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90,
7 100 or more amino acids in length. Portions or fragments in which regions of a polypeptide are
8 deleted can be prepared by recombinant techniques and can be evaluated for one or more functional
9 activities such as the ability to form antibodies specific for a polypeptide. A portion or fragment of a
10 polypeptide may comprise a domain of the polypeptide, in particular an extracellular domain or
11 intracellular domain.

12 **[0121]** An allelic variant may also be created by introducing substitutions, additions, or deletions into
13 a nucleic acid encoding a native polypeptide sequence such that one or more amino acid
14 substitutions, additions, or deletions are introduced into the encoded protein. Mutations may be
15 introduced by standard methods, such as site-directed mutagenesis and PCR-mediated mutagenesis.
16 In an embodiment, conservative substitutions are made at one or more predicted non-essential amino
17 acid residues. A "conservative amino acid substitution" is one in which an amino acid residue is
18 replaced with an amino acid residue with a similar side chain, several of which are known in the art.

19 **[0122]** A naturally occurring allelic variant may contain conservative amino acid substitutions from
20 the native polypeptide sequence or it may contain a substitution of an amino acid from a
21 corresponding position in polypeptide homolog, for example, a murine polypeptide.

22 **[0123]** A polypeptide disclosed herein includes chimeric or fusion proteins. A "chimeric protein" or
23 "fusion protein" comprises all or part (preferably biologically active) of the polypeptide operably linked
24 to a heterologous polypeptide (i.e., a different polypeptide). Within the fusion protein, the term
25 "operably linked" is intended to indicate that the polypeptide and the heterologous polypeptide are
26 fused in-frame to each other. The heterologous polypeptide can be fused to the N-terminus or C-
27 terminus of the polypeptide. A useful fusion protein is a GST fusion protein in which a polypeptide is
28 fused to the C-terminus of GST sequences. Another example of a fusion protein is an immunoglobulin
29 fusion protein in which all or part of a polypeptide is fused to sequences derived from a member of the
30 immunoglobulin protein family. Chimeric and fusion proteins can be produced by standard
31 recombinant DNA techniques.

32 **[0124]** Polypeptides used in the methods disclosed herein may be isolated from a variety of sources,
33 such as from human tissue types or from other sources, or prepared by recombinant or synthetic
34 methods, or by any combination of these and similar techniques.

35 **[0125]** "Polynucleotide" refers to a polymeric form of nucleotides of any length, either ribonucleotides
36 or deoxyribonucleotides. The term includes double- and single-stranded DNA and RNA, modifications
37 such as methylation or capping and unmodified forms of the polynucleotide. The terms

“polynucleotide” and “oligonucleotide” are used interchangeably herein. A polynucleotide may, but need not, include additional coding or non-coding sequences, or it may, but need not, be linked to other molecules and/or carrier or support materials. Polynucleotide Thyroid Cancer Markers for use in the methods of the description may be of any length suitable for a particular method. In certain applications the term refers to antisense nucleic acid molecules (e.g. an mRNA or DNA strand in the reverse orientation to a sense Polynucleotide Thyroid Cancer Markers).

[0126] Polynucleotide Thyroid Cancer Markers include polynucleotides encoding Polypeptide Thyroid Cancer Markers, including a native-sequence polypeptide, a polypeptide variant including a portion of a Polypeptide Thyroid Cancer Marker, an isoform, precursor, a chimeric protein, complexes, homologs, fragments, precursors, and modified forms and derivatives of the markers.

[0127] Polynucleotides used in the methods of the description include complementary nucleic acid sequences, and nucleic acids that are substantially identical to these sequences (e.g. at least about 10%, 20%, 30%, 40%, or 45%, preferably 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% sequence identity).

[0128] Polynucleotides also include sequences that differ from a nucleic acid sequence due to degeneracy in the genetic code. As one example, DNA sequence polymorphisms within the nucleotide sequence of a Thyroid Cancer Marker disclosed herein may result in silent mutations that do not affect the amino acid sequence. Variations in one or more nucleotides may exist among individuals within a population due to natural allelic variation. DNA sequence polymorphisms may also occur which lead to changes in the amino acid sequence of a polypeptide.

[0129] Polynucleotides which may be used in the methods disclosed herein include nucleic acids that hybridize under stringent conditions, preferably high stringency conditions to a nucleic acid sequence of a Polynucleotide Thyroid Cancer Marker. Appropriate stringency conditions which promote DNA hybridization are known to those skilled in the art, or can be found in Ausubel et al., (eds) Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. Generally, stringent conditions may be selected that are about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength, pH, and nucleic acid concentration) at which 50% of the probes complementary to a target sequence hybridize at equilibrium to the target sequence. Generally, stringent conditions will be those in which the salt concentration is less than about 1.0M sodium ion or other salts (e.g. about 0.01 to 1.0M sodium ion) and the temperature is at least about 30°C for short probes, primers or oligonucleotides (e.g. 10-50 nucleotides) and at least 60°C for longer probes, primers and oligonucleotides. For example, a hybridization may be conducted at 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C, or at 42°C in a solution containing 6xSSC, 0.5% SDS and 50% formamide followed by washing in a solution of 0.1x SSC and 0.5% SDS at 68°C.

[0130] Polynucleotide Thyroid Cancer Markers also include truncated nucleic acids or nucleic acid

1 fragments and variant forms of the nucleic acids disclosed or referenced herein that arise by
2 alternative splicing of an mRNA corresponding to a DNA. A fragment of a polynucleotide includes a
3 polynucleotide sequence that comprises a contiguous sequence of approximately at least about 6
4 nucleotides, in particular at least about 8 nucleotides, more particularly at least about 10-12
5 nucleotides, and even more particularly 15-20 nucleotides that correspond to (i.e. identical or
6 complementary to), a region of the specified nucleotide sequence.

7 **[0131]** "Significantly different" levels of markers or a "significant difference" in marker levels in a
8 patient sample compared to a control or standard (e.g. normal levels, levels from a different disease
9 stage, or levels in other samples from a patient) may represent levels that are higher or lower than the
10 standard error of the detection assay, preferably the levels are at least about 1.5, 2, 3, 4, 5, 6, 7, 8, 9
11 or 10 times higher or lower, respectively, than the control or standard.

12 **[0132]** "Microarray" and "array," refer to nucleic acid or nucleotide arrays or protein or peptide arrays
13 that can be used to detect biomolecules associated with thyroid cancer, for instance to measure gene
14 expression. A variety of arrays are available commercially, such as, for example, as the *in situ*
15 synthesized oligonucleotide array GeneChip™ made by Affymetrix, Inc. or the spotted cDNA array,
16 LifeArray™ made by Incyte Genomics Inc..

17 **[0133]** "Binding agent" refers to a substance such as a polypeptide, antibody, ribosome, or aptamer
18 that specifically binds to a Polypeptide Thyroid Cancer Marker. A binding agent, in particular an
19 antibody, that "specifically binds" or "binds" (used interchangeably herein) to a target or an antigen or
20 epitope is a term well understood in the art, and methods to determine specific binding are also well
21 known in the art. A binding agent "specifically binds" to a target if it binds with greater affinity, avidity,
22 more readily, and/or with greater duration than it binds to other substances. It will be appreciated that
23 an antibody that specifically binds to a first target may or may not specifically or preferentially bind to a
24 second target. Thus, "specific binding" does not necessarily require (although it can include) exclusive
25 binding but generally refers to preferential binding. Binding properties may be assessed using an
26 ELISA, which may be readily performed by those skilled in the art (see for example, Newton et al,
27 Develop. Dynamics 197: 1-13, 1993). In an embodiment of the description, antibodies are reactive
28 against a polypeptide marker if they bind with a K_a of greater than or equal to 10^{-7} M.

29 **[0134]** A binding agent may be a ribosome, with or without a peptide component, a RNA or DNA
30 molecule, or a polypeptide. A binding agent may be a polypeptide that comprises a Polypeptide
31 Thyroid Cancer Marker sequence, a peptide variant thereof, or a non-peptide mimetic of such a
32 sequence. By way of example a Polypeptide Thyroid Cancer Marker sequence may be a peptide
33 portion of the polypeptide that is capable of modulating a function mediated by the polypeptide.

34 **[0135]** An aptamer includes a DNA or RNA molecule that binds to nucleic acids and proteins. An
35 aptamer that binds to a Thyroid Cancer Marker can be produced using conventional techniques,
36 without undue experimentation. [For example, see the following publications describing *in vitro*
37 selection of aptamers: Klug et al., Mol. Biol. Reports 20:97-107 (1994); Wallis et al., *Chem. Biol.*

2:543-552 (1995); Ellington, *Curr. Biol.* 4:427-429 (1994); Lato et al., *Chem. Biol.* 2:291-303 (1995); Conrad et al., *Mol. Div.* 1:69-78 (1995); and Uphoff et al., *Curr. Opin. Struct. Biol.* 6:281-287 (1996)].

[0136] Antibodies for use in the presently described methods include but are not limited to synthetic antibodies, monoclonal antibodies, polyclonal antibodies, recombinant antibodies, antibody fragments (such as Fab, Fab', F(ab')₂), dAb (domain antibody; see Ward et al, 1989, *Nature*, 341:544-546), antibody heavy chains, intrabodies, humanized antibodies, human antibodies, antibody light chains, single chain F_{vs} (scFv) (e.g., including monospecific, bispecific etc), anti-idiotypic (ant-Id) antibodies, proteins comprising an antibody portion, chimeric antibodies (for example, antibodies which contain the binding specificity of murine antibodies, but in which the remaining portions are of human origin), derivatives, such as enzyme conjugates or labeled derivatives, diabodies, linear antibodies, disulfide-linked Fvs (sdFv), multispecific antibodies (e.g., bispecific antibodies), epitope-binding fragments of any of the above, and any other modified configuration of an immunoglobulin molecule that comprises an antigen recognition site of the required specificity. An antibody includes an antibody of any type (e.g. IgA, IgD, IgE, IgG, IgM and IgY), any class (e.g. IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2), or any subclass (e.g. IgG2a and IgG2b), and the antibody need not be of any particular type, class or subclass. In certain embodiments of the description the antibodies are IgG antibodies or a class or subclass thereof. An antibody may be from any animal origin including birds and mammals (e.g. human, murine, donkey, sheep, rabbit, goat, guinea pig, camel, horse, or chicken).

[0137] A "recombinant antibody" includes antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies expressed using a recombinant expression vector transfected into a host cell, antibodies isolated from recombinant, combinatorial antibody libraries, antibodies isolated from an animal (e.g. a mouse or cow) that is transgenic and/or transchromosomal for human immunoglobulin genes, or antibodies prepared, expressed, created or isolated by any other means that involves slicing of immunoglobulin gene sequences to other DNA sequences.

[0138] A "monoclonal antibody" refers to an antibody obtained from a population of homogenous or substantially homogenous antibodies. Generally each monoclonal antibody recognizes a single epitope on an antigen. In aspects of the description, a monoclonal antibody is an antibody produced by a single hybridoma or other cell, and it specifically binds to only a Thyroid Cancer Marker as determined, for example by ELISA or other antigen-binding or competitive binding assay known in the art. The term is not limited to a particular method for making the antibody and for example they may be produced by the hybridoma method or isolated from phage libraries using methods known in the art.

[0139] Antibodies, including monoclonal and polyclonal antibodies, fragments and chimeras, may be prepared using methods well known to those skilled in the art. Isolated native or recombinant polypeptides may be utilized to prepare antibodies. See, for example, Kohler et al. (1975) *Nature* 256:495-497; Kozbor et al. (1985) *J. Immunol Methods* 81:31-42; Cote et al. (1983) *Proc Natl Acad Sci* 80:2026-2030; and Cole et al. (1984) *Mol Cell Biol* 62:109-120 for the preparation of monoclonal antibodies; Huse et al. (1989) *Science* 246:1275-1281 for the preparation of monoclonal Fab

1 fragments; and, Pound (1998) Immunochemical Protocols, Humana Press, Totowa, N.J for the
2 preparation of phagemid or B-lymphocyte immunoglobulin libraries to identify antibodies. Antibodies
3 specific for polypeptide markers may also be obtained from scientific or commercial sources.

4 **[0140]** The "status" of a marker refers to the presence, absence or extent/level of the marker or
5 some physical, chemical or genetic characteristic of the marker. Such characteristics include without
6 limitation, expression level, activity level, structure (sequence information), copy number, post-
7 translational modification etc. The status of a marker may be directly or indirectly determined. In some
8 embodiments status is determined by determining the level of a marker in the sample. The "level" of
9 an element in a sample has its conventional meaning in the art, and includes quantitative
10 determinations (e.g. mg/mL, fold change, etc) and qualitative determinations (e.g. determining the
11 presence or absence of a marker or determining whether the level of the marker is high, low or even
12 present relative to a standard).

13 **[0141]** The term "abnormal status" means that a marker's status in a sample is different from a
14 reference status for the marker. A reference status may be the status of the marker in samples from
15 normal subjects, averaged samples from subjects with the condition or sample(s) from the same
16 subject taken at different times. An abnormal status includes an elevated, decreased, present or
17 absent marker(s). Determining the level of a marker in a sample may include determining the level of
18 the marker in a sample and abnormal status could be either lower levels (including undetectable
19 levels) or higher levels (including any amount over zero) compared to a standard. A subject may have
20 an increased likelihood of a condition disclosed herein if the status of a marker in the subject's sample
21 is correlated with the condition (e.g. a level of the marker is closer to a standard or reference or is
22 present in levels that exceed some threshold value where exceeding that value is correlated with the
23 condition). A subject with an increased likelihood of a condition disclosed herein includes a subject
24 with an abnormal status for a marker and as such the subject has a higher likelihood of the condition
25 than if the subject did not have that status.

26 **[0142]** An "elevated status" means one or more characteristics of a marker are higher than a
27 standard. In aspects of the description, the term refers to an increase in a characteristic as compared
28 to a standard. A "low status" means one or more characteristics of a marker are lower than a
29 standard. In aspects of the description, the term refers to a decrease in a characteristic as compared
30 to a standard. A "negative status" means that one or more characteristic of a marker is absent or
31 undetectable.

32 **[0143] General Methods**

33 **[0144]** A variety of methods can be employed for the diagnostic and prognostic evaluation of thyroid
34 cancer involving Thyroid Cancer Markers and the identification of subjects with a predisposition to
35 such disorders. Such methods may, for example, utilize Polynucleotide Thyroid Cancer Markers and
36 fragments thereof, and binding agents (e.g. antibodies) directed against Polypeptide Thyroid Cancer
37 Markers including peptide fragments. In particular, the polynucleotides and antibodies may be used,

for example, for (1) the detection of the presence of polynucleotide mutations, or the detection of either over- or under-expression of mRNA, relative to a non-disorder state or the qualitative or quantitative detection of alternatively spliced forms of polynucleotide transcripts which may correlate with certain conditions or susceptibility toward such conditions; and (2) the detection of either an over- or an under-abundance of polypeptides relative to a non-disorder state or the presence of a modified (e.g., less than full length) polypeptide which correlates with a disorder state, or a progression toward a disorder state.

[0145] The methods described herein may be used to evaluate the probability of the presence of malignant cells, for example, in a group of cells freshly removed from a host. Such methods can be used to detect tumors, quantitate and monitor their growth, and help in the diagnosis and prognosis of disease. For example, significantly different levels of one or more markers in Table 1 are indicative of thyroid cancer.

[0146] The methods of the description require that the amount of Thyroid Cancer Markers quantitated in a sample from a subject being tested be compared to a predetermined standard or cut-off value. A standard may correspond to levels quantitated for another sample or an earlier sample from the subject, or levels quantitated for a control sample, in particular a sample from a subject with a lower grade cancer. Levels for control samples from healthy subjects or cancer subjects may be established by prospective and/or retrospective statistical studies. Healthy subjects who have no clinically evident disease or abnormalities may be selected for statistical studies. Diagnosis may be made by a finding of statistically different levels of Thyroid Cancer Markers compared to a control sample or previous levels quantitated for the same subject.

[0147] The description also contemplates the methods described herein using multiple markers for thyroid cancer. Therefore, the description contemplates a method for analyzing a biological sample for the presence of Thyroid Cancer Markers and other markers that are specific indicators of thyroid cancer. The methods described herein may be modified by including reagents to detect the markers or polynucleotides encoding the markers. Examples of other markers include without limitation the markers listed in Table 2 or markers comprising or selected from the group comprising Galectin-3, thyroglobulin, E-cadherin, and Galectin-3, in particular Galectin-3.

[0148] Nucleic Acid Methods

[0149] As noted herein thyroid cancer, in particular the stage or aggressiveness of a thyroid cancer, may be detected based on the level of Polynucleotide Thyroid Cancer Markers in a sample. Techniques for detecting nucleic acid molecules such as polymerase chain reaction (PCR) and hybridization assays are well known in the art.

[0150] Probes may be used in hybridization techniques to detect polynucleotides. The technique generally involves contacting and incubating nucleic acids obtained from a sample from a patient or other cellular source with a probe under conditions favorable for the specific annealing of the probes to complementary sequences in the nucleic acids (e.g. under stringent conditions as discussed

herein). After incubation, the non-annealed nucleic acids are removed, and the presence of nucleic acids that have hybridized to the probe if any are detected. Nucleotide probes for use in the detection of polynucleotide sequences in samples may be constructed using conventional methods known in the art. The probes may comprise DNA or DNA mimics corresponding to a portion of an organism's genome, or complementary RNA or RNA mimics. The nucleic acids can be modified at the base moiety, at the sugar moiety, or at the phosphate backbone. DNA can be obtained using standard methods such as polymerase chain reaction (PCR) amplification of genomic DNA or cloned sequences. Computer programs known in the art can be used to design primers with the required specificity and optimal amplification properties.

[0151] A nucleotide probe may be labeled with a detectable substance such as a radioactive label which provides for an adequate signal and has sufficient half-life such as ^{32}P , ^3H , ^{14}C or the like. Other detectable substances that may be used include antigens that are recognized by a specific labeled antibody, fluorescent compounds, enzymes, antibodies specific for a labeled antigen, and luminescent compounds. An appropriate label may be selected having regard to the rate of hybridization and binding of the probe to the nucleic acids to be detected and the amount of nucleic acids available for hybridization. Labeled probes may be hybridized to nucleic acids on solid supports such as nitrocellulose filters or nylon membranes as generally described in Sambrook et al., 1989, *Molecular Cloning, A Laboratory Manual* (2nd ed.). The nucleic acid probes may be used to detect Polynucleotide Thyroid Cancer Markers, preferably in human cells. The nucleotide probes may also be useful in the diagnosis of thyroid cancer, involving Polynucleotide Thyroid Cancer Markers in monitoring the progression of thyroid cancer, or monitoring a therapeutic treatment.

[0152] The detection of polynucleotides in a sample may involve the amplification of specific gene sequences using an amplification method such as PCR, followed by the analysis of the amplified molecules using techniques known to those skilled in the art. By way of example, oligonucleotide primers may be employed in a PCR based assay to amplify a portion of a polynucleotide and to amplify a portion of a polynucleotide derived from a sample, wherein the oligonucleotide primers are specific for (i.e. hybridize to) the polynucleotides. The amplified cDNA is then separated and detected using techniques well known in the art, such as gel electrophoresis.

[0153] In order to maximize hybridization under assay conditions, primers and probes employed in the methods of the description generally have at least about 60%, preferably at least about 75% and more preferably at least about 90% identity to a portion of a Polynucleotide Thyroid Cancer Marker; that is, they are at least 10 nucleotides, and preferably at least 20 nucleotides in length. In an embodiment the primers and probes are at least about 10-40 nucleotides in length.

[0154] Hybridization and amplification reactions may also be conducted under stringent conditions as discussed herein. Hybridization and amplification techniques described herein may be used to assay qualitative and quantitative aspects of polynucleotide expression. For example, RNA may be isolated from a cell type or tissue known to express Polynucleotide Thyroid Cancer Markers, and tested utilizing the hybridization (e.g. standard Northern analyses) or PCR techniques.

1 **[0155]** In an aspect of the description, a method is provided employing reverse transcriptase-
2 polymerase chain reaction (RT-PCR), in which PCR is applied in combination with reverse
3 transcription. Generally, RNA is extracted from a sample using standard techniques and is reverse
4 transcribed to produce cDNA. The cDNA is used as a template for a polymerase chain reaction. The
5 cDNA is hybridized to primer sets which are specifically designed against a Polynucleotide Thyroid
6 Cancer Marker. Once the primer and template have annealed a DNA polymerase is employed to
7 extend from the primer, to synthesize a copy of the template. The DNA strands are denatured, and
8 the procedure is repeated many times until sufficient DNA is generated to allow visualization by
9 ethidium bromide staining and agarose gel electrophoresis.

10 **[0156]** Amplification may be performed on samples obtained from a subject with suspected thyroid
11 cancer, an individual who is not afflicted with thyroid cancer or has early stage disease or has
12 aggressive or metastatic disease. The reaction may be performed on several dilutions of cDNA
13 spanning at least two orders of magnitude. A statistically significant difference in expression in several
14 dilutions of the subject sample as compared to the same dilutions of the non-cancerous sample or
15 early-stage cancer sample may be considered positive for the presence of cancer.

16 **[0157]** Oligonucleotides or longer fragments derived from Polynucleotide Thyroid Cancer Markers
17 may be used as targets in a microarray. The microarray can be used to monitor the expression levels
18 of the polynucleotides and to identify genetic variants, mutations, and polymorphisms. The information
19 from the microarray may be used to determine gene function, to understand the genetic basis of a
20 disorder, to diagnose a disorder, and to develop and monitor the activities of therapeutic agents.
21 Thus, the description also includes an array comprising Polynucleotide Thyroid Cancer Markers, and
22 optionally other thyroid cancer markers. The array can be used to assay expression of Polynucleotide
23 Thyroid Cancer Markers in the array. The description allows the quantitation of expression of the
24 polynucleotides.

25 **[0158]** The description provides microarrays comprising Polynucleotide Thyroid Cancer Markers. In
26 one embodiment, the description provides a microarray for distinguishing samples associated with
27 thyroid cancer, in particular aggressive thyroid cancer or thyroid cancer with metastatic potential
28 comprising a positionally-addressable array of polynucleotide probes bound to a support, the
29 polynucleotide probes comprising sequences complementary and hybridizable to Polynucleotide
30 Thyroid Cancer Markers. In an embodiment, the array can be used to monitor the time course of
31 expression of Polynucleotide Thyroid Cancer Markers in the array. This can occur in various biological
32 contexts such as tumor progression. An array can also be useful for ascertaining differential
33 expression patterns of Polynucleotide Thyroid Cancer Markers, and optionally other thyroid cancer
34 markers in normal and abnormal cells. This may provide a battery of nucleic acids that could serve as
35 molecular targets for diagnosis or therapeutic intervention. The preparation, use, and analysis of
36 microarrays are well known to those skilled in the art. (See, for example, Brennan, T. M. et al. (1995)
37 U.S. Pat. No. 5,474,796; Schena, et al. (1996) Proc. Natl. Acad. Sci. 93:10614-10619;
38 Baldeschweiler et al. (1995), PCT Application WO95/251116; Shalon, D. et al. (1995) PCT application

WO95/35505; Heller, R. A. et al. (1997) Proc. Natl. Acad. Sci. 94:2150-2155; and Heller, M. J. et al. (1997) U.S. Pat. No. 5,605,662).

[0159] Protein Methods

[0160] Binding agents may be used for a variety of diagnostic and assay applications. There are a variety of assay formats known to the skilled artisan for using a binding agent to detect a target molecule in a sample. (For example, see Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, NY, 1988). In general, a method of the description may comprise (a) contacting a sample from the subject with a binding agent; (b) detecting in the sample a level of polypeptide that binds to the binding agent; and (c) comparing the level of polypeptide with a predetermined standard or cut-off value. In particular aspects of the description, the binding agent is an antibody.

[0161] In an aspect, the description provides a diagnostic method for monitoring or diagnosing thyroid cancer in a subject by quantitating Polypeptide Thyroid Cancer Markers in a biological sample from the subject comprising reacting the sample with antibodies specific for Polypeptide Thyroid Cancer Markers which are directly or indirectly labeled with detectable substances and detecting the detectable substances.

[0162] In an aspect of the description, a method for detecting or diagnosing thyroid cancer is provided comprising or consisting essentially of: (a) obtaining a sample suspected of containing Polypeptide Thyroid Cancer Markers; (b) contacting said sample with antibodies that specifically bind Polypeptide Thyroid Cancer Markers under conditions effective to bind the antibodies and form complexes; (c) measuring the amount of Polypeptide Thyroid Cancer Markers present in the sample by quantitating the amount of the complexes; and (d) comparing the amount of Polypeptide Thyroid Cancer Markers present in the samples with the amount of Polypeptide Thyroid Cancer Markers in a control, wherein a change or significant difference in the amount of Polypeptide Thyroid Cancer Markers in the sample compared with the amount in the control is indicative of thyroid cancer, stage of thyroid cancer, progression, aggressiveness and/or metastatic potential of the thyroid cancer.

[0163] In an embodiment, the description contemplates a method for monitoring the progression of thyroid cancer in an individual, comprising: (a) contacting antibodies which bind to Polypeptide Thyroid Cancer Markers with a sample from the individual so as to form complexes comprising the antibodies and Polypeptide Thyroid Cancer Markers in the sample; (b) determining or detecting the presence or amount of complex formation in the sample; (c) repeating steps (a) and (b) at a point later in time; and (d) comparing the result of step (b) with the result of step (c), wherein a difference in the amount of complex formation is indicative of disease, disease stage, progression, aggressiveness and/or metastatic potential of the cancer in said individual. The amount of complexes may also be compared to a value representative of the amount of the complexes from an individual not at risk of, or afflicted with thyroid cancer at a different stage.

[0164] Antibodies specifically reactive with Polypeptide Thyroid Cancer Markers or derivatives, such

as enzyme conjugates or labeled derivatives, may be used to detect Polypeptide Thyroid Cancer Markers in various samples (e.g. biological materials, in particular tissue samples). They may be used as diagnostic or prognostic reagents and they may be used to detect abnormalities in the level of Polypeptide Thyroid Cancer Markers or abnormalities in the structure, and/or temporal, tissue, cellular, or subcellular location of Polypeptide Thyroid Cancer Markers. Antibodies may also be used to screen potentially therapeutic compounds *in vitro* to determine their effects on thyroid cancer involving Polypeptide Thyroid Cancer Markers. *In vitro* immunoassays may also be used to assess or monitor the efficacy of particular therapies.

[0165] Antibodies may be used in any immunoassay that relies on the binding interaction between antigenic determinants of Polypeptide Thyroid Cancer Markers and the antibodies. Immunoassay procedures for *in vitro* detection of antigens in samples are also well known in the art. [See for example, Paterson et al., *Int. J. Can.* 37:659 (1986) and Burchell et al., *Int. J. Can.* 34:763 (1984) for a general description of immunoassay procedures]. Qualitative and/or quantitative determinations of Polypeptide Thyroid Cancer Markers in a sample may be accomplished by competitive or non-competitive immunoassay procedures in either a direct or indirect format. Detection of Polypeptide Thyroid Cancer Markers using antibodies can, for example involve immunoassays which are run in either the forward, reverse or simultaneous modes. Examples of immunoassays are radioimmunoassays (RIA), enzyme immunoassays (e.g. ELISA), immunofluorescence, immunoprecipitation, latex agglutination, hemagglutination, histochemical tests, and sandwich (immunometric) assays. Alternatively, the binding of antibodies to Polypeptide Thyroid Cancer Markers can be detected directly using, for example, a surface plasmon resonance (SPR) procedure such as, for example, Biacore®, microcalorimetry or nano-cantilevers. These terms are well understood by those skilled in the art, and they will know, or can readily discern, other immunoassay formats without undue experimentation.

[0166] Antibodies specific for Polypeptide Thyroid Cancer Markers may be labelled with a detectable substance and localised in biological samples based upon the presence of the detectable substance. Examples of detectable substances include, but are not limited to, the following: radioisotopes (e.g., ^3H , ^{14}C , ^{35}S , ^{125}I , ^{131}I), fluorescent labels, (e.g., FITC, rhodamine, lanthanide phosphors), luminescent labels such as luminol; and enzymatic labels (e.g., horseradish peroxidase, beta-galactosidase, luciferase, alkaline phosphatase, acetylcholinesterase), biotinyl groups (which can be detected by marked avidin e.g., streptavidin containing a fluorescent marker or enzymatic activity that can be detected by optical or calorimetric methods), and predetermined polypeptide epitopes recognized by a secondary reporter (e.g., leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags). In some embodiments, labels are attached via spacer arms of various lengths to reduce potential steric hindrance. Antibodies may also be coupled to electron dense substances, such as ferritin or colloidal gold, which are readily visualised by electron microscopy.

[0167] One of the ways an antibody can be detectably labelled is to link it directly to an enzyme. The enzyme when later exposed to its substrate will produce a product that can be detected. Examples of

detectable substances that are enzymes are horseradish peroxidase, beta-galactosidase, luciferase, alkaline phosphatase, acetylcholinesterase, malate dehydrogenase, ribonuclease, urease, catalase, glucose-6-phosphate, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate, triose phosphate isomerase, asparaginase, glucose oxidase, and acetylcholine esterase.

[0168] For increased sensitivity in an immunoassay system a fluorescence-emitting metal atom such as Eu (europium) and other lanthanides can be used. These can be attached to the desired molecule by means of metal-chelating groups such as DTPA or EDTA. A bioluminescent compound may also be used as a detectable substance. Examples of bioluminescent detectable substances are luciferin, luciferase and aequorin.

[0169] Indirect methods may also be employed in which the primary antigen-antibody reaction is amplified by the introduction of a second antibody, having specificity for the antibody reactive against Polypeptide Thyroid Cancer Markers. By way of example, if the antibody having specificity against Polypeptide Thyroid Cancer Markers is a rabbit IgG antibody, the second antibody may be goat anti-rabbit IgG, Fc fragment specific antibody labeled with a detectable substance as described herein.

[0170] Methods for conjugating or labelling the antibodies discussed above may be readily accomplished by one of ordinary skill in the art.

[0171] Cytochemical techniques known in the art for localizing antigens using light and electron microscopy may be used to detect Polypeptide Thyroid Cancer Markers. Generally, an antibody may be labeled with a detectable substance and a Polypeptide Thyroid Cancer Marker may be localized in tissues and cells based upon the presence of the detectable substance.

[0172] In the context of the methods of the description, the sample, binding agents (e.g. antibodies), or Polypeptide Thyroid Cancer Markers may be immobilized on a carrier or support, such as, for example, agarose, cellulose, nitrocellulose, dextran, Sephadex, Sepharose, liposomes, carboxymethyl cellulose, polyacrylamides, polystyrene, filter paper, ion-exchange resin, plastic film, nylon or silk. The support material may have any possible configuration including spherical cylindrical or flat. Thus, the carrier may be in the shape of, for example, a tube, test plate, well, beads, disc, sphere, etc. The immobilized material may be prepared by reacting the material with a suitable insoluble carrier using known chemical or physical methods, for example, cyanogen bromide coupling. Binding agents (e.g. antibodies) may be indirectly immobilized using second binding agents specific for the first binding agent. For example, mouse antibodies specific for Polypeptide Thyroid Cancer Markers may be immobilized using sheep anti-mouse IgG Fc fragment specific antibody coated on the carrier or support.

[0173] Where a radioactive label is used as a detectable substance, a Polypeptide Thyroid Cancer Marker may be localized by radioautography. The results of radioautography may be quantitated by determining the density of particles in the radioautographs by various optical methods, or by counting

1 the grains.

2 **[0174]** Time-resolved fluorometry may be used to detect a fluorescent signal, label, or detectable
3 substance. For example, the method described in Christopoulos TK and Diamandis EP Anal. Chem.,
4 1992:64:342-346 may be used with a conventional time-resolved fluorometer.

5 **[0175]** According to an embodiment of the description, an immunoassay for detecting Polypeptide
6 Thyroid Cancer Markers in a biological sample comprises contacting an amount of a binding agent
7 that specifically binds to Polypeptide Thyroid Cancer Markers in the sample under conditions that
8 allow the formation of a complex(es) comprising the binding agent and Polypeptide Thyroid Cancer
9 Markers and determining the presence or amount of the complex(es) as a measure of the amount of
10 the Polypeptide Thyroid Cancer Markers contained in the sample.

11 **[0176]** In accordance with an embodiment of the description, a method is provided wherein
12 Polypeptide Thyroid Cancer Markers antibodies are directly or indirectly labelled with enzymes,
13 substrates for the enzymes are added wherein the substrates are selected so that the substrates, or a
14 reaction product of an enzyme and substrate, form fluorescent complexes with lanthanide metals,
15 preferably europium and terbium. A lanthanide metal(s) is added and Polypeptide Thyroid Cancer
16 Markers are quantitated in the sample by measuring fluorescence of the fluorescent complexes.
17 Enzymes are selected based on the ability of a substrate of the enzyme, or a reaction product of the
18 enzyme and substrate, to complex with lanthanide metals. Examples of enzymes and substrates for
19 enzymes that provide such fluorescent complexes are described in U.S. Patent No. 5,312,922 to
20 Diamandis. By way of example, when the antibody is directly or indirectly labelled with alkaline
21 phosphatase the substrate employed in the method may be 4-methylumbelliferyl phosphate, 5-
22 fluorosalicyl phosphate, or diflunisal phosphate. The fluorescence intensity of the complexes is
23 typically measured using a time-resolved fluorometer.

24 **[0177]** Antibodies specific for Polypeptide Thyroid Cancer Markers may also be indirectly labelled
25 with enzymes. For example, an antibody may be conjugated to one partner of a ligand binding pair,
26 and the enzyme may be coupled to the other partner of the ligand binding pair. Representative
27 examples include avidin-biotin, and riboflavin-riboflavin binding protein. In embodiments, antibodies
28 specific for Polypeptide Thyroid Cancer Markers are labelled with enzymes.

29 **[0178]** Aspects of the methods of the description involve (a) reacting a biological sample from a
30 subject with antibodies specific for Polypeptide Thyroid Cancer Markers wherein the antibodies are
31 directly or indirectly labelled with enzymes; (b) adding substrates for the enzymes wherein the
32 substrates are selected so that the substrates, or reaction products of the enzymes and substrates
33 form fluorescent complexes; (c) quantitating Polypeptide Thyroid Cancer Markers in the sample by
34 measuring fluorescence of the fluorescent complexes; and (d) comparing the quantitated levels to
35 levels obtained for other samples from the subject patient, or control subjects. In an embodiment, the
36 Polypeptide Thyroid Cancer Markers are set out in Table 1 and the quantitated levels are compared
37 to levels quantitated for normal subjects or subjects with an early stage of disease wherein a

significant difference in the levels of the markers compared with the control subjects is indicative of thyroid cancer, or stage or aggressiveness of thyroid cancer.

[0179] A particular embodiment of the description comprises the following steps: (a) incubating a biological sample with a first antibody specific for Polypeptide Thyroid Cancer Markers which is directly or indirectly labeled with a detectable substance, and a second antibody specific for Polypeptide Thyroid Cancer Markers which is immobilized; (b) separating the first antibody from the second antibody to provide a first antibody phase and a second antibody phase; (c) detecting the detectable substance in the first or second antibody phase thereby quantitating Polypeptide Thyroid Cancer Markers in the biological sample; and (d) comparing the quantitated Polypeptide Thyroid Cancer Markers with levels for a predetermined standard. The standard may correspond to levels quantitated for samples from control subjects with no disease or early stage disease or from other samples of the subject including earlier samples of the subject.

[0180] In accordance with an embodiment, the present description provides means for determining Polypeptide Thyroid Cancer Markers in a sample by measuring Polypeptide Thyroid Cancer Markers by immunoassay. It will be evident to a skilled artisan that a variety of competitive or non-competitive immunoassay methods can be used to measure Polypeptide Thyroid Cancer Markers in serum. Competitive methods typically employ immobilized or immobilizable antibodies to Polypeptide Thyroid Cancer Markers and labeled forms of Polypeptide Thyroid Cancer Markers. Sample Polypeptide Thyroid Cancer Markers and labeled Polypeptide Thyroid Cancer Markers compete for binding to antibodies specific for Polypeptide Thyroid Cancer Markers. After separation of the resulting labeled Polypeptide Thyroid Cancer Markers that have become bound to antibody (bound fraction) from that which has remained unbound (unbound fraction), the amount of the label in either bound or unbound fraction is measured and may be correlated with the amount of Polypeptide Thyroid Cancer Markers in the test sample in any conventional manner, e.g., by comparison to a standard curve.

[0181] In another aspect, a non-competitive method is used for the determination of Polypeptide Thyroid Cancer Markers with the most common method being the "sandwich" method. In this assay, two antibodies specific for a Polypeptide Thyroid Cancer Marker are employed. One of the antibodies is directly or indirectly labeled (the "detection antibody"), and the other is immobilized or immobilizable (the "capture antibody"). The capture and detection antibodies can be contacted simultaneously or sequentially with the test sample. Sequential methods can be accomplished by incubating the capture antibody with the sample, and adding the detection antibody at a predetermined time thereafter or the detection antibody can be incubated with the sample first and then the capture antibody added. After the necessary incubation(s) have occurred, to complete the assay, the capture antibody may be separated from the liquid test mixture, and the label may be measured in at least a portion of the separated capture antibody phase or the remainder of the liquid test mixture. Generally it is measured in the capture antibody phase since it comprises Polypeptide Thyroid Cancer Marker "sandwiched" between the capture and detection antibodies. In another embodiment, the label may be measured without separating the capture antibody and liquid test mixture.

1 **[0182]** In particular sandwich immunoassays of the description mouse polyclonal/monoclonal
2 antibodies specific for Polypeptide Thyroid Cancer Markers and rabbit polyclonal/monoclonal
3 antibodies specific for Polypeptide Thyroid Cancer Markers are utilized.

4 **[0183]** In a typical two-site immunometric assay for Polypeptide Thyroid Cancer Markers one or both
5 of the capture and detection antibodies are polyclonal antibodies or one or both of the capture and
6 detection antibodies are monoclonal antibodies (i.e. polyclonal/polyclonal, monoclonal/monoclonal, or
7 monoclonal/polyclonal). The label used in the detection antibody can be selected from any of those
8 known conventionally in the art. The label may be an enzyme or a chemiluminescent moiety, but it
9 can also be a radioactive isotope, a fluorophor, a detectable ligand (e.g., detectable by a secondary
10 binding by a labeled binding partner for the ligand), and the like. In an aspect, the antibody is labelled
11 with an enzyme which is detected by adding a substrate that is selected so that a reaction product of
12 the enzyme and substrate forms fluorescent complexes. The capture antibody may be selected so
13 that it provides a means for being separated from the remainder of the test mixture. Accordingly, the
14 capture antibody can be introduced to the assay in an already immobilized or insoluble form, or can
15 be in an immobilizable form, that is, a form which enables immobilization to be accomplished
16 subsequent to introduction of the capture antibody to the assay. An immobilized capture antibody may
17 comprise an antibody covalently or noncovalently attached to a solid phase such as a magnetic
18 particle, a latex particle, a microtiter plate well, a bead, a cuvette, or other reaction vessel. An
19 example of an immobilizable capture antibody is antibody which has been chemically modified with a
20 ligand moiety, e.g., a hapten, biotin, or the like, and which can be subsequently immobilized by
21 contact with an immobilized form of a binding partner for the ligand, e.g., an antibody, avidin, or the
22 like. In an embodiment, the capture antibody may be immobilized using a species specific antibody
23 for the capture antibody that is bound to the solid phase.

24 **[0184]** The description also contemplates diagnostic methods employing mass spectrometry. In an
25 aspect, the description relates to a method for diagnosing or screening for thyroid cancer in a subject
26 comprising: (a) extracting proteins from a sample from the subject and producing a profile of the
27 proteins by subjecting the proteins to mass spectrometry; and (b) comparing the profile with a profile
28 for a reference comprising Thyroid Cancer Marker sets of the description.

29 **[0185]** Proteins may be extracted from the samples in a manner known in the art. For example,
30 proteins may be extracted by ultra-centrifugation or other standard techniques. The separated
31 proteins may be digested into peptides, in particular using proteolytic enzymes such as trypsin,
32 pepsin, subtilisin, and proteinase. For example, proteins may be treated with trypsin which cleaves at
33 the sites of lysine and arginine, to provide doubly-charged peptides with a length of from about 5 to 50
34 amino acids. Such peptides may be particularly appropriate for mass spectrometry analysis,
35 especially electrospray ionization mass spectrometry. Chemical reagents including cyanogen bromide
36 may also be utilized to digest proteins.

37 **[0186]** Mass spectrometers that may be used to analyze the peptides or proteins include a Matrix-
38 Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometer ("MALDI-TOF") (e.g. from

PerSeptive Biosystems, Framingham, Mass.); an Electrospray Ionization ("ESI") ion trap spectrometer, (e.g. from Finnigan MAT, San Jose, Calif.), an ESI quadrupole mass spectrometer (e.g. from Finnigan or Perkin-Elmer Corporation, Foster City, Calif.), a quadrupole/TOF hybrid tandem mass spectrometer, QSTAR XL (Applied Biosystems/MDS Sciex), or a Surface Enhanced Laser Desorption/Ionization (SELDI-TOF) Mass Spectrometer (e.g. from Ciphergen Biosystems Inc.).

[0187] Screening Methods

[0188] The description contemplates a method of assessing the potential of a test compound to contribute to thyroid cancer comprising: (a) maintaining separate aliquots of thyroid cancer cells in the presence and absence of the test compound; and (b) comparing the levels of Thyroid Cancer Markers associated with the thyroid cancer in each of the aliquots. A significant difference between the levels of Thyroid Cancer Markers in an aliquot maintained in the presence of (or exposed to) the test compound relative to the aliquot maintained in the absence of the test compound, indicates that the test compound potentially contributes to thyroid cancer.

[0189] The description also contemplates methods for evaluating test agents or compounds for their potential efficacy in treating thyroid cancer. Test agents and compounds include but are not limited to peptides such as soluble peptides including Ig-tailed fusion peptides, members of random peptide libraries and combinatorial chemistry-derived molecular libraries made of D- and/or L-configuration amino acids, phosphopeptides (including members of random or partially degenerate, directed phosphopeptide libraries), antibodies [e.g. polyclonal, monoclonal, humanized, anti-idiotypic, chimeric, single chain antibodies, fragments, (e.g. Fab, F(ab)₂, and Fab expression library fragments, and epitope-binding fragments thereof)], polynucleotides (e.g. antisense, siRNA), and small organic or inorganic molecules. The agents or compounds may be endogenous physiological compounds or natural or synthetic compounds.

[0190] The description provides a method for assessing the potential efficacy of a test agent for potential efficacy in treating thyroid cancer in a patient the method comprising comparing: (a) levels of one or more Thyroid Cancer Markers, and optionally other markers of thyroid cancer, in a first sample obtained from a patient and exposed to the test agent; and (b) levels of one or more Thyroid Cancer Markers, and optionally other markers, in a second sample obtained from the patient, wherein the sample is not exposed to the test agent, wherein a significant difference in the levels of expression of one or more Thyroid Cancer Markers, and optionally the other markers, in the first sample, relative to the second sample, is an indication that the test agent is potentially efficacious for treating thyroid cancer in the patient. The first and second samples may be portions of a single sample obtained from a patient or portions of pooled samples obtained from a patient.

[0191] In an aspect, the description provides a method of selecting an agent for treating thyroid cancer, in particular aggressive thyroid cancer in a patient comprising: (a) obtaining a sample from the patient; (b) separately maintaining aliquots of the sample in the presence of a plurality of test agents; (c) comparing one or more Thyroid Cancer Markers, and optionally other markers, in each of the

1 aliquots; (d) selecting one of the test agents which alters the levels of one or more Thyroid Cancer
2 Markers, and optionally other markers in the aliquot containing that test agent, relative to other test
3 agents; and (e) optionally administering the selected test to the patient.

4 **[0192]** The description further relates to a method of assessing the efficacy of a therapy for
5 modulating thyroid cancer in a patient. A method of the description comprises comparing: (a) levels of
6 Thyroid Cancer Markers in a first sample from the patient obtained from the patient prior to providing
7 at least a portion of the therapy to the patient; and (b) levels of Thyroid Cancer Markers in a second
8 sample obtained from the patient following therapy. In an embodiment, a significant difference
9 between the levels of Thyroid Cancer Markers in the second sample relative to the first sample or an
10 abnormal state is an indication that the therapy is efficacious for modulating the thyroid cancer. In a
11 particular embodiment, the method is used to assess the efficacy of a therapy for treating a thyroid
12 cancer where lower levels of Thyroid Cancer Markers in the second sample relative to the first
13 sample, is an indication that the therapy is efficacious. The "therapy" may be any therapy for treating
14 thyroid cancer including but not limited to therapeutics, gene therapy, and surgery. Therefore, the
15 method can be used to evaluate a patient before, during, and after therapy.

16 **[0193]** The description contemplates a method for determining the effect of an environmental factor
17 on thyroid cancer comprising comparing Thyroid Cancer Markers in the presence and absence of the
18 environmental factor.

19 **[0194] Kits**

20 **[0195]** The description contemplates kits for carrying out the methods of the description to diagnosis
21 thyroid cancer or stage of thyroid cancer, and to detect the aggressiveness or metastatic potential of a
22 thyroid cancer. Such kits typically comprise two or more components required for performing a
23 diagnostic assay. Components include but are not limited to compounds, reagents, containers,
24 and/or equipment. Accordingly, the methods described herein may be performed by utilizing pre-
25 packaged test or diagnostic kits comprising at least agents (e.g. antibodies, probes, primers, etc)
26 described herein, which may be conveniently used, e.g., in clinical settings, to diagnose patients, in
27 particular patients afflicted with thyroid cancer, suspected of having thyroid cancer, or at risk of thyroid
28 cancer or exhibiting a predisposition to developing thyroid cancer, and more particularly to determine
29 the aggressiveness or metastatic potential of a thyroid cancer.

30 **[0196]** The description contemplates a kit with a container comprising a binding agent(s) as
31 described herein for characterizing a thyroid condition, such as cancer. By way of example, the kit
32 may contain antibodies specific for one or more of the Polypeptide Thyroid Cancer Markers,
33 antibodies against the antibodies labelled with enzymes, and substrates for the enzymes. The kit
34 may also contain microtiter plate wells, standards, assay diluent, wash buffer, adhesive plate covers,
35 reagents and/or instructions for carrying out a method of the description using the kit.

36 **[0197]** In an aspect, the description provides a test kit for diagnosing thyroid cancer in a subject
37 which comprises an antibody that binds to a Polypeptide Thyroid Cancer Marker(s) and/or

1 polynucleotides that hybridize to or amplify Polynucleotide Thyroid Cancer Marker(s). In another
2 aspect the description relates to use of an antibody that binds to a Polypeptide Thyroid Cancer
3 Marker and/or a polynucleotide that hybridizes to or amplifies a Polynucleotide Thyroid Cancer
4 Marker, in the manufacture of a composition for detecting or characterizing a thyroid cancer.

5 **[0198]** In a further aspect of the description, the kit includes antibodies or antibody fragments which
6 bind specifically to epitopes of Polypeptide Thyroid Cancer Marker(s) and means for detecting binding
7 of the antibodies to their epitopes associated with thyroid cancer cells, either as concentrates
8 (including lyophilized compositions), which may be further diluted prior to testing. In particular, the
9 description provides a kit for diagnosing or characterizing thyroid cancer comprising a known amount
10 of a first binding agent that specifically binds to a Polypeptide Thyroid Cancer Marker(s) wherein the
11 first binding agent comprises a detectable substance, or it binds directly or indirectly to a detectable
12 substance.

13 **[0199]** A kit may be designed to detect the levels of Polynucleotide Thyroid Cancer Markers in a
14 sample. Such kits generally comprise oligonucleotide probes or primers, as described herein, which
15 hybridize to or amplify Polynucleotide Thyroid Cancer Markers. Oligonucleotides may be used, for
16 example, within PCR or hybridization procedures. Test kits useful for detecting target Polynucleotide
17 Thyroid Cancer Markers are also provided which comprise a container containing a Polynucleotide
18 Thyroid Cancer Marker, and fragments or complements thereof.

19 **[0200]** The kits of the description can further comprise containers with tools useful for collecting test
20 samples (e.g. serum) including lancets and absorbent paper or cloth for collecting and stabilizing
21 blood. In one example, the kits may include such tissue collection means as needle and/or syringes
22 for obtaining the fine needle or core biopsies. The kits may also comprise slides and tissue collection
23 devices for collecting tissue samples and for staining the slides, such as by immunostaining. The
24 slides can then be read either quantitatively or qualitatively, and either visually (i.e. manually) or in an
25 automated manner (such as by using a scanning device or the like). In the latter case, the scanning
26 device may be connected to a computer system (such as described below) or the like for conducting
27 the analysis.

28 **[0201] Computer Systems**

29 **[0202]** Analytic methods contemplated herein can be implemented by use of computer systems and
30 methods described below and known in the art. Thus, the description provides computer readable
31 media comprising one or more Thyroid Cancer Markers. "Computer readable media" refers to any
32 medium that can be read and accessed directly by a computer, including but not limited to magnetic
33 storage media, such as floppy discs, hard disc storage medium, and magnetic tape; optical storage
34 media such as CD-ROM; electrical storage media such as RAM and ROM; and hybrids of these
35 categories such as magnetic/optical storage media. Thus, the description contemplates computer
36 readable medium having recorded thereon markers identified for patients and controls.

37 **[0203]** "Recorded" refers to a process for storing information on computer readable medium. The

1 skilled artisan can readily adopt any of the presently known methods for recording information on
2 computer readable medium to generate manufactures comprising information on one or more markers
3 disclosed herein.

4 **[0204]** A variety of data processor programs and formats can be used to store information on one or
5 more Thyroid Cancer Markers. For example, the information can be represented in a word processing
6 text file, formatted in commercially-available software such as WordPerfect and Microsoft Word, or
7 represented in the form of an ASCII file, stored in a database application, such as DB2, Sybase,
8 Oracle, or the like. Any number of data processor structuring formats (e.g., text file or database) may
9 be adapted in order to obtain computer readable medium having recorded thereon the marker
10 information.

11 **[0205]** By providing the marker information in computer readable form, one can routinely access the
12 information for a variety of purposes. For example, one skilled in the art can use the information in
13 computer readable form to compare marker information obtained during or following therapy with the
14 information stored within the data storage means.

15 **[0206]** The description provides a medium for holding instructions for performing a method for
16 determining whether a patient has thyroid cancer, in particular aggressive thyroid cancer, or a pre-
17 disposition to such condition, comprising determining the presence or absence of one or more Thyroid
18 Cancer Markers, and based on the presence or absence of the markers, determining the condition or
19 a pre-disposition to the condition, optionally recommending a procedure or treatment.

20 **[0207]** The description also provides in an electronic system and/or in a network, a method for
21 determining whether a subject has a condition disclosed herein, or a pre-disposition to a condition
22 disclosed herein, comprising determining the presence or absence of one or more markers, and
23 based on the presence or absence of the markers, determining whether the subject has the condition
24 or a pre-disposition to the condition, and optionally recommending a procedure or treatment.

25 **[0208]** The description further provides in a network, a method for determining whether a subject has
26 a condition disclosed herein or a pre-disposition to a condition disclosed herein comprising: (a)
27 receiving phenotypic information on the subject and information on one or more markers disclosed
28 herein associated with samples from the subject; (b) acquiring information from the network
29 corresponding to the markers; and (c) based on the phenotypic information and information on the
30 markers, determining whether the subject has the condition or a pre-disposition to the condition, and
31 (d) optionally recommending a procedure or treatment.

32 **[0209]** The description still further provides a system for identifying selected records that identify a
33 diseased cell or tissue. A system of the description generally comprises a digital computer; a
34 database server coupled to the computer; a database coupled to the database server having data
35 stored therein, the data comprising records of data comprising one or more markers disclosed herein,
36 and a code mechanism for applying queries based upon a desired selection criteria to the data file in
37 the database to produce reports of records which match the desired selection criteria.

1 **[0210]** The description contemplates a business method for determining whether a subject has a
2 condition disclosed herein or a pre-disposition to a condition disclosed herein comprising: (a)
3 receiving phenotypic information on the subject and information on one or more markers disclosed
4 herein associated with samples from the subject; (b) acquiring information from a network
5 corresponding to the markers; and (c) based on the phenotypic information, information on the
6 markers and acquired information, determining whether the subject has the condition or a pre-
7 disposition to the condition, and optionally recommending a procedure or treatment.

8 **[0211]** In an aspect of the description, the computer systems, components, and methods described
9 herein are used to monitor a condition or determine the stage of a condition.

10 **[0212] Therapeutic Applications**

11 **[0213]** The description contemplates therapeutic applications associated with the Thyroid Cancer
12 Markers disclosed herein including thyroid cancer. Thyroid Cancer Markers may be a target for
13 therapy. For example, markers in Table 1 can be a target for treatment of thyroid cancers.

14 **[0214]** Therapeutic methods include immunotherapeutic methods including the use of antibody
15 therapy. In one aspect, the description provides one or more antibodies that may be used to prevent
16 thyroid cancer. In another aspect, the description provides a method of preventing, inhibiting or
17 reducing thyroid cancer comprising administering to a patient an antibody which binds to a Thyroid
18 Cancer Marker in an amount effective to prevent, inhibit, or reduce the condition or the onset of the
19 condition. The description also contemplates a method of treating thyroid cancer in a subject,
20 comprising delivering to the subject in need thereof, an antibody specific for a Thyroid Cancer Marker
21 in Table 1, in particular Thyroid Cancer Marker in Table 1 that is upregulated in thyroid cancer or a
22 stage of thyroid cancer. According to one aspect of the description, there is provided a method of
23 treating a subject having thyroid cancer wherein an antibody specific for a marker in Table 1 is
24 administered in a therapeutically effective amount. In a further aspect, the antibody is provided in a
25 pharmaceutically acceptable form.

26 **[0215]** An antibody which binds to a Thyroid Cancer Marker may be in combination with a label, drug
27 or cytotoxic agent, a target-binding region of a receptor, an adhesion molecule, a ligand, an enzyme,
28 a cytokine, or a chemokine. In aspects of the description, the Thyroid Cancer Marker may be
29 conjugated to cytotoxic agents (e.g., chemotherapeutic agents) or toxins or active fragments thereof.
30 Examples of toxins and corresponding fragments thereof include diphtheria A chain, exotoxin A chain,
31 ricin A chain, abrin A chain, curcin, crotoxin, phenomycin, enomycin and the like. A cytotoxic agent may
32 be a radiochemical prepared by conjugating radioisotopes to antibodies, or binding of a radionuclide
33 to a chelating agent that has been covalently attached to the antibody. An antibody may also be
34 conjugated to one or more small molecule toxins, such as a calicheamicin, a maytansine, a
35 trichothene, and CC1065 (see U.S. Pat. No. 5,208,020).

36 **[0216]** The methods of the description contemplate the administration of single antibodies as well as
37 combinations, or "cocktails", of different individual antibodies such as those recognizing different

1 epitopes of other markers. Such cocktails may have certain advantages inasmuch as they contain
2 antibodies that bind to different epitopes of Thyroid Cancer Markers and/or exploit different effector
3 mechanisms. Such antibodies in combination may exhibit synergistic therapeutic effects. In addition,
4 the administration of one or more marker specific antibodies may be combined with other therapeutic
5 agents. The specific antibodies may be administered in their "naked" or unconjugated form, or may
6 have therapeutic agents conjugated to them.

7 **[0217]** In an aspect, the description provides a pharmaceutical composition for the treatment of
8 thyroid cancer characterized in that the composition comprises an antibody specific for a marker in
9 Table 1, in particular a Thyroid Cancer Marker that is upregulated in thyroid cancer or a type of thyroid
10 cancer, together with a pharmaceutically acceptable carrier, excipient or vehicle.

11 **[0218]** Antibodies used in the methods of the description may be formulated into pharmaceutical
12 compositions comprising a carrier suitable for the desired delivery method. Suitable carriers include
13 any material which when combined with the antibodies retains the function of the antibody and is non-
14 reactive with the subject's immune systems. Examples include any of a number of standard
15 pharmaceutical carriers such as sterile phosphate buffered saline solutions, bacteriostatic water, and
16 the like (see, generally, Remington's Pharmaceutical Sciences 16th Edition, A. Osal., Ed., 1980).

17 **[0219]** One or more marker specific antibody formulations may be administered via any route
18 capable of delivering the antibodies to the site or injury. Routes of administration include, but are not
19 limited to, intravenous, intraperitoneal, intramuscular, intradermal, and the like. Antibody preparations
20 may be lyophilized and stored as a sterile powder, preferably under vacuum, and then reconstituted in
21 bacteriostatic water containing, for example, benzyl alcohol preservative, or in sterile water prior to
22 injection.

23 **[0220]** Treatment will generally involve the repeated administration of the antibody preparation via an
24 acceptable route of administration at an effective dose. Dosages will depend upon various factors
25 generally appreciated by those of skill in the art, including the etiology of the condition, stage of the
26 condition, the binding affinity and half life of the antibodies used, the degree of marker expression in
27 the patient, the desired steady-state antibody concentration level, frequency of treatment, and the
28 influence of any therapeutic agents used in combination with a treatment method of the description. A
29 determining factor in defining the appropriate dose is the amount of a particular antibody necessary to
30 be therapeutically effective in a particular context. Repeated administrations may be required to
31 achieve a desired effect. Direct administration of one or more marker antibodies is also possible and
32 may have advantages in certain situations.

33 **[0221]** Patients may be evaluated for Thyroid Cancer Markers in order to assist in the determination
34 of the most effective dosing regimen and related factors. The assay methods described herein, or
35 similar assays, may be used for quantitating marker levels in patients prior to treatment. Such assays
36 may also be used for monitoring throughout therapy, and may be useful to gauge therapeutic success
37 in combination with evaluating other parameters such as levels of markers.

1 **[0222]** Polynucleotide Thyroid Cancer Markers disclosed herein can be turned off by transfecting a
2 cell or tissue with vectors that express high levels of the polynucleotides. Such constructs can
3 inundate cells with untranslatable sense or antisense sequences. Even in the absence of integration
4 into the DNA, such vectors may continue to transcribe RNA molecules until all copies are disabled by
5 endogenous nucleases. Vectors derived from retroviruses, adenovirus, herpes or vaccinia viruses, or
6 from various bacterial plasmids, may be used to deliver polynucleotides to a targeted organ, tissue, or
7 cell population. Methods well known to those skilled in the art may be used to construct recombinant
8 vectors that will express polynucleotides such as antisense. (See, for example, the techniques
9 described in Sambrook et al (supra) and Ausubel et al (supra).)

10 **[0223]** Methods for introducing vectors into cells or tissues include those methods known in the art
11 which are suitable for *in vivo*, *in vitro* and *ex vivo* therapy. For example, delivery by transfection or by
12 liposomes is well known in the art.

13 **[0224]** Modifications of gene expression can be obtained by designing antisense molecules, DNA,
14 RNA or PNA, to the regulatory regions of a Polynucleotide Thyroid Cancer Marker, i.e., the promoters,
15 enhancers, and introns. Preferably, oligonucleotides are derived from the transcription initiation site,
16 e.g. between -10 and +10 regions of the leader sequence. The antisense molecules may also be
17 designed so that they block translation of mRNA by preventing the transcript from binding to
18 ribosomes. Inhibition may also be achieved using "triple helix" base-pairing methodology. Triple helix
19 pairing compromises the ability of the double helix to open sufficiently for the binding of polymerases,
20 transcription factors, or regulatory molecules. Therapeutic advances using triplex DNA are reviewed
21 by Gee J E et al (In: Huber B E and B I Carr (1994) Molecular and Immunologic Approaches, Futura
22 Publishing Co, Mt Kisco N.Y.).

23 **[0225]** Ribozymes are enzymatic RNA molecules that catalyze the specific cleavage of RNA.
24 Ribozymes act by sequence-specific hybridization of the ribozyme molecule to complementary target
25 RNA, followed by endonucleolytic cleavage. The description therefore contemplates engineered
26 hammerhead motif ribozyme molecules that can specifically and efficiently catalyze endonucleolytic
27 cleavage of a polynucleotide marker.

28 **[0226]** Specific ribozyme cleavage sites within any potential RNA target may initially be identified by
29 scanning the target molecule for ribozyme cleavage sites which include the following sequences,
30 GUA, GUU and GUC. Once the sites are identified, short RNA sequences of between 15 and 20
31 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be
32 evaluated for secondary structural features which may render the oligonucleotide inoperable. The
33 suitability of candidate targets may also be determined by testing accessibility to hybridization with
34 complementary oligonucleotides using ribonuclease protection assays.

35 **[0227]** In some aspects one or more Polypeptide Thyroid Cancer Markers and polynucleotides
36 encoding the markers, and fragments thereof, may be used in the treatment of a thyroid cancer in a
37 subject. In an aspect the Thyroid Cancer Marker is down-regulated in thyroid cancer. The markers

may be formulated into compositions for administration to subjects suffering from a thyroid cancer. Therefore, the present description also relates to a composition comprising one or more Thyroid Cancer Markers, preferably a Thyroid Cancer Marker downregulated in thyroid cancer, and a pharmaceutically acceptable carrier, excipient or diluent. A method for treating or preventing a thyroid cancer in a subject is also provided comprising administering to a patient in need thereof, one or more one or more Polypeptide Thyroid Cancer Markers and polynucleotides encoding the markers, or a composition of the description.

[0228] An active therapeutic substance described herein may be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application, or rectal administration. Depending on the route of administration, the active substance may be coated in a material to protect the substance from the action of enzymes, acids and other natural conditions that may inactivate the substance. Solutions of an active compound as a free base or pharmaceutically acceptable salt can be prepared in an appropriate solvent with a suitable surfactant. Dispersions may be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof, or in oils.

[0229] A composition described herein can be prepared by known methods for the preparation of pharmaceutically acceptable compositions which can be administered to subjects, such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in *Remington: The Science and Practice of Pharmacy* (21st Edition, 2005, University of the Sciences in Philadelphia (Editor), Mack Publishing Company), and in *The United States Pharmacopeia: The National Formulary* (USP 24 NF19) published in 1999. On this basis, the compositions include, albeit not exclusively, solutions of the active substances in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids.

[0230] A composition is indicated as a therapeutic agent either alone or in conjunction with other therapeutic agents or other forms of treatment. The compositions of the description may be administered concurrently, separately, or sequentially with other therapeutic agents or therapies.

[0231] The therapeutic activity of compositions and agents/compounds identified using a method of the description and may be evaluated *in vivo* using a suitable animal model.

[0232] The following non-limiting examples are illustrative of the present description:

[0233] Example 1

[0234] Proteins that are secreted by cultured cancer cells into the media of their cell culture plates (i.e. "secretome" proteins) make especially appealing targets for study because they may be detectable in bodily fluids. The study described in this example examines the secretome of seven thyroid cancer cell lines: TPC-1, BCPAP, CAL 62, SW1736, C643, MRO, and WRO. Proteomic analysis of the conditioned serum-free media of these cells using LC-MS/MS allows for identification of proteins that these cancer cells secrete. This serves as a surrogate for proteins that human thyroid

cancer cells secrete *in vivo*. Identification of secretome proteins has lead to the discovery of numerous potential thyroid cancer biomarkers that may be used to predict aggressiveness of thyroid cancers. Furthermore, the study independently validates selected secretome proteins in the sera of thyroid cancer patients versus cancer-free individuals using Western blots.

[0235] The following materials and methods were employed in the Study described in this Example.

[0236] Materials and Methods

[0237] Cell Culture and Serum Free Media Collection. Seven thyroid cancer cell lines are used in this study TPC-1 (papillary), BCPAP (papillary), CAL62 (anaplastic), SW1736 (anaplastic), C643 (anaplastic), MRO (follicular), and WRO (follicular). The cells were grown in 25 mL of conditioned RPMI-1640 cell culture media (containing antibodies and supplemented with 10% fetal bovine serum) in 150 mm dishes to approximately 65% confluence. Cells were kept at 37°C in a humidified atmosphere of 5% CO₂ / 95% air. The conditioned media was then aspirated and cells were washed three times with phosphate-buffered saline (PBS). Thereafter, cells were washed once with serum-free media (SFM) that was collected as a time 0 h control. Cells were incubated in the SFM for 48 hours. Following 48 h, the SFM was collected, centrifuged at 2200 RPM for 5 minutes at 4°C, and filtered using a 0.2 µm nylon filter. Upon filtration collected SFM samples were immediately frozen at -80°C until later processing. SFM was collected from sixty 150 mm plates for TPC-1, SW1736 and CAL 62, and from twenty-five 150 mm BCPAP, C643, WRO and MRO plates.

[0238] Protein Precipitation from collected SFM and Preparation for LC-MS/MS analysis.

Proteins were isolated from SFM using 0.2% sodium deoxycholate (Sigma Aldrich, MO) and 10% trichloroacetic acid (Sigma Aldrich, MO) as described earlier. [4] Following 2 h incubation on ice, the samples were centrifuged at 11 000 g for 30 minutes and washed two times with ice-cold acetone. The precipitated proteins were then dissolved in 50 mM NaHCO₃ buffer. Protein concentration was later determined using the Bradford assay (Bio-Rad, CA). Protein samples were then heated for 1 h at 65°C in the presence of 5 mM dithiothreitol, cooled to room temperature, and incubated in the dark for 1 h with 10 mM iodoacetamide to allow for alkylation. Sequencing grade trypsin (Promega, WI) at 1:20 (w/w) in 50 mM ammonium bicarbonate was subsequently added and the samples were incubated at 37°C overnight. Digested samples were then dried under vacuum and redissolved in 10 µL of 0.1% formic acid.

[0239] Liquid Chromatography-MS/MS analysis. Samples were analyzed by online LC-MS/MS in triplicates. The nanobore LC system and MS/MS setup was followed and has been described earlier [5]. The liquid chromatograph used in the experiment was an LC Packings Ultimate unit (Amsterdam, The Netherlands). The mass spectrometer used was a QSTAR Pulsar-i hybrid quadrupole/time-of-flight (QqTOF) instrument (Applied Biosystems/MDS SCIEX, CA). An autosampler was used to load 1 µL of sample onto a C18 reverse-phase precolumn (LC Packings: 300 µm x 5 mm). Subsequently, reverse-phase chromatography on an analytical column (75 µm x 150 mm packed in-house with 3-µm Kromasil C18 beads with 100 Å pores, The Nest Group) was used. For separation, a nonlinear binary

gradient was used: eluant A consisting of 94.9% deionized water, 5.0% acetonitrile, and 0.1% formic acid (pH 3); and eluant B consisting of 5.0% deionized water, 94.9% acetonitrile, and 0.1% formic acid. During the first 5 min of the LC run, eluant A at a flow rate of $25 \mu\text{L min}^{-1}$ was used to load peptides from the sample onto the C18 precolumn. Desalting continued for two additional min. At the 8th min, the C18 precolumn was switched in-line with the reverse-phase analytical column; separation was performed at 200 nL min^{-1} using a 180-min binary gradient shown below.

Time (min)	0	5	10	120	140	145	155	157	189
B (%)	5	5	15	35	60	80	80	5	Stop

[0240] MS/MS Settings and Data Collection. Data was collected in information-dependent acquisition (IDA) mode using Analyst QS 1.1 and Bioanalyst Extension 1.1 software (Applied Biosystems/MDS SCIEX). MS cycles consisted of a TOF MS survey scan with an m/z range of 400-1500 Th for 1 s. This was followed by five product-ion scans with an m/z range of 80-2000 Th for 2 s each. IDA CE Parameters script was used to control the collision energy (CE). Switching criteria were set to ions with $m/z \geq 400$ and ≤ 1500 Th, charge states of 2-4, and abundances of ≥ 10 counts. Former target ions were excluded for 30 s, and ions within a 6-Th window were ignored. Additionally, the IDA Extensions II script was set to "no repetition" before dynamic exclusion and to select a precursor ion nearest to a threshold of 10 counts on every fourth cycle. LC-MS/MS data were searched using the ProteinPilot software (Applied Biosystems, Foster City, CA) and a Celera human protein database (CDS KBMS 20041109) containing 178239 protein sequences. The cutoff for significance used for this search was set for a score of 1.3, which corresponds to a confidence score of 95%.

[0241] Secretion features of identified proteins. To analyze identified proteins' secretion features, Signal Peptide Predictor (SignalIP, <http://www.cbs.dtu.dk/services/SignalP> 3.0) was used. SignalIP uses amino-acid sequences to predict the existence and location of signal peptide cleavage sites. SignalIP determines the likelihood a protein is a signaling peptide by using numerous artificial neural networks and hidden Markov model algorithms to detect signal peptides from protein sequences. A protein is considered classically secreted if it receives a signal peptide probability ≥ 0.900 . In order to identify non-classical, or leaderless, protein secretion SecretomeP (<http://www.cbs.dtu.dk/services/SecretomeP> 2.0) was used. SecretomeP uses a neural network that combines six protein characteristics to determine if a protein is non-classically secreted. The protein characteristics include: the number of atoms, number of positively charged residues, presence of transmembrane helices, presence of low-complexity regions, presence of pro-peptides, and subcellular localization. A protein is considered non-classically secreted if it receives an NN-score ≥ 0.500 (note: only proteins that were not considered classically secreted, i.e. received SignalP scores < 0.900 , were analyzed using SecretomeP).

[0242] Western Blot: Verification of Biomarkers in Sera. Western Blots were used to verify the expression of selected secretory proteins, E-cadherin, Nucleolin, CYR61 (cysteine rich angiogenic

inducer, 61 variant), Prothomyosin alpha, α -Enolase, Biotinidase, Clusterin, Tyrosine-protein kinase receptor UFO (AXL), amyloid precursor protein (APP), amyloid precursor protein like protein 2 (APLP2), Pyruvate kinase M2 (PKM2), α -MCFD2, α -NPC2, 14-3-3 zeta, SET and calsynenin-1 in thyroid cancer patients' blood. Patient serum samples were depleted of the 20 most abundant blood proteins using the Proteoprep 20 Plasma Immunodepletion kit (Sigma-Aldrich, MO) according to manufacturer's specifications.

[0243] For Western Blot analysis, 12% SDS-PAGE gels were used as described earlier. Proteins were transferred from the gel to a polyvinylidenedifluoride (PVDF) membrane, that was blocked using 5% non-fat milk in Tris-buffered saline (TBS, 0.1 M, pH = 7.2). Blots were incubated using monoclonal or polyclonal antibodies against E-cadherin, Nucleolin, CYR61 (cysteine rich angiogenic inducer, 61 variant), Prothomyosin alpha, α -Enolase, Biotinidase, Clusterin, Tyrosine-protein kinase receptor UFO (AXL), amyloid precursor protein (APP), amyloid precursor protein like protein 2 (APLP2), Pyruvate kinase M2 (PKM2), α -MCFD2, α -NPC2, 14-3-3 zeta, SET and calsynenin-1 at the appropriate dilutions at 4°C for 2 hours. The membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (DAKO Cytomation, Denmark), diluted to an appropriate concentration in 1% BSA, and room-temperature incubated for 2 h. Following each step, blots were washed three times with Tween (0.1%)-Tris-buffer saline (TTBS). Protein bands were detected by the enhanced chemiluminescence method (ECL, Santa Cruz Biotechnology, CA) on XO-MAT film.

[0244] Results

[0245] The results are discussed below and aspects are illustrated in Figures 1-11.

[0246] Optimization of Cell Culture Conditions for SFM Collection. While cells are normally grown in media that contains serum, the high abundance proteins found in serum would interfere with the detection of secretome proteins. For this reason, cell culture conditions needed to be optimized for SFM collection. To avoid this interference, the cells were washed thoroughly four times (three times with PBS and once with serum-free media) and then grown in serum-free media for 48 h, allowing secretome proteins to accumulate. To limit cellular stress under these conditions, cells were only placed in SFM when they reached 60% confluence. Trypan blue staining was performed following collection of the SFM at 48 h to estimate the number of dead cells. Since >95% of cells were viable at 48 h, the release of non-secretory proteins into the media is considered to be minimal, but cannot be completely ruled out.

[0247] Proteins Released by TPC-1, CAL 62, MRO, WRO, BCPAP, SW1736 and C643 Thyroid Cancer Cell Lines. A total of 233 proteins were initially identified in the four thyroid cancer cell lines. The subcellular localization and biological functions of the proteins were determined using Ingenuity Pathway Analysis (IPA, Ingenuity Systems, www.ingenuity.com). In all cell lines, membrane and extracellular proteins were predominantly identified. Additionally, proteins associated with cellular metabolism were common to all cell lines. Numerous signal transduction and cell cycle proteins were also identified in WRO and TPC-1 cells. In order to become a candidate for further verification,

proteins must have been identified from MS spectrum data with at least 2 high-confidence peptides with a confidence level $\geq 95\%$. Proteins identified with at least two high-confidence peptides are considered high-confidence identifications. Proteins were not identified from the 0 h controls, except for blood albumin and globulins, which were removed from the identified proteins list. After applying the high confidence threshold to the identified protein list, 83 proteins remained as candidates for independent verification (see Table 1). Protein sequences were obtained for these proteins and inputted into SignalP and/or SecretomeP to obtain the reported score. Literature searches were performed on each protein to identify its cellular localization, and whether it has been reported to be present in exosomes or in patient blood/tissue samples. Nearly all of these high-confidence identifications were determined to be secretory proteins according to their SignalP and SecretomeP scores.

[0248] Verification of Selected Secretome Proteins in Human Sera by Western Blotting. The presence of select proteins were independently verified by Western Blot in thyroid cancer patients' sera, and in the SFM (see Figures 1A-D, 3A-C, 4A,B and 6). The proteins that have been verified are E-cadherin, Nucleolin, CYR61 (cysteine rich angiogenic inducer, 61 variant), Prothomyosin alpha, α -Enolase, Biotinidase, Clusterin, Tyrosine-protein kinase receptor UFO (AXL), amyloid precursor protein (APP), amyloid precursor protein like protein 2 (APLP2), Pyruvate kinase M2 (PKM2), α -MCFD2, α -NPC2, 14-3-3 zeta, SET, and calyntenin-1. Proteins were selected for verification were based upon SecretomeP scores, SignalP scores, IPA database information, along with information from literature searches (i.e. whether the protein has also been reported to be present in the blood of thyroid cancer patients in other studies, whether it has been reported to be found in exosomes, and/or the role the protein may play in disease progression). The justification for selection of these proteins lies in the fact that they were high-confidence identifications, are suggested to be secreted proteins based upon their SignalP and/or SecretomeP scores, and/or have been reported in the literature to be secreted or to play a role in cancer pathogenesis.

[0249] Verification of Selected Secretome Proteins in Human Sera by ELISA.

[0250] The presence of select proteins were independently verified by ELISA in thyroid cancer patients' sera (see Figures 4G and H, 5F and 8). The proteins that have been verified are clusterin, ALCAM/CD166 and AXL tyrosine kinase.

[0251] Verification of Selected Secretome Proteins in Human Thyroid Carcinoma and Normal Tissues by Immunohistochemistry. The presence of select proteins were independently verified by immunohistochemistry in thyroid carcinoma, benign thyroid nodules and/or normal tissues (see Figures 1E-G, 2A-H, 3D-I, 4C-F, 5A-E, 7A-G,9 and 10). The proteins that have been verified are α -Enolase, Prothomyosin alpha, Nucleolin, Biotinidase, Clusterin, ALCAM/CD166, amyloid precursor protein like protein 2 (APLP2), amyloid precursor protein (APP), 14-3-3 zeta, Tyrosine-protein kinase receptor UFO (AXL), SET, Pyruvate kinase M2 (PKM2), and Heterogeneous ribonucleoprotein K (hnRNP K).

[0252] Verification of Selected Secretome Proteins in Mouse Xenografts of Human Thyroid

Carcinoma Cell Lines. The presence of select proteins were independently verified by immunohistochemistry in tissue sections of xenografts of human thyroid carcinoma cell lines, BCPAP (papillary thyroid carcinoma) and C643 (anaplastic thyroid carcinoma) in immunocompromised mice (NOD/Scid/gamma) (see Figure11). The proteins that have been verified are amyloid precursor protein (APP), Tyrosine-protein kinase receptor UFO (AXL), Pyruvate kinase M2 (PKM2), and SET. The expression patterns and subcellular localization of these proteins in mouse xenografts were similar to those observed in cultured thyroid cancer cells and in human thyroid carcinomas confirming that these proteins retain their characteristics in xenografts.

[0253] Discussion

[0254] The study revealed a total of 233 proteins in the secretome of TPC-1, BCPAP, CAL 62, SW1736, WRO, and MRO cells, of which 83 are considered high-confidence identifications due to the numerous peptides used in their identification. Nearly all identified high-confidence proteins were deemed to be secretory according to their SignalP and SecretomeP scores, lending additional support to the hypothesis that the proteins identified in the study are secretory proteins. There were far more proteins unique to TPC-1 and SW1736 cells than the other cell lines due to the fact that three-times as many TPC-1 and SW1736 cells were used for SFM collection.

[0255] Nucleolin. Nucleolin is a nuclear protein involved in numerous cell cycle processes. It does not have a known classical secretion signal and is not suggested to be a secretory protein based upon its SecretomeP and SignalP scores. SecretomeP and SignalP scores cannot completely rule out the possibility a protein is in-fact secretory, and numerous studies have demonstrated that nucleolin is in-fact, present on the cellular surface of proliferating cells [6]. It remains unclear how nucleolin is transported from the nuclear membrane to the cell surface. It has been shown that the use of antagonists to surface nucleolin suppresses tumour growth and angiogenesis, suggesting an important role between cell-surface nucleolin expression and tumour progression [7]. Confirmatory western blotting revealed nucleolin to be present in all of the cell lysates, but only in the SFM of WRO and MRO cells. It was also detectable in all 5 thyroid cancer patient samples, but not in the normal blood sample. The detectability of nucleolin in patient blood samples suggests it may be a useful thyroid cancer biomarker.

[0256] Cysteine Rich Angiogenic Inducer 61 (CYR61). CYR61 belongs to the CCN family of proteins, initially identified as secretory proteins whose production is induced by oncogenes [8]. Paradoxically, CYR61, while having demonstrated importance in cancer cell proliferation, has also been shown to play an important role in the induction of apoptosis [9]. The secretome analysis revealed CYR61 to be secreted by TPC-1 cells. As with nucleolin, it was present in all thyroid cancer patient blood samples, but not in the normal. Interestingly, CYR61 was only found in the whole cell lysate of TPC-1 cells. This illustrates the potential for secretome analysis to reveal markers that may be used to distinguish between different thyroid cancer types.

- 1 **[0257] E-Cadherin.** The cadherins are a family of proteins responsible for cell-cell adhesion.
2 Studies have shown that loss of E-cadherin mediated cell adhesion is associated with increased
3 tumour aggressiveness and patient mortality [10]. E-Cadherin expression was noticed in the cell
4 lysate and SFM of WRO and MRO cells, but not TPC-1. It was also present in all thyroid cancer
5 blood samples, but not in the normal controls.
- 6 **[0258] Prothymyosin alpha.** Prothymyosin alpha is a heterochromatin remodelling protein whose
7 expression has previously been shown to be significantly elevated in well-differentiated thyroid
8 carcinomas compared to adenomas and goitres. [11] While it was present in the cell lysate of all three
9 cell-lines, it was found to be secreted only in TPC-1 cells. As with nucleolin, CYR61, and E-Cadherin,
10 prothymyosin alpha may serve as a potential thyroid cancer biomarker as it was found in all thyroid
11 cancer patient blood samples but not in the normal controls.
- 12 **[0259] Activated leukocyte cell adhesion molecule (ALCAM/CD166).** Activated leukocyte cell
13 adhesion molecule (ALCAM/CD166) is usually expressed in cells that are involved in growth and
14 migration, including neural development, immune response, and tumor formation. [12, 13] It is an
15 adhesion molecule that is located at intercellular junctions and is involved in tumor cell adhesion,
16 which is necessary for primary tumor formation and metastasis. ALCAM binds to CD6 on T-cells and
17 mediates T-cell activation and proliferation. ALCAM was identified in four thyroid cancer cell lines,
18 including TPC-1, BCPAP, CAL62, and SW1736.
- 19 **[0260] AXL.** AXL is a receptor tyrosine kinase, ubiquitously expressed transmembrane protein, that
20 binds to growth factors and transduces signals from the extracellular matrix to the cytoplasm. It is
21 involved in stimulating cell proliferation and aggregation through hemophilic binding. AXL
22 overexpression plays a role in cell adhesion and overexpression of this protein has been found in
23 several cancers. [14]
- 24 **[0261] APP.** Amyloid beta (A4) protein- is a cell surface receptor and transmembrane precursor
25 protein that is cleaved by different secretases to form a variety of peptides which can bind to
26 complexes for transcriptional activation. APP plays a role in development of the adult nervous system,
27 cell adhesion, neuronal survival, neurite outgrowth, synaptogenesis, vesicular transport, neuronal
28 migration, modulation of synaptic plasticity, and insulin and glucose homeostasis. [15]
- 29 **[0262] PKM2.** Normal cells express the pyruvate kinase M1 isoform (PKM1), tumor cells
30 predominantly express the M2 isoform (PKM2). Switching from PKM1 to PKM2 promotes aerobic
31 glycolysis and provides a selective advantage for tumor formation. The PKM1/M2 isoforms are
32 generated through alternative splicing of two mutually exclusive exons. A recent study shows that the
33 alternative splicing event is controlled by heterogeneous nuclear ribonucleoprotein (hnRNP) family
34 members hnRNPA1, hnRNPA2, and polypyrimidine tract binding protein (PTB; also known as
35 hnRNPI). [16]
- 36 **[0263] APLP2.** Amyloid-like protein 2 (APLP2) is a paralogue of APP and is similarly cleaved by
37 secretases to form peptides which may have similar functions to APP cleaved domains, including cell

adhesion, migration, cell signaling, and cell cycle regulation. Increased expression of APLP2 has been reported in some tumours. APLP2 was identified in seven cell lines, including TPC-1, BCPAP, CAL62, SW1736, C643, MRO, and WRO. [17]

[0264] Clusterin. Clusterin is a glycoprotein that has many biological functions of which are not well understood. It appears to be involved in cell death, tumour progression, tissue differentiation, cell-cell interactions, cell proliferation, lipid transportation, and neurodegenerative disorders. Clusterin was identified in seven cell lines, including TPC-1, CAL62, SW1736, , MRO, and WRO. [18]

[0265] In summary, by verifying the above protein biomarkers in the sera and tissues of thyroid cancer patients, the feasibility of using a secretome approach to identify potential thyroid cancer biomarkers has been illustrated. The findings herein also reveal the potential for secretome analysis to identify proteins that may help to distinguish between benign and premalignant neoplastic lesions as well as aggressive and non-aggressive carcinomas.

[0266] Table 1: Thyroid Cancer Markers

ID	SEQ ID No.	Thyroid Cancer Marker	Accession No.
1.	SEQ ID NO: 31	Versican	trm Q59FG9
2.	SEQ ID NO: 6	Clusterin	spt P10909
3.	SEQ ID NO: 91	Vacuolar proton pump subunit S1	spt Q15904
4.	SEQ ID NO: 32	Gamma-glutamyl hydrolase*	spt Q92820
5.	SEQ ID NO: 66	Insulin-like growth factor binding protein 7	spt Q16270
6.	SEQ ID NO: 21	α -Enolase***	trm Q53FT9
7.	SEQ ID NO: 82	Stem cell growth Factor	trm Q5U0B9
8.	SEQ ID NO: 34	Syndecan-4	trm Q53FN9 Q53FN9_HUMAN; spt P31431 SDC4_HUMAN
9.	SEQ ID NO: 28	Fibronectin	trm Q6N025
10.	SEQ ID NO: 70	Nucleophosmin	spt P06748
11.	SEQ ID NO: 29	Ubiquitin-A 52-residue ribosomal protein fusion product	trm Q3MIH3
12.	SEQ ID NO: 33	Lysyl oxidase-like 2*	trm Q53HV3 Q53HV3_HUMAN; spt Q9Y4K0 LOXL2_HUMAN
13.	SEQ ID NO: 18	Nucleobindin-1 variant	trm Q53GX6
14.	SEQ ID NO: 14	Calsyntenin-1	trm Q5UE58
15.	SEQ ID NO: 3	Prothymosin- α	trm Q9NYD3
16.	SEQ ID NO: 40	Agrin	spt O00468
17.	SEQ ID NO: 7	Amyloid-like protein 2 (APLP2)***	trm Q9BT36
18.	SEQ ID NO: 43	Beta-2-microglobulin	trm Q6IAT8 Q6IAT8_HUMAN; spt P61769 B2MG_HUMAN
19.	SEQ ID NO: 27	CD44 antigen	spt P16070

20.	SEQ ID NO: 22	dystroglycan 1	trm Q969J9
21.	SEQ ID NO: 17	Gelsolin**	spt P06396
22.	SEQ ID NO: 35	hnRNP A2/B1**	spt P22626
23.	SEQ ID NO: 8	Nucleolin	spt P19338
24.	SEQ ID NO: 11	SET protein	trm Q6FHZ5
25.	SEQ ID NO: 5	Biotinidase*	spt P43251
26.	SEQ ID NO: 16	Nidogen-1*	spt P14543
27.	SEQ ID NO: 15	Dickkopf-related protein 3	trm Q6PQ81
28.	SEQ ID NO: 30	Basement membrane specific heparin sulfate core protein**	spt P98160
29.	SEQ ID NO: 45	Cadherin 2***	spt P19022
30.	SEQ ID NO: 62	Granulins (proepithelin)***	spt P28799
31.	SEQ ID NO: 2	Activated Leukocyte Cell Adhesion Molecule (ALCAM)	trm Q1HGM9
32.	SEQ ID NO: 48	Cathepsin Z***	trm Q5U000
33.	SEQ ID NO: 64	Hypothetical protein (belongs to the actin family)***	trm Q8WVW5
34.	SEQ ID NO: 73	Peptidylprolyl isomerase A	trm Q3KQW3
35.	SEQ ID NO: 65	Insulin-like growth factor binding protein 6***	spt P24592
36.	SEQ ID NO: 1	AXL receptor tyrosine kinase***	trm Q8N5L2 Q8N5L2_HUMAN
37.	SEQ ID NO: 38	14-3-3 protein epsilon (14-3-3E)	spt P62258
38.	SEQ ID NO: 10	14-3-3 protein zeta/delta (Protein kinase C inhibitor protein 1)***	spt P63104
39.	SEQ ID NO: 39	60S acidic ribosomal protein P2 (NY-REN-44 antigen)***	spt P05387
40.	SEQ ID NO: 41	Alpha-actinin-1***	spt P12814
41.	SEQ ID NO: 42	Alpha-actinin-4	spt O43707
42.	SEQ ID NO: 44	C4B1 (Complement component C4B)***	trm Q6U2E9
43.	SEQ ID NO: 26	Calmodulin (CaM)***	trm Q9BRL5 Q9BRL5_HUMAN; spt P62158 CALM_HUMAN
44.	SEQ ID NO: 46	Calreticulin (CRP55)	trm Q53G71
45.	SEQ ID NO: 47	Cathepsin C***	trm Q8WY99
46.	SEQ ID NO: 49	CDNA FLJ45706 fis, clone FEBRA2028457, highly similar to Nucleolin***	trm Q6ZS99
47.	SEQ ID NO: 50	Chaperonin 10-related protein***	trm Q9UNM1
48.	SEQ ID NO: 51	Cofilin-129	spt P23528
49.	SEQ ID NO: 52	Collagen alpha-1 (V) chain***	spt P20908
51.	SEQ ID NO: 54	Collagen alpha-1(XII) chain***	spt Q99715
52.	SEQ ID NO: 55	Collagen, type I, alpha 2***	trm Q7Z5S6

53.	SEQ ID NO: 56	Colony stimulating factor 1 (Macrophage)***	trm Q5VVF4
54.	SEQ ID NO: 57	EGF-containing fibulin-like extracellular matrix protein 1***	spt Q12805
55.	SEQ ID NO: 58	Filamin A***	trm Q60FE6
56.	SEQ ID NO: 59	Follistatin-related protein 1***	spt Q12841
57.	SEQ ID NO: 60	Fructose-bisphosphate aldolase***	trm Q6FI10
58.	SEQ ID NO: 63	Heat shock protein (HSP 90-alpha 2)***	trm Q5CAQ7
59.	SEQ ID NO: 61	Glucose-6-phosphate isomerase	spt P06744
60.	SEQ ID NO: 13	HNRPK protein (Heterogeneous nuclear ribonucleoprotein K)***	trm Q5T6W2
61.	SEQ ID NO: 80	Secretogranin 2	trm Q53T11
62.	SEQ ID NO: 67	L-lactate dehydrogenase A chain	spt P00338
63.	SEQ ID NO: 68	Matrix metalloproteinase 1 (MMP-1)	trm Q5TZP0
64.	SEQ ID NO: 69	Matrix metalloproteinase 1 preprotein variant	trm Q53G75
65.	SEQ ID NO: 37	Niemann-Pick disease, type C2 variant***	trm Q53HV6
66.	SEQ ID NO: 71	Nucleoside diphosphate kinase (NME1-NME2)***	trm Q32Q12
67.	SEQ ID NO: 72	OAF homolog***	trm Q86UD1
68.	SEQ ID NO: 74	Phosphoglycerate kinase 1 (PGK1)***	trm Q5J7W1
69.	SEQ ID NO: 75	pyruvate kinase 3 isoform 1 variant protein (PKM2)***	trm Q8WUW7
70.	SEQ ID NO: 85	Tissue-type plasminogen activator (PLAT protein)***	trm Q6PJA5
71.	SEQ ID NO: 76	Protein CutA***	spt O60888
72.	SEQ ID NO: 77	Protein FAM3C***	spt Q92520
73.	SEQ ID NO: 78	Protein S100A9***	spt P06702
74.	SEQ ID NO: 12	Pyruvate kinase isozymes M1/M2***	trm Q53GK4
75.	SEQ ID NO: 79	Ribosomal protein S27a***	spt P62979
76.	SEQ ID NO: 81	SPARC (Secreted protein acidic and rich in cysteine) (Osteonectin)	spt P09486
77.	SEQ ID NO: 83	Sulfhydryl oxidase 1 (Quiescin Q6) (hQSOX)***	spt O00391
78.	SEQ ID NO: 84	Thrombospondin 2***	trm Q5RI52
79.	SEQ ID NO: 86	Transforming growth factor, beta-induced, 68kDa variant	trm Q53GU8
80.	SEQ ID NO: 87	Transketolase (TK)***	trm Q53EM5
81.	SEQ ID NO: 88	Translation elongation factor 1 alpha 1-like 14***	trm Q96RE1
82.	SEQ ID NO: 89	Triosephosphate isomerase	trm Q6FHP9
83.	SEQ ID NO: 90	UV excision repair protein RAD23 homolog B***	spt P54727

84.	SEQ ID NO: 19	Cysteine rich angiogenic inducer, 61 variant (CYR61)	trm Q53FA4 Q53FA4_HUMAN; spt Q00622 CYR61_HUMAN
85.	SEQ ID NO: 23	Melanoma associated antigen	rm Q92626 Q92626_HUMAN
86.	SEQ ID NO: 24	Osteopontin	trm Q0JV14 Q0JV14_HUMAN; spt P10451 OSTP_HUMAN
87.	SEQ ID NO: 25	Plasminogen activator, urokinase activator	trm Q5SWW9 Q5SWW9_HUMAN ; trm Q5SWW8 Q5SWW8_HUMAN ; trm Q5PY49 Q5PY49_HUMAN; trm Q53XS3 Q53XS3_HUMAN; spt P00749 UROK_HUMAN
88.	SEQ ID NO: 36	MCFD2***	trm Q68D61
89.	SEQ ID NO: 4	Galectin-3	spt Q08380
90.	SEQ ID NO: 96	S100A6	spt P06703
91.	SEQ ID NO: 97	Profilin-1	spt P07737
92.	SEQ ID NO: 98	Cyclin D1	spt Q9H014
93.	SEQ ID NO: 99	Phosphatase and tensin homolog (PTEN)	spt P60484

*Novel Papillary Cancer Markers (TPC-1/BCPAP);

** Novel Follicular Cancer Markers (MROWRO);

*** Novel Aggressive/Metastatic Thyroid Cancer Markers (CAL62, SW1736, C643)

[0249] Table 2: Known Thyroid Cancer Markers

S.No	SEQ ID#	Protein	Protein ID
1.	SEQ ID NO: 4	Galectin-3	spt Q08380
2.	SEQ ID NO: 92	Serum thyroglobulin	spt p01266
3.	SEQ ID NO: 93	BRAF mutation	spt p15056
4.	SEQ ID NO: 20	E-Cadherin	spt P12830
5.	SEQ ID NO: 94	Vimentin	trm Q5JVT0
6.	SEQ ID NO: 95	Galectin-1	spt p09382
7.	SEQ ID NO: 9	Amyloid precursor protein (APP)	AAB19991

[0250] Example 2: Molecular Signature for Prediction of Benign and Malignant Thyroid Tumors using Fine Needle Aspiration (FNA) Biopsies

[0251] Summary

[0252] Currently, there are no protein biomarkers in clinical use that can accurately distinguish benign from malignant thyroid tumors prior to surgery. In this study, we explored the potential of a biomarker signature based on alterations in sub-compartmental expression analyses of a panel of

seven proteins identified by secretome proteomics to distinguish between thyroid benign tissues, adenomas and thyroid cancers (TC). In this example, seven proteins were selected to determine their potential, alone or in combination, in pre-surgical diagnosis of thyroid cancer (TC). The seven proteins were: PGK1, PKM2, Cyclin D1, Galectin-3, PTEN, S100A6, and Profilin-1. The expression patterns or levels of these proteins were investigated for distinguishing thyroid cancer (TC) from benign thyroid tissues and adenomas based on sub-cellular expression patterns, in particular from thyroid tissues and FNA biopsies (or FNAB) and cytospins.

[0253] Immunohistochemical analyses of PGK1, PKM2, Cyclin D1, Galectin-3, PTEN, S100A6, and Profilin-1 was carried out in 115 non-malignant tissues (53 benign, 62 adenomas) and 114 TC and in 35 fine needle aspiration biopsies (FNABs) and cytospins. The cytoplasmic and nuclear immunostaining were scored and compared and statistical analysis was carried out using R package.

[0254] Model selection using the seven proteins resulted in nuclear PGK1 loss and nuclear Galectin-3 overexpression based identification of TC from non-malignant tissues. Nuclear cyclin D1 and cytoplasmic PTEN overexpression identified adenoma from benign. Malignancy Score based on Nuclear PGK1 achieved high clinical utility in identifying TC from non-malignant tissues. Adenoma Score based on nuclear cyclin D1 achieved high clinical utility in identifying adenoma from benign tissues. Importantly, this malignancy score has been validated in fine-needle aspiration biopsies (FNAB) and cytospins to identify TC from non-malignant thyroid nodules.

[0255] The molecular signatures described herein have the potential to serve as a diagnostic tool in conjunction with FNAB to identify TC, adenoma and benign tissues.

[0256] Materials and Methods

[0257] Patient specimens

[0258] The study was approved by the Mount Sinai Hospital (MSH) Research Ethics Board (REB), Toronto, Canada (REB guideline #07-0212-E). Informed consent for scientific use of anonymous patients' data and tumor tissues had been obtained from all patients as per REB guidelines. All data were analyzed anonymously. Archived formalin-fixed paraffin-embedded (FFPE) tissue blocks from the MSH tumor bank were retrieved and reviewed by a pathologist. Clinico-pathological parameters were obtained from histopathological analyses and the clinical database. Diagnoses at the time of surgery were used to stratify patients. Fifty three benign thyroid nodules, 62 adenomas and 114 TC tissues were analyzed for protein expression of all the seven proteins. Benign nodules were obtained from patients with multinodular goiters, Graves' disease, Hashimoto's thyroiditis, lymphocytic thyroiditis, or hyperplastic nodules. Thirty five FNA biopsies and cytospins were also collected for marker analyses.

[0259] Immunohistochemical analysis in human thyroid tissues

[0260] Formalin fixed and paraffin embedded (FFPE) tissue sections (4 μ m thickness) were deparaffinized in xylene and hydrated with graded alcohol series as described previously (Chaker et

al., 2013). For antigen retrieval for proteins PGK1, PKM2, Cyclin D1, Galectin-3, S100A6, and PTEN, slides were immersed in Tris-EDTA buffer (10 mM Tris base, 1 mM EDTA, 0.05% Tween 20, pH 9.0, TBS) and pre-treated in a 900-watt microwave oven for 20 minutes. Antigen retrieval for Profilin-1 was similarly performed using Sodium Citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) in place of TBS buffer. All further incubations were conducted at room temperature. The VECTASTAIN™ rapid protocol was followed for immunostaining. Non-specific binding was blocked by incubating the slides with 10% horse serum for anti-mouse secondary antibodies and goat serum for anti-rabbit secondary antibodies for 20 minutes. Thereafter, the sections were incubated with the following anti-human antibodies from AbCAM, (Cambridge, MA); mouse monoclonal PGK1 (1:750 dilution), rabbit polyclonal PKM2 (1:100), rabbit monoclonal Cyclin D1 (1:100), mouse monoclonal Galectin-3 (1:200), mouse monoclonal S100A6 (1:600), mouse monoclonal Profilin-1 (1:1500), or mouse monoclonal PTEN (1:200) for 1 h. Tissues were then treated with 3% H₂O₂ in TBS for 5 minutes to block the endogenous peroxidase activity and subsequently incubated with biotinylated anti-mouse or anti-rabbit secondary antibody for 20 minutes. The sections were finally incubated with VECTASTAIN™ Elite ABC Reagent (Vector labs, Burlingame, CA) for 30 minutes and diaminobenzidine was used as the chromogen. Negative control tissues were incubated with biotinylated horse anti-mouse (or goat anti-rabbit) secondary antibody following the same protocol. Slides were counterstained with hematoxylin and viewed using a light microscope. The FNA biopsies and cytospins were immunostained following a modified protocol.

[0261] Evaluation of immunohistochemistry

[0262] Immunostaining scoring as based on percentage positivity and staining intensity. Sections were scored as positive if epithelial cells showed immunoreactivity in the cytoplasm and/or nucleus when observed by two evaluators. Percentage positive scores were assigned according to the following scale: 0 (< 10% cells); 1 (10-30% cells); 2 (31-50% cells); 3 (51-70% cells); and 4 (> 70%). Staining intensity was scored semi-quantitatively as follows: 0 (none); 1 (mild); 2 (moderate); and 3 (intense). A total score for each of cytoplasmic and nuclear staining was then obtained (ranging from 0 to 7) by adding the percentage positivity scores and intensity scores for each section. Three fields for each tissue were scored and an average of the fields was calculated. Average scores of the two evaluators were used for subsequent analyses.

[0263] Statistical analyses

[0264] All statistical analyses were carried out in R version 3.10. Multiple Imputations by Chained Equations (MICE) was used to impute missing data and generate 30 complete data sets to limit loss of power to less than 1% (White et al.; 2011). Imputations were done using the predictive mean matching method and were carried out using the MICE R package (Buuren & Groothuis-Oudshoorn, 2011). Univariate and multivariable logistic regression analyses were used to assess the individual and cumulative predictive value of biomarkers for cancer vs. benign and adenoma, as well as for benign vs. adenoma tissues. Similar analyses were done using the receiver operating characteristic (ROC) curve to assess the discriminatory value of biomarkers summarized by the area-under-curve

(AUC). ROC curve analyses were performed using pROC package in R (Robin et al., 2011). A two-step approach was used for model selection under multiple imputed data (Wood et al., 2008). Backward selection was used to derive the final model. The cumulative predictive value of biomarkers that correlated positively and negatively with cancer was assessed in a similar manner for exploring their biological relevance. The clinical utility of biomarkers was assessed using sensitivity, specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV), and AUC of the ROC curves between cancer vs. benign or adenoma, and adenoma vs. benign tissues. The optimal cut-off value was chosen as the threshold that maximized the AUC. All analyses were internally validated using random split-sample. The predictive value of biomarkers was assessed in both a Test set, and a Validation set. Final models and optimal cut-off values were derived from the Test set, and verified in the Validation set.

[0265] Results

[0266] Immunohistochemical analysis of PGK1, PKM2, Cyclin D1, Galectin-3, PTEN, S100A6, and Profilin-1 was carried out to determine differences in their total cellular expressions and in subcellular localization (cytoplasmic and nuclear levels) in benign thyroid tissues, adenomas and TC. Biomarker expression levels (% positivity score + intensity) in benign, adenoma, and cancer tissues are summarized in Table 3. Representative photomicrographs of tissue sections showing the immunostaining expression patterns of all seven proteins in benign thyroid nodules, adenomas and TC are shown in Figure 12. All seven proteins were detected in thyroid tissues though differential subcellular localization was observed in non-malignant thyroid tissues (benign and adenomas, Fig. 12 panels A and B respectively) and TC (Fig. 12 panel C and Table 3). Reduced PGK1 nuclear expression was observed in TC (i.e. malignant tissue) as compared to benign nodules and adenomas (Fig. 12 (1st row), Table 3). Both nuclear and cytoplasmic staining was observed for PKM2 with a significant decrease in nuclear expression in TC (Fig. 12 (2nd row), Table 3). An increase in nuclear expression of Cyclin D1 was observed in cancer as compared to benign nodules and adenomas (Fig. 12 (3rd row), Table 3). Both nuclear and cytoplasmic expression of Galectin-3 was increased in TC as compared to non-malignant tissues (Fig. 12 (4th row), Table 3). Nuclear PTEN expression was significantly downregulated in malignant tissue as compared to benign tissues and adenomas (Fig. 12 (5th row), Table 3). Expression of cytoplasmic and nuclear S100A6 was decreased in malignant tissue as compared to benign tissue and adenomas (Fig. 12 (6th row), Table 3). A decrease in nuclear expression of Profilin-1 was also observed in TCs as compared to benign tissues (Fig. 12 (7th row), Table 3).

[0267] Table 3: Biomarker expression in cancer, adenoma and benign tissues

Biomarkers	Cancer (n=114)	Adenoma (n=62)	Benign (n=53)
Cytoplasmic PTEN	1.88 (1.78)	4.49 (1.33)	2.31 (1.67)
Nuclear PTEN	3.78 (2.05)	5.61 (0.95)	5.85 (1.11)

Cytoplasmic Profilin1	4.46 (1.66)	4.05 (2.19)	4.59 (1.92)
Nuclear Profilin1	2.77 (2.20)	5.27 (1.41)	6.13 (1.03)
Cytoplasmic S100A6	3.91 (2.00)	4.05 (2.13)	4.63 (1.52)
Nuclear S100A6	4.04 (1.98)	4.84 (2.20)	5.84 (1.77)
Nuclear CyclinD1	5.07 (1.56)	4.46 (1.61)	1.98 (1.63)
Cytoplasmic Galectin-3	3.71 (2.19)	1.91 (2.52)	0.89 (1.28)
Nuclear Galectin-3	0.90 (1.11)	0.14 (0.35)	0.05 (0.18)
Cytoplasmic PGK1	4.77 (1.30)	5.73 (0.93)	6.19 (0.67)
Nuclear PGK1	3.57 (2.17)	6.51 (0.47)	6.64 (0.36)
Cytoplasmic PKM2	3.38 (2.00)	4.20 (1.59)	3.80 (1.68)
Nuclear PKM2	0.40 (1.05)	1.33 (1.76)	3.76 (2.05)

1 **[0268]** In Table 3, expression levels are summarized as “mean (standard deviation)”.

2 **[0269]** Biomarker predictive values for cancer vs. non-malignant (benign or adenoma) thyroid tissues

3 **[0270]** The predictive value of the biomarkers tested to distinguish TC from non-malignant tissues
 4 (benign and adenomas) was determined. Cases were divided into a Test set and a Validation set
 5 using random split-sample. Sub-cellular compartmental expression of each biomarker with the
 6 exception of cytoplasmic Profilin-1 and cytoplasmic S100A6 were associated with TC (Table 4).

7 **[0271]** Nuclear PGK1 emerged as the strongest predictor of cancer in comparison with non-
 8 malignant tissues (benign or adenomas) [Test set: Odds ratio (OR) = 0.05 (95% confidence interval
 9 (CI) = 0.01, 0.17), $p < 0.0001$, AUC = 0.93; Validation set: OR = 0.05 (95% CI = 0.01, 0.21), $p <$
 10 0.0001 , AUC = 0.96; Table 4] underscoring its potential clinical applicability. It was contemplated that
 11 a model using a panel of two or more of the biomarkers would better identify cancer tissues compared
 12 to nuclear PGK1 alone. To test this hypothesis, a two-step approach for model selection under
 13 multiple imputed data was used, resulting in a final model comprising loss of nuclear PGK1 and
 14 overexpression of nuclear Galectin-3 in the Test set (Table 5). The predictive values of panels of
 15 overexpressed biomarkers and downregulated biomarkers were further explored (Table 6).
 16 Downregulation of cytoplasmic PTEN and nuclear PGK1 was found to be associated with cancer. A
 17 model based on these biomarkers achieved a discriminatory value AUC of 0.94, and 0.99 in the Test
 18 and Validation sets respectively (Table 6). Further, overexpression of nuclear PTEN, nuclear Galectin-
 19 3, and nuclear PKM2 in combination as a panel was found to be associated with cancer and achieved
 20 a discriminatory value, AUC of 0.92 in both the Test and Validation sets (Table 6).

21

22 **[0272]** Table 4: Univariate logistic regression analyses

	Test set (n= 27 benign, 31 adenoma, 57 cancer)			Validation set (n = 26 benign, 31 adenoma, 57 cancer)		
Biomarker	OR [95% CI]	p	AUC	OR [95% CI]	p	AUC
Non-malignant (Benign and Adenoma) vs. Cancer						
Cytoplasmic PTEN	0.65 [0.50, 0.84]	0.0009	0.73	0.62 [0.48, 0.80]	0.0002	0.74
Nuclear PTEN	0.38 [0.23, 0.65]	0.0003	0.83	0.31 [0.17, 0.56]	0.0001	0.89

Cytoplasmic Profilin1	1.07 [0.84, 1.35]	0.5909	0.52	1.04 [0.84, 1.29]	0.7369	0.51
Nuclear Profilin1	0.54 [0.42, 0.71]	< 0.0001	0.80	0.34 [0.22, 0.53]	< 0.0001	0.90
Cytoplasmic S100A6	0.88 [0.71, 1.10]	0.2639	0.57	0.91 [0.73, 1.15]	0.4460	0.56
Nuclear S100A6	0.77 [0.63, 0.95]	0.0157	0.68	0.68 [0.53, 0.87]	0.0024	0.74
Nuclear Cyclin D1	1.65 [1.27, 2.15]	0.0002	0.77	1.66 [1.28, 2.15]	0.0001	0.73
Cytoplasmic Galectin-3	1.46 [1.22, 1.76]	< 0.0001	0.73	1.66 [1.34, 2.08]	< 0.0001	0.79
Nuclear Galectin-3	12.1 [3.49, 41.7]	0.0001	0.80	7.1 [1.98, 25.49]	0.0027	0.74
Cytoplasmic PGK1	0.31 [0.17, 0.57]	0.0002	0.79	0.24 [0.13, 0.44]	< 0.0001	0.85
Nuclear PGK1	0.05 [0.01, 0.17]	< 0.0001	0.93	0.05 [0.01, 0.21]	< 0.0001	0.96
Cytoplasmic PKM2	0.77 [0.62, 0.96]	0.0231	0.61	0.88 [0.71, 1.10]	0.2746	0.55
Nuclear PKM2	0.47 [0.32, 0.69]	0.0002	0.79	0.50 [0.35, 0.72]	0.0002	0.77
Benign vs. Adenoma						
Cytoplasmic PTEN	2.55 [1.61, 4.05]	0.0001	0.87	2.04 [1.34, 3.10]	0.0009	0.80
Nuclear PTEN	0.95 [0.55, 1.63]	0.8380	0.59	0.61 [0.28, 1.34]	0.2188	0.66
Cytoplasmic Profilin1	0.91 [0.07, 1.19]	0.4950	0.57	0.84 [0.64, 1.11]	0.2183	0.62
Nuclear Profilin1	0.66 [0.42, 1.03]	0.0660	0.67	0.28 [0.11, 0.69]	0.0056	0.78
Cytoplasmic S100A6	0.90 [0.68, 1.20]	0.4730	0.54	0.82 [0.59, 1.15]	0.2471	0.56
Nuclear S100A6	0.83 [0.62, 1.09]	0.1837	0.62	0.68 [0.43, 1.09]	0.1123	0.69
Nuclear Cyclin D1	2.07 [1.36, 3.15]	0.0007	0.82	2.57 [1.59, 4.15]	0.0001	0.89
Cytoplasmic Galectin-3	1.40 [1.03, 1.90]	0.0308	0.59	1.19 [0.88, 1.61]	0.2478	0.55
Nuclear Galectin-3	2.65 [0.3, 23.34]	0.3799	0.53	8.95 [0.2, 394.1]	0.2561	0.55
Cytoplasmic PGK1	0.55 [0.25, 1.23]	0.1461	0.61	0.34 [0.13, 0.90]	0.0301	0.73
Nuclear PGK1	1.15 [0.31, 4.32]	0.8359	0.54	0.19 [0.04, 0.96]	0.0444	0.66
Cytoplasmic PKM2	1.21 [0.84, 1.75]	0.2997	0.59	1.14 [0.83, 1.56]	0.4279	0.57
Nuclear PKM2	0.61 [0.46, 0.81]	0.0006	0.77	0.53 [0.39, 0.74]	0.0001	0.80

1 **[0273] Table 5: Multivariable analyses**

	Test Set		Validation Set	
	OR [95% CI]	p	OR [95% CI]	p
Non-malignant (Benign and Adenoma) vs. Cancer				
Nuclear Galectin-3	12.09 [1.48, 98.39]	0.0201	2.23 [0.54, 9.21]	0.2656
Nuclear PGK1	0.03 [0.01, 0.25]	0.0009	0.07 [0.02, 0.27]	0.0001
Model AUC	0.95		0.96	
Benign vs. Adenoma				
Nuclear Cyclin D1	1.8 [1.13, 2.86]	0.0131	2.33 [1.42, 3.81]	0.0008
Cytoplasmic PTEN	2.37 [1.45, 3.88]	0.0007	1.51 [0.94, 2.43]	0.0887
Model AUC	0.92		0.91	

2

3 **[0274] Table 6: Multivariable logistic regression analyses of downregulated and**
4 **overexpressed biomarkers**

	Test Set		Validation Set	
	OR [95% CI]	p	OR [95% CI]	p
Non-malignant (Benign and Adenoma) vs Cancer				
Downregulated biomarkers				
Cytoplasmic PTEN	0.61 [0.39, 0.98]	0.0391	0.34 [0.15, 0.79]	0.0124
Nuclear PGK1	0.04 [0.01, 0.19]	2.67E-05	0.02 [0, 0.21]	0.0011
Model AUC	0.94		0.99	
Non-malignant (Benign and Adenoma) vs Cancer				
Overexpressed biomarkers				
Nuclear PTEN	0.52 [0.31, 0.87]	0.0136	0.41 [0.24, 0.71]	0.0014
Nuclear Galectin-3	6.9 [1.67, 28.54]	0.0077	4.65 [1.28, 16.84]	0.0192
Nuclear PKM2	0.53 [0.33, 0.86]	0.0097	0.61 [0.39, 0.94]	0.0245
Model AUC	0.92		0.92	

5

6 **[0275] Biomarkers predictive for adenoma vs. benign thyroid tissues**

7 **[0276]** Among the biomarkers analyzed, cytoplasmic PTEN, nuclear Cyclin D1 and cytoplasmic
8 Galectin-3 were significant predictors for stratifying adenoma from benign thyroid tissues (Table 4).
9 The two most predictive biomarkers in the Test set and Validation set were cytoplasmic PTEN and
10 nuclear Cyclin D1 (Table 5). Using the two-step approach for model selection under multiple imputed
11 data, it was found that these two biomarkers can be used to identify adenoma from benign tissues
12 with an AUC of 0.92 in the Test set and 0.91 in Validation set (Table 5).

13 **[0277] Clinical Utility of Biomarkers for identifying thyroid cancer from non-malignant tissues**

14 **[0278]** Based on the high predictive and discriminatory values nuclear PGK1 and nuclear Cyclin D1
15 risk scores were used in a two-step approach to differentiate cancer, adenoma, and benign tissues. A
16 risk score model was developed in the Test set, and an optimal cut-off value which maximizes the
17 AUC was chosen. The risk score model based on regression estimates as weights is given as

Malignancy Score = $18.13 - 3.05 \times \text{nuclear PGK1}$; the optimal cut-off was 0.86 (IHC score of PGK1 = 5.67), and AUC of 0.91 (Table 7). Notably, 98% of non-malignant tissues were correctly identified in the Test set. The clinical applicability of this cut-off value was verified in the Validation set, as it achieved an AUC of 0.92, with similar sensitivity (0.89), and specificity (0.96) (Table 7). Furthermore, analysis for the clinical utility of panels of downregulated and overexpressed biomarkers demonstrated similar precision for stratification of cancer and non-cancer tissues (Table 8).

[0279] Table 7: Clinical utility of nuclear PGK1 and nuclear Cyclin D1 in identifying cancer, adenoma, and benign tissues

	Sens.	Spec.	PPV	NPV	AUC	Size of High Risk Group
Non-malignant (Benign and Adenoma) vs. Cancer (PGK1)						
Test set	0.84	0.98	0.98	0.86	0.91	42 %
Validation set	0.89	0.96	0.96	0.90	0.92	46 %
Benign vs. Adenoma (Cyclin D1)						
Test set	0.68	0.92	0.90	0.71	0.80	40 %
Validation set	0.76	0.90	0.90	0.76	0.83	46 %

[0280] In Table 7, the optimal cut-off were as follows: (i) (Benign or Adenoma) vs. Cancer, PGK1 of 0.86 (IHC score = 5.67); (ii) Benign vs. Adenoma, Cyclin D1 of 0.66 (IHC score = 3.9).

[0281] Table 8: Clinical utility of downregulated or overexpressed biomarkers in identifying cancer from non-malignant (benign and adenoma) tissues

	Sens.	Spec.	PPV	NPV	AUC	Size of High Risk Group
Non-malignant (Benign and Adenoma) vs Cancer Downregulated biomarkers: cytoplasmic PTEN and nuclear PGK1						
Test set	0.84	0.97	0.97	0.86	0.91	43 %
Validation set	0.89	0.96	0.96	0.90	0.93	46 %
Non-malignant (Benign and Adenoma) vs Cancer Overexpressed biomarkers: nuclear PTEN, nuclear Galectin-3, and nuclear PKM2						
Test set	0.87	0.87	0.87	0.88	0.87	50 %
Validation set	0.85	0.85	0.85	0.85	0.85	50 %

[0282] In Table 8, the optimal cut-off were as follows for (Benign or Adenoma) vs Cancer:

- Downregulated biomarkers: 0.60 (Score = $20.82 - 0.58 \times \text{cytoplasmic PTEN} - 3.26 \times \text{nuclear PGK1}$);
- Overexpressed biomarkers: 0.15 (Score = $-3.52 + 0.70 \times \text{nuclear PTEN} - 1.97 \times \text{nuclear Galectin-3} + 0.63 \times \text{nuclear PKM2}$).

1 **[0283]** Clinical Utility of Biomarkers for identifying benign thyroid tissues from adenoma

2 **[0284]** The clinical utility of nuclear Cyclin D1 for use in identifying adenoma from benign tissues was
3 assessed in a similar manner. A risk score model Adenoma Score = $-2.18 + 0.73 \times \text{nuclear Cyclin D1}$
4 was developed in the Test set with an optimal cut-off value 0.66 that achieved an AUC of 0.80 and
5 92% of benign tissues were correctly identified in the Test set. These results were verified in the
6 Validation set, with an AUC of 0.83 and specificity of 0.90.

7
8 **[0285] Discussion**

9 **[0286]** This study evaluated expression of a panel of seven proteins in human thyroid tissues, the
10 proteins having been postulated to potentially serve as a diagnostic tool for identifying thyroid cancer
11 (TC) from non-malignant tissues (benign and adenomas) as well as for distinguishing between
12 adenomas and benign thyroid tissues. This is believed to be the first report to detect the expression of
13 Profilin-1 in thyroid tissues as a cancer biomarker.

14 **[0287]** PGK1, PKM2, S100A6 and Galectin-3 have previously been identified by us in thyroid cancer
15 cell lines in secretome analyses (Chaker et al., 2013; Kashat et al., 2010). Other proteins, cyclin D1
16 and PTEN were investigated in our selected panel of candidates based on their important biological
17 role in head and neck cancer and/or TC (Seybt, Ramalingam, Huang, Looney, & Reid, 2012). PGK1
18 and PKM2 are both metabolic proteins that have been shown to have different roles in cancer
19 progression. PGK1 affects DNA replication and repair in the nucleus (J. Wang et al., 2007); PKM2
20 promotes tumor cell proliferation by binding to and trans-activating Y333-phosphorylated β -catenin
21 (Wong, De Melo, & Tang, 2013). Thus, PGK1 and PKM2 have dual roles that are essential for
22 tumorigenesis: regulating cancer cell metabolism and gene transcription. In prostate cancer,
23 overexpression of PGK1 reduces VEGF and increases angiostatin, a vascular inhibitor (J. Wang et
24 al., 2007). Therefore, it was suggested that PGK1 over-expression may restrict tumor growth by
25 limiting angiogenesis and that reduced PGK1 expression promotes angiogenesis and metastasis (J.
26 Wang et al., 2007). PGK1 is expressed in several cancers such as breast, ovarian, pancreatic, and
27 gastric cancer (Hwang, Liang, Chien, & Yu, 2006; J. Wang et al., 2007; Zieker et al., 2010). PKM2
28 phosphorylates histone H3 and promotes gene transcription and tumorigenesis (Yang et al., 2012).
29 Upon EGFR activation, PKM2 is upregulated in colorectal and gastric cancer (Yang et al., 2011; Zhou
30 et al., 2012). Abberant overexpression of PKM2 was associated with aggressive tumor characteristics
31 and BRAF mutation in PTC (Feng et al., 2013). Cyclin D1 is a member of the family of cyclins that
32 function as regulators of cyclin-dependent kinases for controlling cell proliferation. Deregulation of
33 cyclin expression leads to abnormal cell growth and tumorigenesis. Overexpression of cyclin D1 has
34 been observed in thyroid (Brzezińska, Cyniak-Magierska, Sporny, Pastuszek-Lewandoska, &
35 Lewiński, 2007; Lazzereschi et al., 1998; Seybt et al., 2012; Temmim, Ebraheem, Baker, & Sinowatz,
36 2006; S. Wang, Wu, Savas, Patwardhan, & Khan, 1998), breast (Buckley et al., 1993), and head and
37 neck squamous carcinoma (Michalides et al., 1995). Galectin-3 is a member of a family of

carbohydrate binding proteins and is known to have roles in apoptosis, cell adhesion, innate immunity and T-cell regulation. In support of our findings are the reports from other groups that demonstrated expression of Galectin-3 in TC (Cui et al., 2012; de Matos et al., 2012; Rossi et al., 2013). Galectin-3 positivity in FNA samples from PTC was associated with aggressive pathological features such as extrathyroidal extension, lymph node metastasis, and thyroid capsular invasion (Kim et al., 2012). Nevertheless, the translation of Galectin-3 as a TC biomarker into clinical use has not been successful. Therefore, we explored its potential in combination with other proteins as a panel of biomarkers.

[0288] PTEN is a tumor suppressor gene that is mutated in several cancers (Dean et al., 2014; Li et al., 1997; Marzese et al., 2014; Maxwell, Neisen, Messenger, & Waugh, 2014; Squarize et al., 2013). It negatively regulates signaling of the PI3K pathway. Profilin-1 is a ubiquitously expressed actin binding protein which regulates actin polymerization in response to extracellular signals, thereby affecting the cytoskeletal structure and its role in cell migration and proliferation. Ding et al. (2013) reported profilin-1 to be downregulated in breast cancers with propensity to metastasize. However, contrary to its dissemination promoting activities, loss of profilin-1 inhibited metastatic outgrowth of disseminated breast cancer cells, thereby revealing a dichotomous role for profilin-1 in early versus late stages of breast cancer metastasis.

[0289] Immunohistochemical analysis of the selected panel of proteins studied herein showed differential expression between non-malignant and malignant thyroid tissues. The significant decrease in nuclear and/or cytoplasmic expression of PGK1, PKM2, PTEN, Profilin-1 and S100A6, and increase in nuclear and/or cytoplasmic expression of Cyclin D1 and Galectin-3, in association with thyroid malignancy suggest the potential for analyses of these proteins in FNAB and cytosmeas as a valuable diagnostic tool. Use of the above biomarkers would aid in more accurately characterizing biopsy results and prevent unnecessary thyroidecotomies and the inherent complications and morbidity risk associated with such surgical interventions.

[0290] Example 3: Immunohistochemical Subcellular Protein Biomarkers Localization Distinguishes Benign from Malignant Thyroid Nodules

[0291] Summary

[0292] Accurate pre-surgical distinction between benign, indeterminate and malignant thyroid nodules is of critical clinical importance to avoid unnecessary surgery in non-malignant patients. Using alterations in sub-cellular localization for seven putative biomarker proteins in surgical tissues (identified by proteomics), we aimed to define a specific combination of proteins which could distinguish benign from malignant nodules to assist in future surgical selection by fine needle aspiration biopsy (FNAB).

[0293] Immunohistochemical subcellular localization (IHC) analyses of 7 proteins were

retrospectively performed on surgical tissues and a risk model biomarker panel was developed and validated. The biomarker panel efficacy was verified in 50 FNAB formalin fixed and paraffin embedded cell blocks and 26 cytosmears prepared from fresh surgically resected thyroid nodules.

[0294] Selection modeling using these seven proteins resulted in nuclear phosphoglycerate kinase 1 (PGK1) loss and nuclear Galectin-3 overexpression as the best combination for distinguishing TC from benign nodules. A computed Malignancy Score also accurately identified TC in benign and indeterminate nodules. Its efficacy was confirmed in FNAB cell blocks and cytosmears prepared from fresh surgical thyroid samples immediately after resection.

[0295] In this study we have shown that a combination of PGK1 and Galectin-3 using surgical tissues demonstrated a high efficacy for recognizing benign, malignant and indeterminate thyroid nodules which could improve surgical selection. These findings illustrate the potential clinical application of using the above method with FNAB.

[0296] Materials and Methods

[0297] Patient specimens

[0298] The same patient specimens, protocols and results from Example 2 were used. As mentioned above, 115 non-malignant thyroid tissues 53 benign non-neoplastic thyroid nodules and 62 follicular adenomas) and 114 thyroid cancer (TC) tissues were analyzed for protein expression.

[0299] The frequency of follicular carcinomas seen in our hospital is low; hence we could not include these in this analysis. However, our study included 33 follicular variants of PTC and 9 oncocytic (Hurthle cell variant of PTC); these cases often pose a challenge in FNAB diagnosis and IHC markers are needed to improve their diagnosis. Anaplastic carcinomas are rarely indeterminate on aspiration, nevertheless these constitute aggressive cancers and it is important to know the status of our biomarkers in these cases, hence these were included in our study. Fifty FNABs were collected from surgically resected fresh thyroid tissues using a 22 gauge needle in formalin, and used for preparation of FFPE cell blocks for IHC analysis. FNAB FFPE cell blocks were used to cut 4 µm sections; one section was stained with hematoxylin and eosin and serial sections were used for IHC. Twenty-six cytosmears were made from the FNAB taken from fresh tissues of thyroidectomy specimens and included the clinical index nodules as well as tissue distant from the nodule. Cells were fixed using Cytology Fixative spray (Leica Biosystems). One slide was stained with hematoxylin and eosin while the others were used for IHC. The pre-surgical FNAB cytology and surgical pathology obtained in this study cohort was compared to the IHC results for the FNAB FFPE and cytosmears obtained at surgery.

[0300] Immunohistochemical analysis in thyroid tissues

[0301] The IHC data from Example 2 were used in this study. However, surgical cytosmears used in this study were incubated in TBS-T (Tris-buffered saline with 0.025% Triton) for 5 min. No antigen retrieval treatment was performed for cytosmears.

[0302] The IHC analysis further included an immunofluorescence assay to determine sub-cellular expression of PGK1 and Galectin-3. The following procedure was followed on tissue samples:

[0303] Thin Preps of Thyroid Fine Needle Aspiration (FNAB) from benign or papillary thyroid carcinoma (PTC) patients were prepared in the Mount Sinai Hospital (MSH) Cytology Core facility. Slides were immersed in 95% ethanol for 10 minutes before these were washed three times with Washing Buffer [Tris-buffered saline pH 7.6 (TBS), 0.025% Triton X-100 (TX-100)] for 5 min. Cells were permeabilized in 0.1% TX-100 in TBS for 5 min. and blocked in TBS containing 0.1% TX-100 and 4% Normal Goat Serum (NGS, Vector Laboratories, S-100) or 125 µl/slide of Background Punisher™ blocking solution (Biocare Medical, Vector Labs, BP974L) for 30 minutes at room temperature. Slides were washed three times with Washing Buffer, and were immunostained with primary antibody in Suspension Buffer (TBS, 0.1% TX-100, 2% NGS) containing either anti-human Galectin 3 (clone B2C10, Santa Cruz Biotechnology, 1/100 dilution) or anti-human PGK1 (Santa Cruz Biotechnology, clone 14, 1/50 dilution) for 1 hour at room temperature. Slides were washed in Wash Buffer three times before these were incubated with TBS containing AF488-conjugated goat anti-mouse antibodies (A11029, 1/100 dilution, Life Technologies) for 1 hour at room temperature. Slides were washed three times in Washing Buffer and then mounted with 20 µl of ProLong Gold Antifade Reagent (Life Technologies). Slides were dried overnight in the dark. Images were taken the next day.

[0304] Evaluation of immunohistochemistry

[0305] The evaluation of IHC data from Example 2 was used in this study.

[0306] **Results**

[0307] Immunohistochemical analysis findings

[0308] The IHC analysis results from Example 2 were used in this study. Reference is therefore made to Table 3 and Figure 12 and the associated discussion above.

[0309] Figures 15-18 (described above) illustrate the results from the immunofluorescence assays.

[0310] Biomarker predictive values derived from surgical formalin -fixed paraffin embedded (FFPE) benign vs. malignant thyroid nodules

[0311] The predictive value of the biomarkers to distinguish TC from benign thyroid nodules (non-neoplastic nodules and follicular adenoma) was determined. The cases were divided into a Test set (n = 27 benign, 31 adenoma, 57 cancer) and a Validation set (n = 26 benign, 31 adenoma, 57 cancer) using random split-sample. With the exception of cytoplasmic Profilin-1 and cytoplasmic S100A6, all the biomarkers had sub-cellular compartmental nuclear expressions associated with TC (Table 9). Nuclear PGK1 emerged as the strongest predictor of cancer in comparison with non-malignant tissues [Test set: Odds ratio (OR) = 0.05 (95% CI = 0.01, 0.17), p < 0.0001, AUC = 0.93, Validation set: OR =

0.05 (95% CI = 0.01, 0.21), $p < 0.0001$, AUC = 0.96; Table 2] underscoring its potential clinical applicability. We hypothesized that a model developed from a panel of these biomarkers could be more predictive of cancer compared to nuclear PGK1 alone. To test this hypothesis, model selection under multiple imputed data was used (23) to achieve an optimal final Test set model of reduced nuclear PGK1 and overexpressed nuclear Galectin-3 (Table 9).

[0312] Table 9: Logistic regression analyses

Univariate	Test set (n= 27 benign, 31 adenoma, 57 cancer)			Validation set (n = 26 benign, 31 adenoma, 57 cancer)		
Biomarker	OR [95% CI]	p	AUC	OR [95% CI]	p	AUC
Benign (non-neoplastic nodules and follicular adenoma) vs. Cancer						
Cytoplasmic PTEN	0.65 [0.50, 0.84]	0.0009	0.73	0.62 [0.48, 0.80]	0.0002	0.74
Nuclear PTEN	0.38 [0.23, 0.65]	0.0003	0.83	0.31 [0.17, 0.56]	0.0001	0.89
Nuclear Profilin1	0.54 [0.42, 0.71]	< 0.0001	0.8	0.34 [0.22, 0.53]	< 0.0001	0.9
Nuclear S100A6	0.77 [0.63, 0.95]	0.0157	0.68	0.68 [0.53, 0.87]	0.0024	0.74
Nuclear Cyclin D1	1.65 [1.27, 2.15]	0.0002	0.77	1.66 [1.28, 2.15]	0.0001	0.73
Cytoplasmic Galectin-3	1.46 [1.22, 1.76]	< 0.0001	0.73	1.66 [1.34, 2.08]	< 0.0001	0.79
Nuclear Galectin-3	12.1 [3.49, 41.7]	0.0001	0.8	7.1 [1.98, 25.49]	0.0027	0.74
Cytoplasmic PGK1	0.31 [0.17, 0.57]	0.0002	0.79	0.24 [0.13, 0.44]	< 0.0001	0.85
Nuclear PGK1	0.05 [0.01, 0.17]	< 0.0001	0.93	0.05 [0.01, 0.21]	< 0.0001	0.96
Cytoplasmic PKM2	0.77 [0.62, 0.96]	0.0231	0.61	0.88 [0.71, 1.10]	0.2746	0.55
Nuclear PKM2	0.47 [0.32, 0.69]	0.0002	0.79	0.50 [0.35, 0.72]	0.0002	0.77
Multivariable analyses						
Benign (non-neoplastic nodules and follicular adenoma) vs. Cancer						
Nuclear Galectin-3	8.04 [0.96, 73.09]		0.05	3.38 [1.05, 14.32]		0.04
Nuclear PGK1	0.04 [0.01, 0.22]		0.0003	0.08 [0.02, 0.28]		0.0001
Model AUC	0.96			0.95		

[0313] Surgical FFPE Malignancy Score based discrimination of benign vs. malignant thyroid nodules

[0314] Based on the high predictive and discriminatory values nuclear PGK1 and nuclear Galectin-3 risk scores were used to differentiate TC from benign thyroid nodules. A risk score model was developed in the Test set, and an optimal cut-off value which maximizes the AUC was chosen. The risk score model based on regression estimates as weights is given as Malignancy Score = 19.92 + (2.128 x Nuclear Galectin-3 score) – (3.322 x Nuclear PGK1 score; the optimal cut-off was 0.86 (IHC

score of 5.67), and AUC of 0.94 (Table 10). Notably, 98% (specificity) of non-malignant tissues were correctly identified in the Test set with a sensitivity of 90%. The clinical applicability of this cut-off value was verified in the Validation set, as it achieved an AUC of 0.90, with sensitivity (80%), and specificity (99%) (Table 10).

[0315] Table 10: Clinical utility of nuclear PGK1 and nuclear Galectin-3 in identifying thyroid cancer from Benign thyroid nodules (non-neoplastic nodules and follicular adenoma)

	Sens.	Spec.	PPV	NPV	AUC	Size of High Risk Group
Benign (non-neoplastic nodules and follicular adenoma) vs. Cancer (PGK1 and Galectin-3)						
Test set	0.90	0.98	0.97	0.91	0.94	45 %
Validation set	0.80	0.99	0.99	0.84	0.90	39 %
FFPE Benign (non-neoplastic nodules and follicular adenoma) vs. Cancer -PGK1 and Galectin-3						
FNAB FFPE (n=50)	0.97	0.64	0.90	0.88	0.81	83.67%
Cytosmears Benign nodules vs. Cancer- PGK1						
Cytosmears (n=26)	1.00	0.90	0.94	1.00	0.95	65%

[0316] Optimal cut-off: Nuclear PGK1= 5.6; Nuclear Galectin-3 = 1. Benign (non-neoplastic nodules and follicular adenoma) vs. Cancer: Malignancy Score = $19.92 + (2.128 \times \text{Nuclear Galectin-3 score}) - (3.322 \times \text{Nuclear PGK1 score})$.

[0317] Biomarker Predictive values for distinguishing benign from malignant nodules in surgical FNAB-FFPE cell blocks

[0318] To test the efficacy of PGK1 and Galectin-3 for identifying TC from benign thyroid nodules (non-neoplastic nodules and follicular adenoma), we prepared FNAB FFPE cell blocks from 50 fresh surgically resected thyroid nodules, and performed IHC for these proteins and correlated the FNAB findings with surgical pathology diagnosis. Representative photomicrographs depicting immunostaining for PGK1 and Galectin-3 in FNAB FFPE cell blocks are shown in Figure 13. Based on the PGK1 Nuclear IHC score positivity cut off value of < 5.6 for TC, diagnosis was made and compared to the surgical diagnosis for each case to assess concordance between surgical pathology diagnosis and biomarker based diagnosis. The PGK1 IHC based diagnosis matched surgical diagnosis in 34/50 (68%) of FNAB cases (Tables 11 and 12). Based on the nuclear Galectin-3 IHC score positivity cut off value of > 1 for TC and < 1 for benign tissues IHC diagnosis matched the surgical diagnosis in 41/50 (82%) cases (Tables 11 and 12). Importantly, using a combination of PGK1 and Galectin-3 all the 50 FNAB cases were classified accurately (Tables 11 and 12). Among the 9 cases that were non-diagnostic in pre-surgical FNAB cytological diagnosis, our biomarkers results classified 3 cases as adenomatous nodules and 6 cases as PTC including one

microcarcinoma and the surgical diagnoses were concordant with the biomarker results in all the 9 cases (Tables 11 and 12). Notably, for all the 19 indeterminate cases in pre-surgical FNAB cytological diagnosis (which included 13 Atypia of undetermined significance (AUS)/FLUS, 2 Follicular Neoplasm (FN)/ Suspicious for a Follicular Neoplasm (SFN) and 4 suspicious for malignancy), the biomarker diagnosis were concordant with the surgical diagnosis (Tables 11 and 12). Additional biomarker diagnoses identified 2 follicular carcinomas which had been classified as FN/SFN and suspicious for malignancy based on pre-surgical FNAB cytology (Table 12).

[0319] Table 11: Correlation of Molecular diagnosis with surgical diagnosis in FNAB FFPE cell blocks and Cytosmears

Pre-surgical Diagnosis Bethesda Category FNAB FFPE	Number of Cases (N – 50)	PGK-1 Molecular diagnostic Concordance with Surgical diagnosis YES (n)	Galectin 3 Molecular Diagnostic Concordance with Surgical diagnosis YES (n)	Combined markers Molecular Diagnostic Concordance with Surgical diagnosis YES (n)
I, Nondiagnostic	9	8	5	9
II, Benign	15	9	12	15
III, AUS/FLUS	13	6	12	13
IV, FNS/SFN	2	2	2	2
V, Suspicious for malignancy	4	4	3	4
VI, malignant	7	5	7	7
Cytosmears	N - 26	PGK-1 Diagnostic Concordance with Surgical diagnosis YES (n)		
Cancer	13	13		
Benign	13	12		

[0320] SD, surgical diagnosis. Patients included in FFPE FNA: Age, Range 25-78 years, Median 53 years; male -12, female – 88 cases. Patients included in Cytosmears Age, Range 25-77 years, Median 48 years; male - 6, female – 20 cases.

[0321] Table 12: FNAB FFPE Cell Blocks Molecular Diagnostic Concordance with Surgical Diagnosis

Bethesda Category	Pre-surgical Cytological diagnosis	Surgical diagnosis (SD)	PGK-1 Diagnostic Concordance with SD	Galectin-3 Diagnostic Concordance with SD	Combined Diagnostic Concordance with SD
I	nondiagnostic	Papillary Thyroid Carcinoma	YES	NO	YES
	nondiagnostic	Adenomatous nodule	NO	YES	YES
	nondiagnostic	Papillary Thyroid Carcinoma	YES	YES	YES
	nondiagnostic	Papillary Thyroid Carcinoma	YES	YES	YES
	nondiagnostic	Papillary Microcarcinoma	YES	NO	YES

	nondiagnostic	Papillary Thyroid Carcinoma	YES	NO	YES
	nondiagnostic	Multiple hyperplastic adenomatous colloid nodules	YES	YES	YES
	nondiagnostic	Nodular Hyperplasia with Adenomatous Nodule	YES	YES	YES
	nondiagnostic	Papillary Thyroid Carcinoma	YES	NO	YES
II	benign	Papillary Thyroid Carcinoma	YES	YES	YES
	benign	Papillary Microcarcinoma	YES	NO	YES
	benign	Papillary Microcarcinoma	YES	NO	YES
	benign	Nodular Follicular Thyroid Disease	YES	YES	YES
	benign	Benign	YES	YES	YES
	benign	Benign	YES	YES	YES
	benign	Papillary Thyroid Carcinoma	YES	NO	YES
	benign	Multiple hyperplastic adenomatous colloid nodules	NO	YES	YES
	benign	Multinodular Colloid Goiter with Degenerative Features	NO	YES	YES
	benign	Multiple hyperplastic adenomatous colloid nodules	NO	YES	YES
	benign	Multiple hyperplastic adenomatous nodules	NO	YES	YES
	benign	Benign	NO	YES	YES
	benign	Nodular Hyperplasia	NO	YES	YES
	benign	Papillary Microcarcinoma	YES	YES	YES
	benign	Papillary Microcarcinoma** Benign region taken	YES	YES	YES
III	AUS/FLUS	Papillary Thyroid Carcinoma	YES	YES	YES
	AUS/FLUS	Papillary Thyroid Carcinoma	YES	YES	YES
	AUS/FLUS	Adenomatous hyperplastic nodule	NO	YES	YES
	AUS/FLUS	Papillary Thyroid Carcinoma** Benign region taken	NO	YES	YES
	AUS/FLUS	Papillary Thyroid Carcinoma	NO	YES	YES
	AUS/FLUS	Lymphocytic thyroiditis (Hashimotos)	NO	YES	YES
	AUS/FLUS	Papillary Thyroid Carcinoma	YES	NO	YES
	AUS/FLUS	Papillary Microcarcinoma	YES	YES	YES

	AUS/FLUS	Multiple hyperplastic adenomatous colloid nodules	NO	YES	YES
	AUS/FLUS	Papillary Thyroid Carcinoma	YES	YES	YES
	AUS/FLUS	Papillary Thyroid Carcinoma	YES	YES	YES
	AUS/FLUS	Nodular Hyperplasia with Adenomatous Nodule	NO	YES	YES
	AUS/FLUS	Multiple colloid nodules	NO	YES	YES
IV	FNS/SFN	Follicular Carcinoma	YES	YES	YES
	FNS/SFN	Multiple Hyperplastic and Colloid Nodules	YES	YES	YES
V	Suspicious for malignancy	Papillary Thyroid Carcinoma	YES	YES	YES
	Suspicious for malignancy	Papillary Thyroid Carcinoma	YES	NO	YES
	Suspicious for malignancy	Follicular Carcinoma	YES	YES	YES
	Suspicious for malignancy	Papillary Thyroid Carcinoma	YES	YES	YES
VI	malignant	Papillary Thyroid Carcinoma	YES	YES	YES
	malignant	Papillary Thyroid Carcinoma	YES	YES	YES
	malignant	Medullary Thyroid Carcinoma	YES	YES	YES
	malignant	Papillary Thyroid Carcinoma	YES	YES	YES
	malignant	Papillary Thyroid Carcinoma	YES	YES	YES
	malignant	Papillary Thyroid Carcinoma	NO	YES	YES
	malignant	Papillary Thyroid Carcinoma	NO	YES	YES

1

2 **[0322]** Efficacy of biomarkers for identifying thyroid cancer using surgical cytosemears

3 **[0323]** The efficacy of PGK1 and Galectin-3 for detecting TC using cytosemears obtained at surgery
4 was evaluated in 26 cases. Representative photomicrographs depicting immunostaining for PGK1
5 and Galectin-3 in cytosemears is shown in Figure 14. Based on the PGK1 Nuclear IHC score positivity
6 cut off value of < 4 for TC and > 4 for benign cases IHC based diagnosis was made and compared to
7 the surgical diagnosis for each case to assess the degree of correspondence between the surgical
8 pathology diagnosis and biomarker based diagnosis. The PGK1 IHC based diagnosis matched the
9 surgical diagnosis in 25/26 (96%) of cytosemears (Tables 11 and 13).

10

11 **[0324]** **Table 13. FNAB Cytosemears Molecular Diagnostic Concordance with Tissue Source**

Serial #	Case	Surgical diagnosis (SD)	Tissue Source	Concordance with TS
S1	1	Papillary Microcarcinoma	benign	YES

S2		Papillary Microcarcinoma	index nodule	YES
S3	2	Papillary Thyroid Carcinoma	benign	YES
S4		Papillary Thyroid Carcinoma	index nodule	YES
S5	3	Papillary Thyroid Carcinoma	index nodule	YES
S6		Papillary Thyroid Carcinoma	index nodule	YES
S7	4	Medullary Thyroid Carcinoma	benign	YES
S8		Medullary Thyroid Carcinoma	index nodule	YES
S9	5	Papillary Thyroid Carcinoma	index nodule	YES
S10		Papillary Thyroid Carcinoma	index nodule	YES
S11	6	Papillary Thyroid Carcinoma	benign	YES
S12		Papillary Thyroid Carcinoma	index nodule	YES
S13	7	Papillary Thyroid Carcinoma	benign	YES
S14		Papillary Thyroid Carcinoma	index nodule	YES
S15	8	Papillary Thyroid Carcinoma	benign	YES
S16		Papillary Thyroid Carcinoma	index nodule	YES
S17	9	Papillary Thyroid Carcinoma	benign	YES
S18		Papillary Thyroid Carcinoma	index nodule	YES
S19	10	Papillary Thyroid Carcinoma	index nodule	YES
S20		Papillary Thyroid Carcinoma	index nodule	YES
S21	11	Papillary Thyroid Carcinoma	benign	YES
S22		Papillary Thyroid Carcinoma	index nodule	YES
S23	12	Papillary Thyroid Carcinoma	benign	YES
S24		Papillary Thyroid Carcinoma	index nodule	YES
S25	13	Papillary Thyroid Carcinoma	index nodule	YES
S26		Papillary Thyroid Carcinoma	benign	NO

1

2 **[0325] Discussion**

3 **[0326]** In Example 2 and in our previously studies [47, 49] we have investigated the functional
4 relevance of seven proteins, PGK1, PKM2, Cyclin D1, Galectin-3, PTEN, S100A6, and Profilin-1, in
5 human cancers, particularly in thyroid cancer (TC). The current study evaluated the expression of a
6 panel of these proteins in human thyroid tissues, and examined their potential to serve as a diagnostic
7 tool for distinguishing TC from benign thyroid nodules (non-neoplastic nodules and follicular
8 adenoma). To our knowledge, this is the first report to detect the expression of Profilin-1 in thyroid
9 tissues as a cancer biomarker.

10 **[0327]** In the present study, we found that using PGK1 and Galectin-3 proteins together in a panel
11 provides an effective biomarker combination for distinguishing benign thyroid nodules from TC in a
12 clinical setting. The malignancy score based on regression estimates as weights resulted in AUC of
13 0.94 with high specificity (98%), PPV (97%), sensitivity (90%) and NPV (91%) in a Test set and similar
14 performance in a validation set (AUC of 0.90, with high specificity (99%), PPV (99%), sensitivity
15 (80%) and NPV (84%)).

16 **[0328]** Importantly, all the surgical FNAB FFPE cell blocks were accurately classified using a
17 combination of nuclear PGK1 and nuclear Galectin-3. Nine of 50 cases were non-diagnostic in pre-
18 surgical FNAB cytology; the protein biomarker analysis classified 3 cases as adenomatous nodules
19 and 6 cases as papillary TC including one microcarcinoma and these findings were supported by the
20 surgical pathology. Further, 19 indeterminate cases in pre-surgical FNAB cytology (13 (AUS)/FLUS, 2

1 FN/SFN, and 4 suspicious for malignancy), were also accurately classified based on our molecular
2 diagnosis concordant with their surgical pathology. Importantly, IHC biomarker analyses identified 2
3 follicular carcinomas which had been classified as FN/SFN and suspicious for malignancy based on
4 pre-surgical FNAB cytology.

5 **[0329]** Notably, we found that 96% of cytosmears could be accurately classified using nuclear PGK1
6 alone, obviating the use of Galectin-3 in these cases.

7 **[0330]** These preliminary data from direct FNAB from the surgical thyroidectomy specimens has
8 overcome the potential sampling errors.

9 **[0331]** The results from the present study show that the use of the described methods can improve
10 pre-operative surgical selection particularly in patients with FNAB cytology showing features of atypia,
11 suspicious for malignancy or indeterminate findings.

12 **[0332]** In conclusion, IHC analyses of our selected panel of seven proteins on archived surgical
13 tissues showed differential subcellular localization between benign and malignant thyroid tissues. In
14 depth data analyses suggested a combination of nuclear PGK1 loss alone or in combination with an
15 overexpression of nuclear Galectin-3 can be used to differentiate benign thyroid nodules from overt
16 and indeterminate TC with high sensitivity and specificity. These findings show the potential utility of
17 these proteins in pre-surgical FNABs or cytosmears as a diagnostic strategy to reduce the number of
18 indeterminate biopsy results and improve surgical selection. This conclusion can also be extended to
19 other non-surgical sampling techniques, such as core biopsies etc. This is of paramount importance in
20 not only avoiding unnecessary surgery on benign nodules, with its associated morbidity and other
21 risks, but also in reducing healthcare costs and improving selection of those nodules at risk for
22 malignancy.

23
24 **[0333]** Although the above description includes reference to certain specific embodiments, various
25 modifications thereof will be apparent to those skilled in the art. Any examples provided herein are
26 included solely for the purpose of illustration and are not intended to be limiting in any way. Any
27 drawings provided herein are solely for the purpose of illustrating various aspects of the description
28 and are not intended to be limiting in any way. The scope of the claims appended hereto should not
29 be limited by the preferred embodiments set forth in the above description, but should be given the
30 broadest interpretation consistent with the present specification as a whole. The disclosures of all
31 prior art recited herein are incorporated herein by reference in their entirety.

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WE CLAIM:

1. A method for diagnosing thyroid cancer, or for differentiating malignant or pre-malignant thyroid tissue from non-malignant thyroid tissue in a subject, the method comprising:
 - comparing a detected amount of PGK1 in a thyroid tissue sample from the subject with a control amount of PGK1 in non-cancerous tissue;
 - and diagnosing thyroid cancer or identifying malignant thyroid tissue in the subject when the detected amount of PGK1 is less than the control amount.
2. The method of claim 1, wherein the amount of PGK1 in the thyroid tissue sample is detected by contacting the sample with a binding agent that specifically binds to PGK1 and the amount of PGK1 in the sample comprises the amount of PGK1 that binds to the binding agent.
3. The method of claim 2, wherein the binding agent is an antibody and/or includes a detectable label.
4. The method of claim 1 or 2, wherein the amount of PGK1 in the thyroid tissue sample is detected by immunohistochemical analysis.
5. The method of any one of claims 1 to 4, wherein the non-malignant thyroid tissue is benign tissue or an adenoma.
6. The method of any one of claims 1 to 5, wherein the amount of PGK1 is the amount of nuclear or cytoplasmic PGK1.
7. The method of any one of claims 1 to 6, wherein the thyroid tissue sample is a fine needle aspirate biopsy, a core biopsy or a cytospin.
8. A method for diagnosing thyroid cancer, or for differentiating malignant or pre-malignant thyroid tissue from non-malignant thyroid tissue in a subject, the method comprising:
 - comparing detected amounts of PGK1 and Galectin-3 in a thyroid tissue sample from the subject with control amounts of PGK1 and Galectin-3 in non-cancerous tissue;
 - and diagnosing thyroid cancer or identifying malignant thyroid tissue in the subject when the detected amount of PGK1 is less than the control amount of PGK1 and the detected amount of Galectin-3 is greater than the control amount of Galectin-3.
9. The method of claim 8, wherein the amounts of PGK1 and Galectin-3 in the thyroid tissue samples are detected by contacting the sample with first and second binding agents, wherein the first binding agent specifically binds to PGK1 and the second binding agent specifically binds to Galectin-

3, and wherein the amount of PGK1 in the sample comprises the amount of PGK1 that binds to the first binding agent and the amount of Galectin-3 in the sample comprises the amount of Galectin-3 that binds to the second binding agent.

10. The method of claim 9, wherein first and second binding agents are antibodies and/or include respective detectable labels.

11. The method of claim 9 or 10, wherein the amounts of PGK1 and Galectin-3 in the thyroid tissue sample are detected by immunohistochemical analysis.

12. The method of any one of claims 8 to 11, wherein the non-malignant thyroid tissue is benign tissue or an adenoma.

13. The method of any one of claims 8 to 12, wherein the amounts of PGK1 and Galectin-3 are the amounts of nuclear or cytoplasmic PGK1 and Galectin-3.

14. The method of any one of claims 8 to 13, wherein the thyroid tissue sample is a fine needle aspirate biopsy, a core biopsy or a cytospin smear.

15. A method for diagnosing thyroid cancer, or for differentiating malignant or pre-malignant thyroid tissue from non-malignant thyroid tissue in a subject, the method comprising:

- comparing detected amounts of two or more biomarkers in a thyroid tissue sample from the subject with control amounts of the biomarkers in non-cancerous tissue, the biomarkers comprising PGK1, PKM2, PTEN, Profilin-1, Galectin-3 and Cyclin D1;
- and diagnosing thyroid cancer or identifying malignant thyroid tissue in the subject when (i) the detected amounts of PGK1, PKM2, PTEN, Profilin-1 or S100A6 are less than the respective control amounts and/or (ii) the detected amounts of Cyclin D1 or Galectin-3 are greater than the respective control amounts.

16. The method of claim 15, wherein the amounts of the two or more biomarkers in the thyroid tissue sample are detected by contacting the sample with binding agents that specifically bind to PGK1, PKM2, PTEN, Profilin-1, S100A6, Galectin-3 and/or Cyclin D1 and the amounts of PGK1, PKM2, PTEN, Profilin-1, S100A6, Galectin-3 and/or Cyclin D1 in the sample comprise the amounts of PGK1, PKM2, PTEN, Profilin-1, S100A6, Galectin-3 and/or Cyclin D1 that bind to the respective binding agents.

17. The method of claim 16, wherein binding agents are antibodies and/or include detectable labels.

18. The method of claim 16 or 17, wherein the amounts of the two or more biomarkers in the thyroid tissue sample is detected by immunohistochemical analysis.
19. The method of any one of claims 15 to 18, wherein the non-malignant thyroid tissue is benign tissue or an adenoma.
20. The method of any one of claims 15 to 19, wherein the amounts of PGK1, PKM2, PTEN, Profilin-1, S100A6, Galectin-3 and/or Cyclin D1 are the amounts of nuclear or cytoplasmic PGK1, PKM2, PTEN, Profilin-1, S100A6, Galectin-3 and/or Cyclin D1.
21. The method of any one of claims 15 to 20, wherein the thyroid tissue sample is a fine needle aspirate biopsy, a core biopsy or a cytospin.
22. A method for diagnosing thyroid cancer, or for differentiating malignant or pre-malignant thyroid tissue from non-malignant thyroid tissue in a subject, the method comprising:
- comparing an amount of Profilin-1 detected in a thyroid tissue sample from the subject with a control amount of Profilin-1 in non-cancerous tissue;
 - and diagnosing thyroid cancer or identifying malignant thyroid tissue in the subject when the detected amount of Profilin-1 is less than the control amount.
23. The method of claim 22, wherein the amount of Profilin-1 in the thyroid tissue sample is detected by contacting the sample with a binding agent that specifically binds to Profilin-1 and the amount of Profilin-1 in the sample comprises the amount of Profilin-1 that binds to the binding agent.
24. The method of claim 23, wherein binding agent is an antibody and/or includes a detectable label.
25. The method of claim 23 or 24, wherein the amount of Profilin-1 in the thyroid tissue sample is detected by immunohistochemical analysis.
26. The method of any one of claims 22 to 25, wherein the amount of Profilin-1 is the amount of nuclear or cytoplasmic Profilin-1.
27. The method of any one of claims 22 to 26, wherein the thyroid tissue sample is a fine needle aspirate biopsy, a core biopsy or a cytospin.

Fig.1

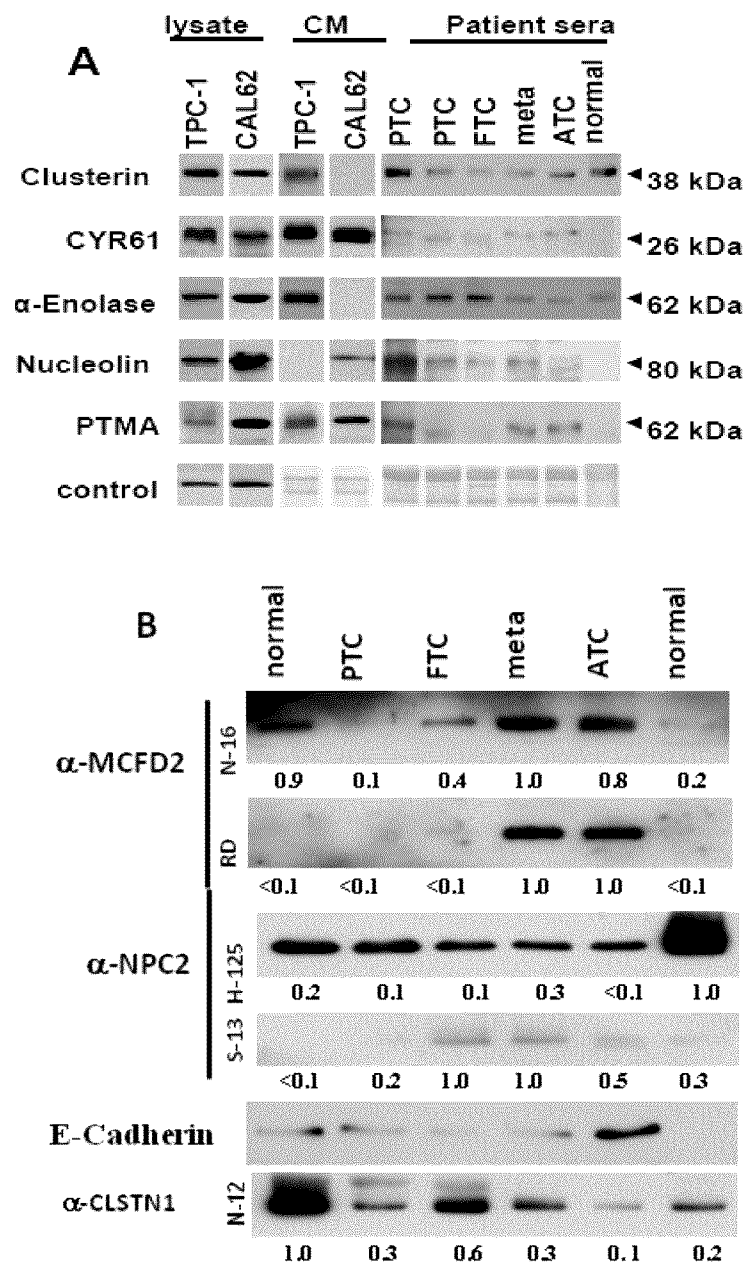
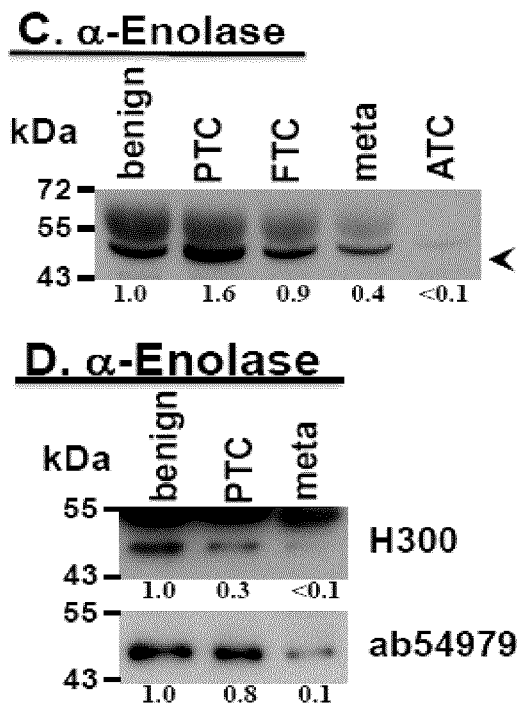


Fig.1 (Cont'd)



α -Enolase

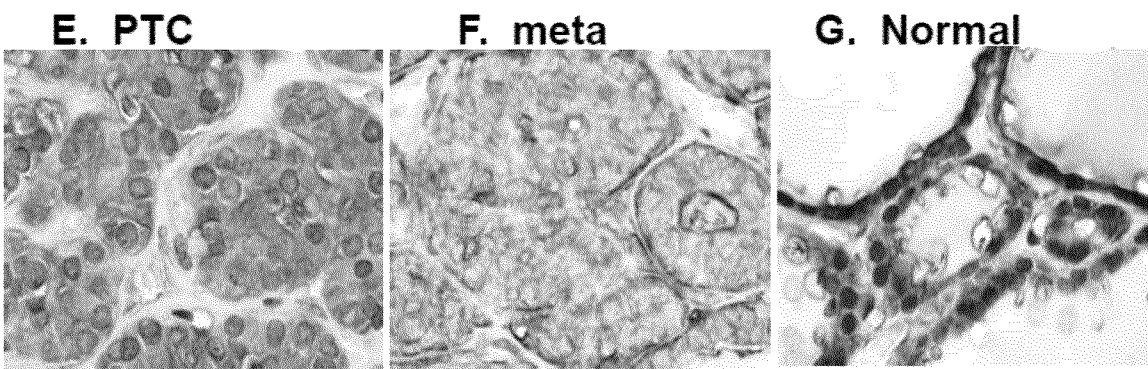


Fig. 2

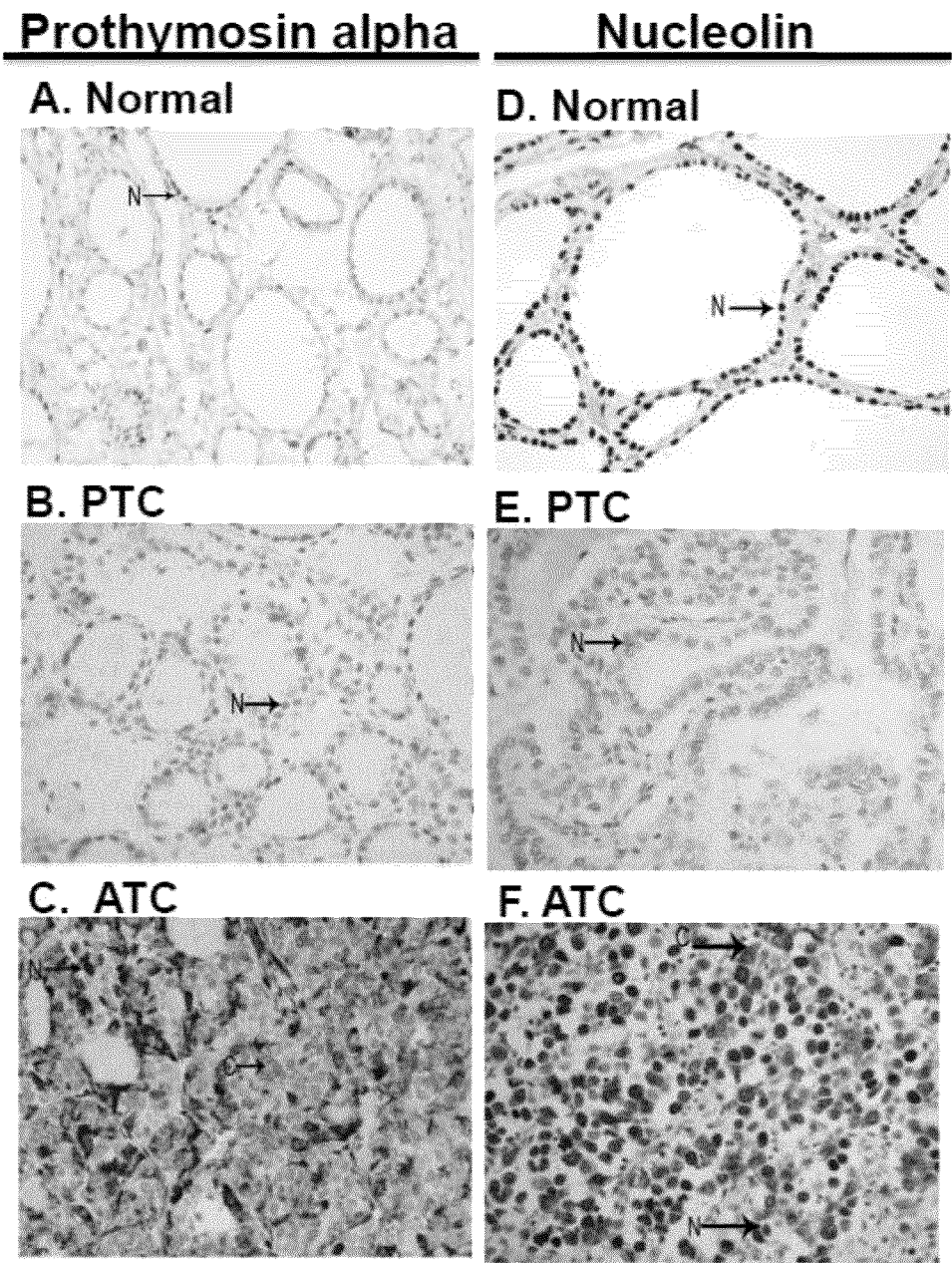
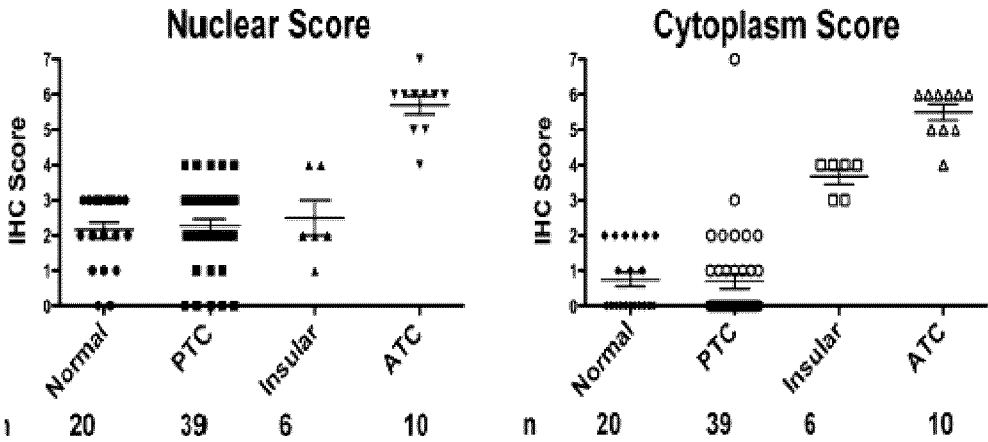


Fig.2 (Cont'd)

G. PTMA



H. Nucleolin

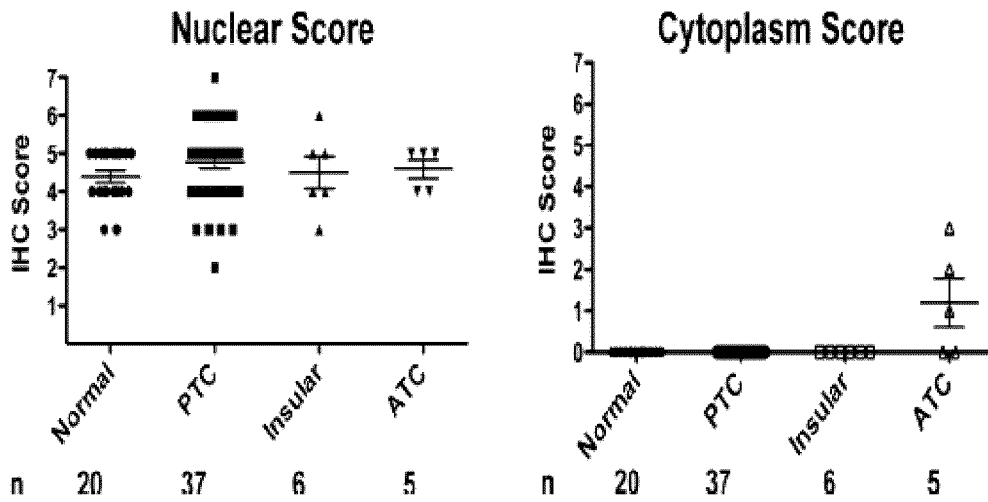


Fig.3

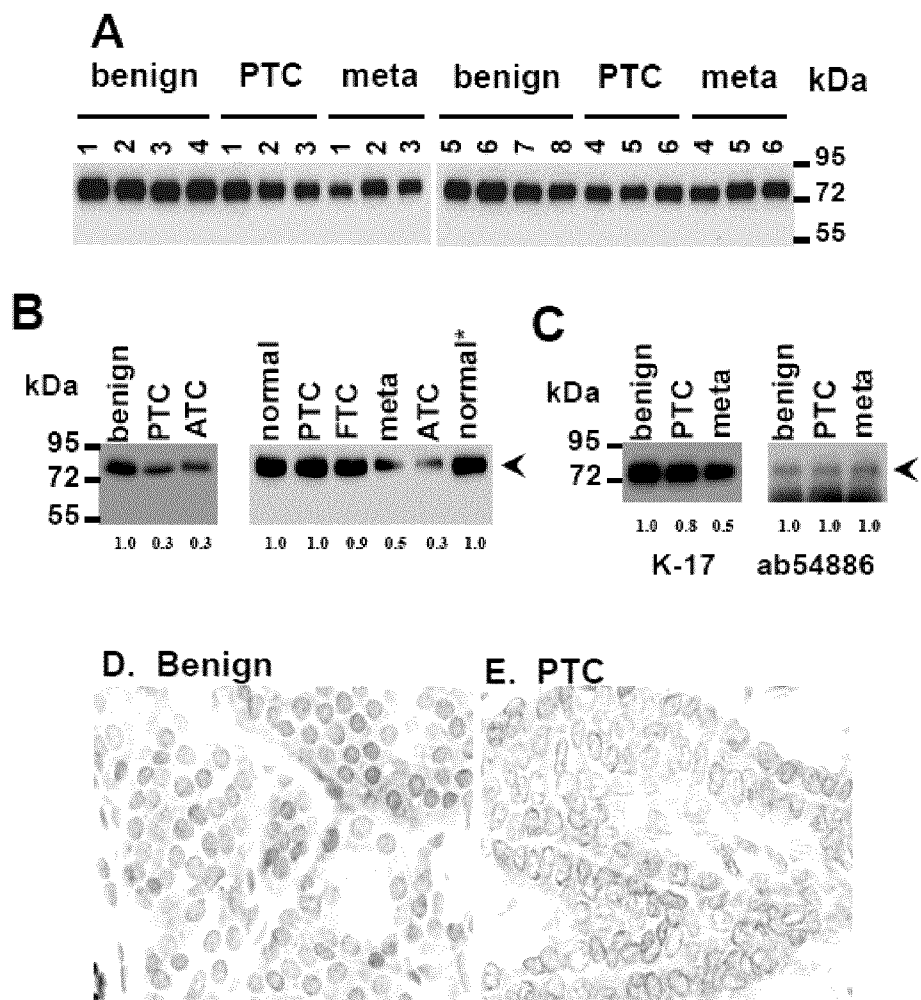


Fig.3 (Cont'd)

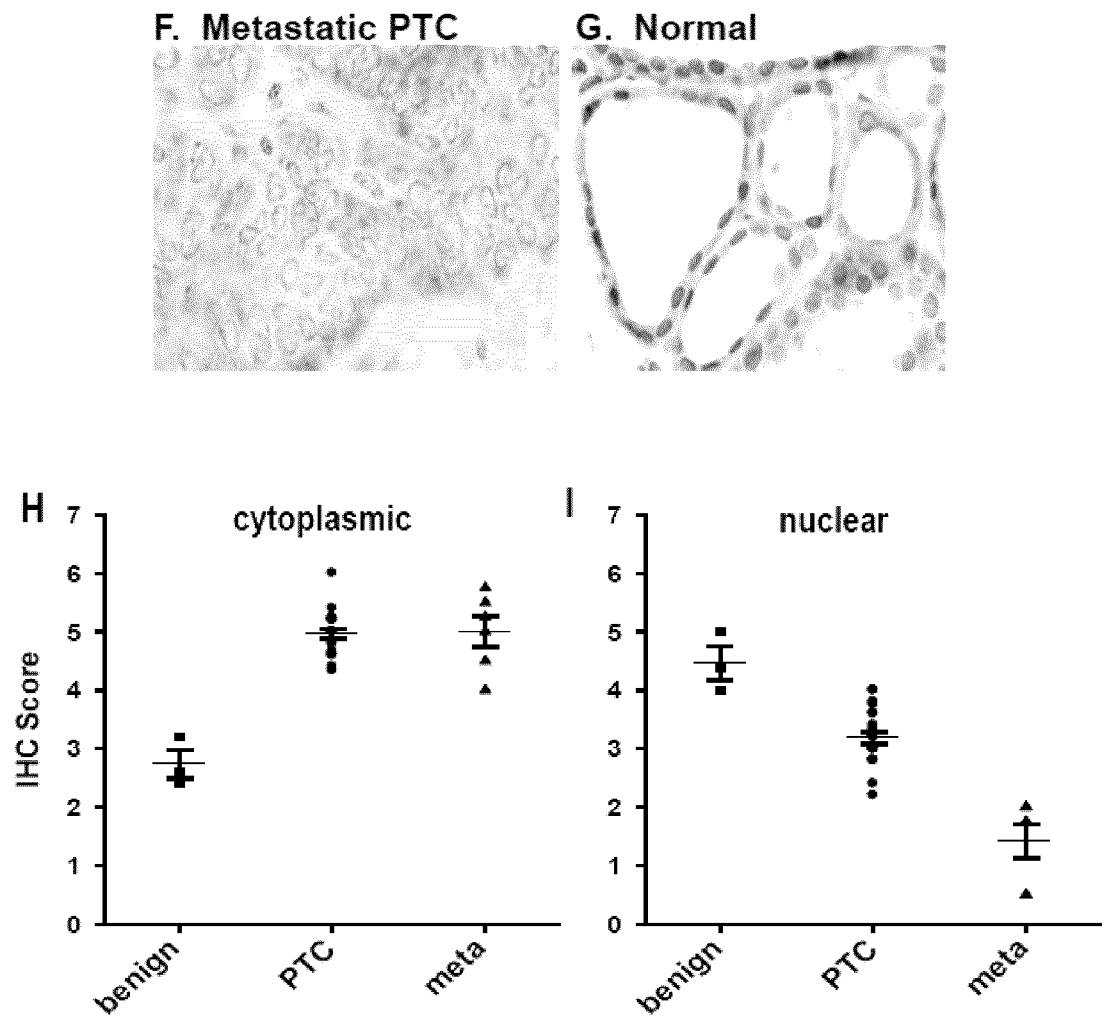


Fig.4

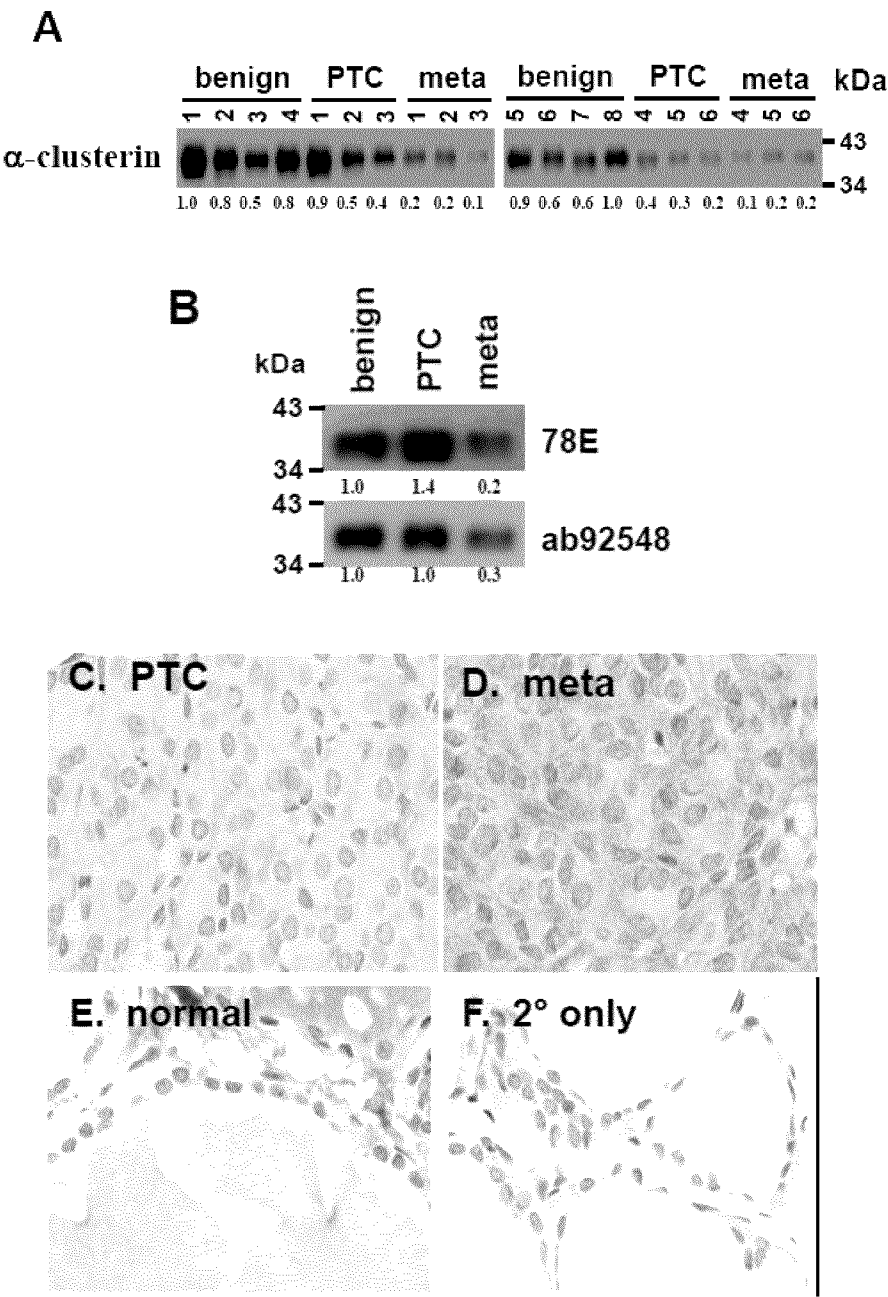


Fig.4 (Cont'd)

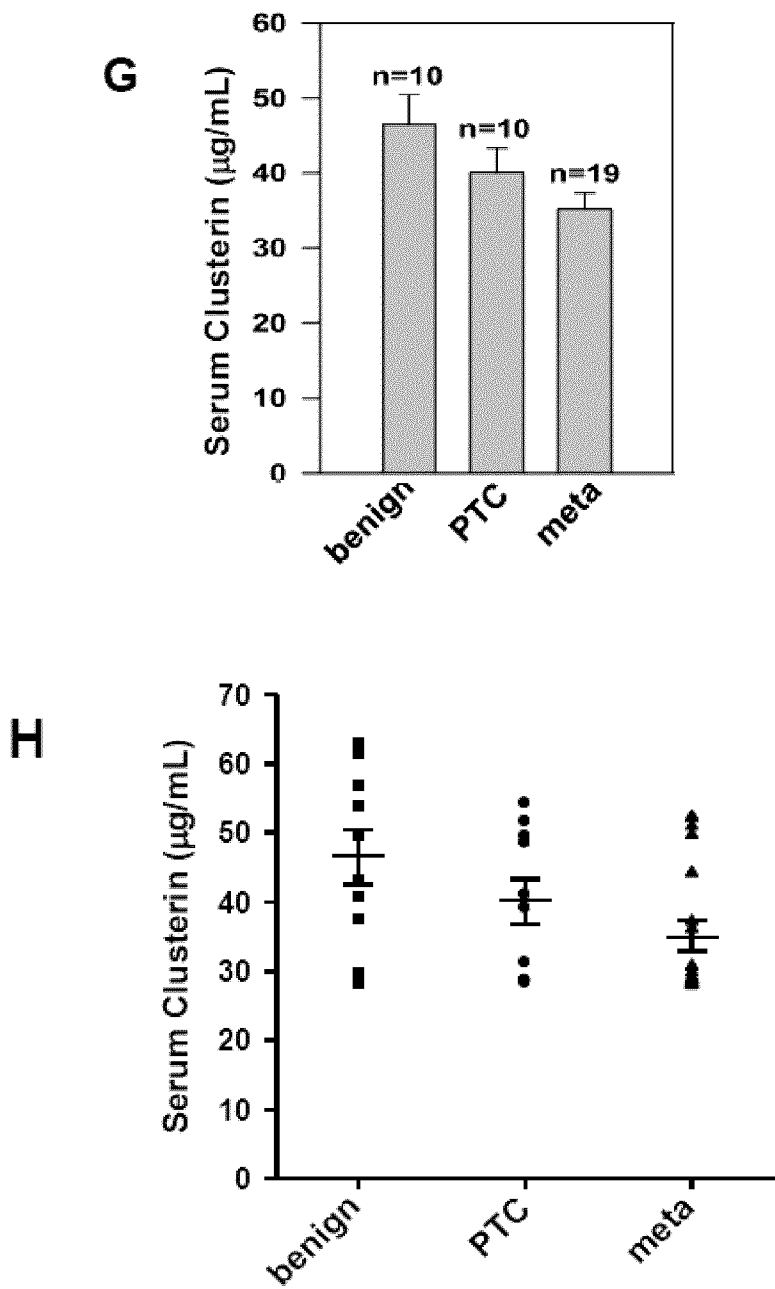


Fig.5

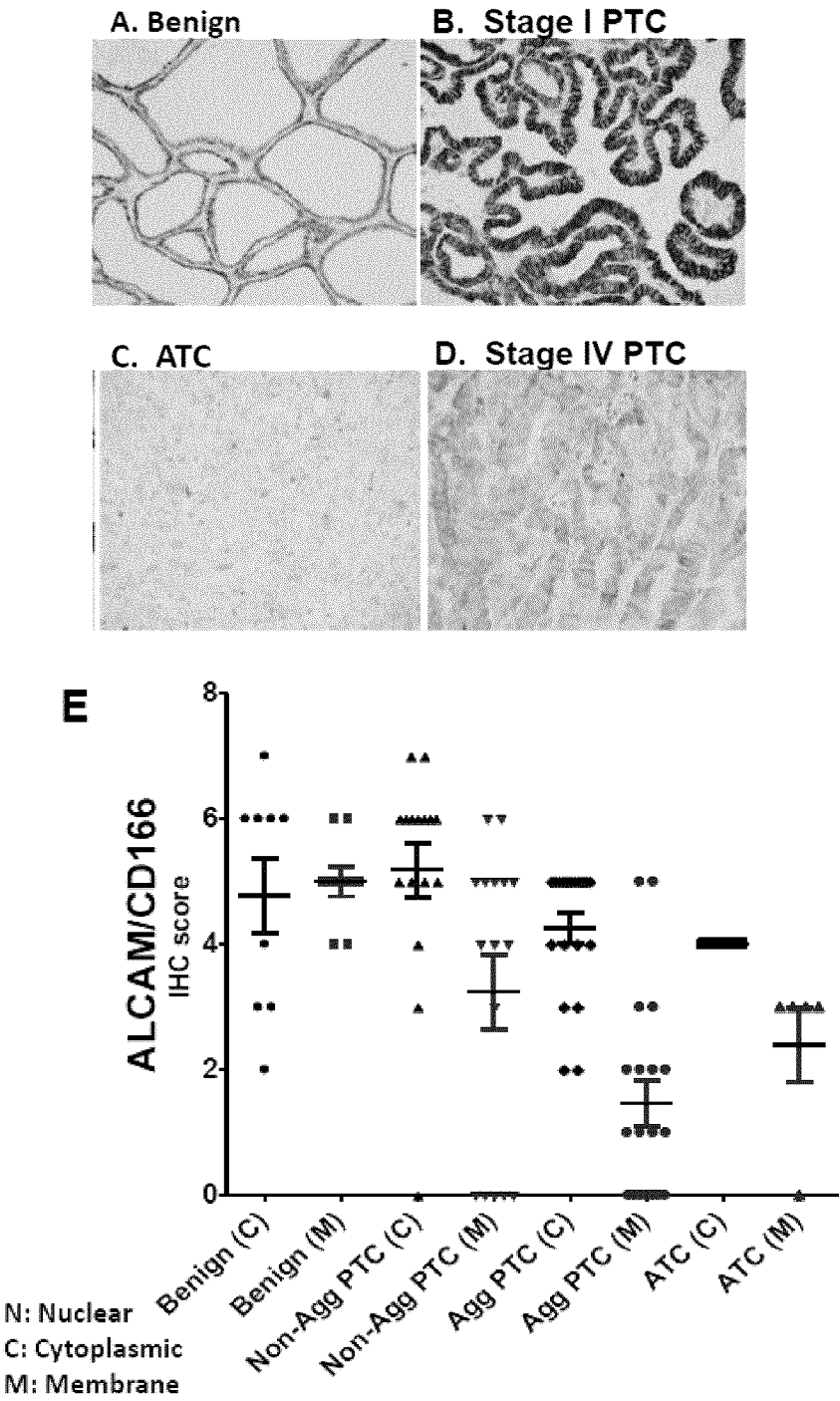


Fig.5 (Cont'd)

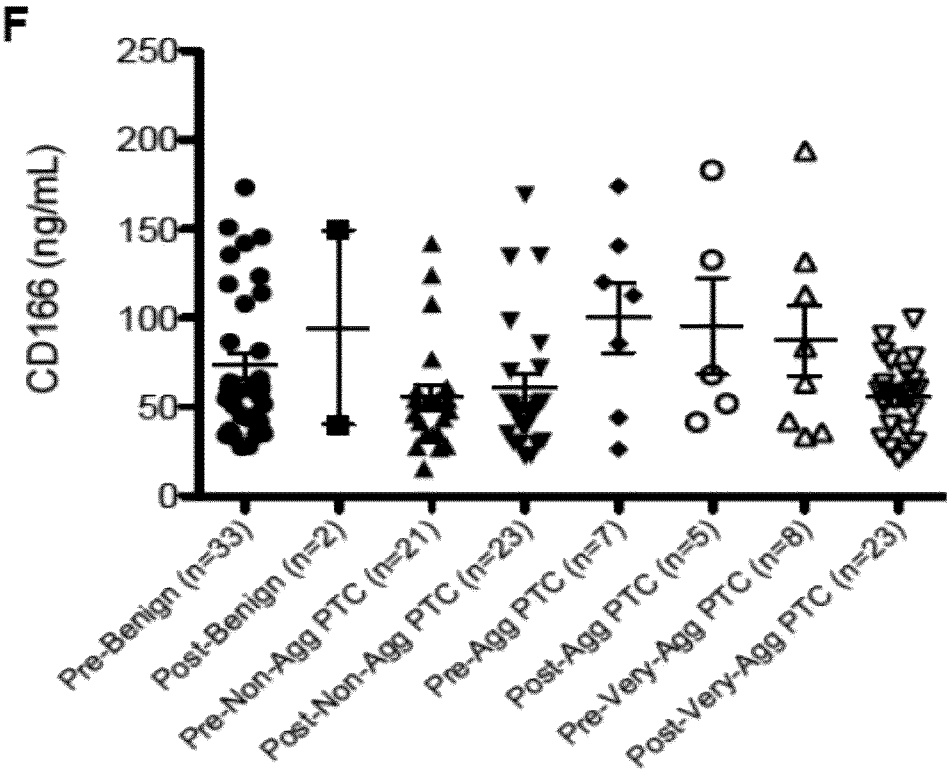


Fig. 6

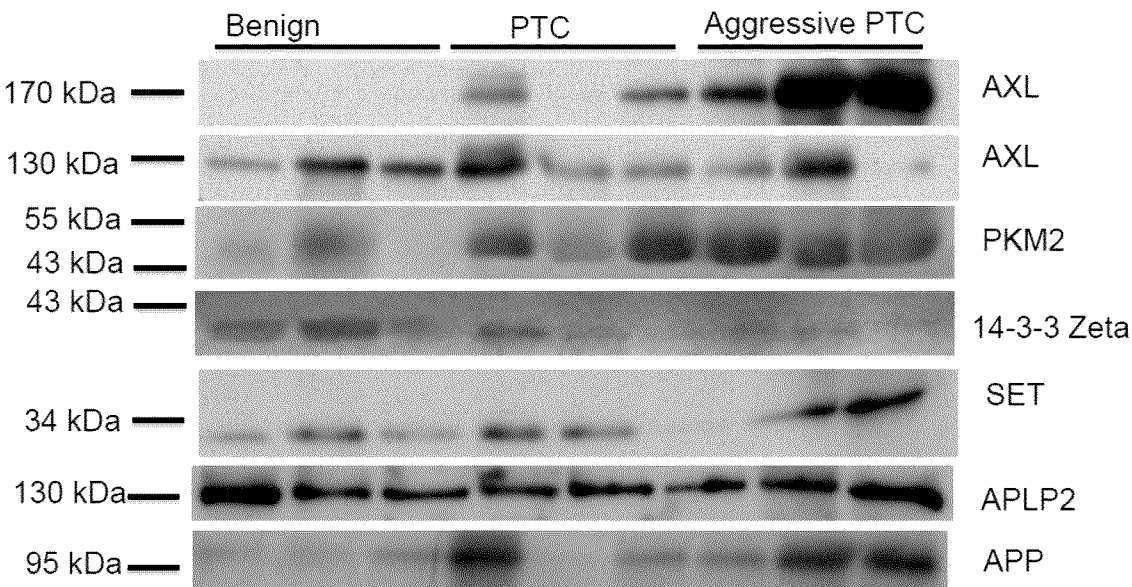


Fig.7

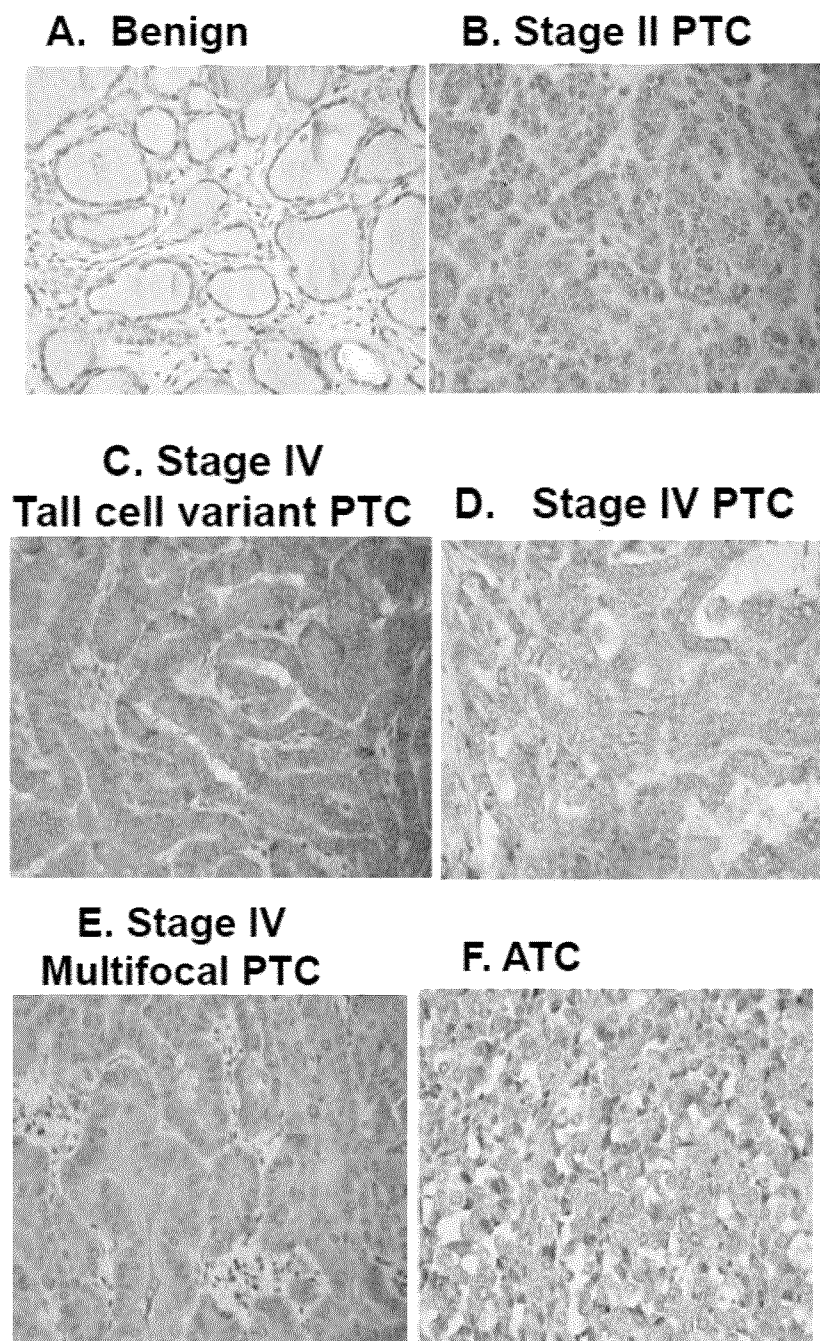


Fig.7 (Cont'd)

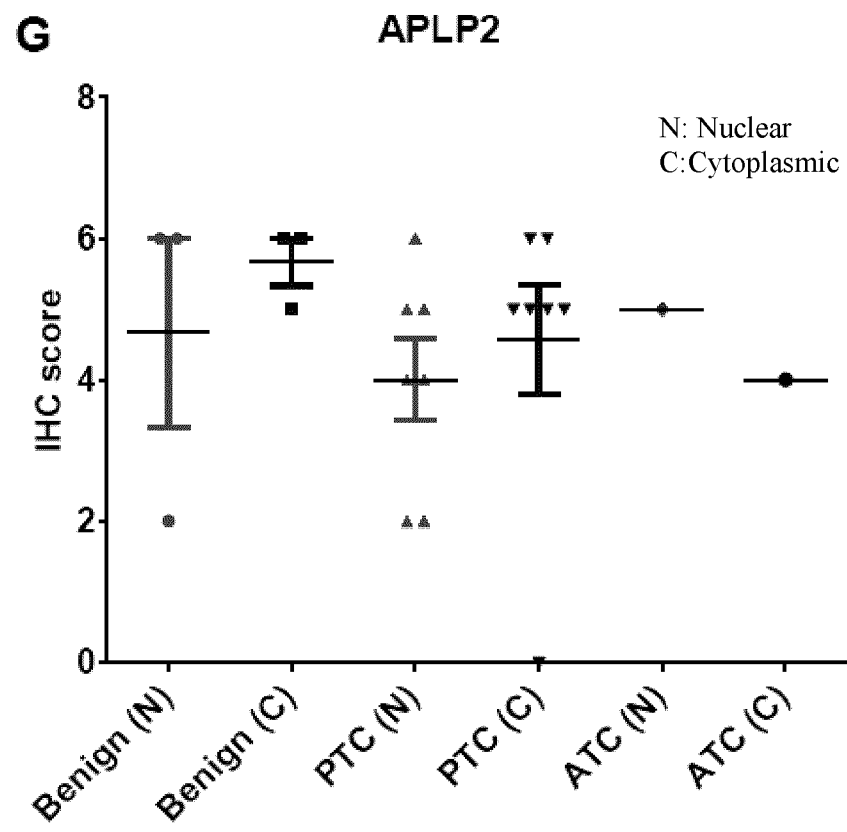


Fig. 8

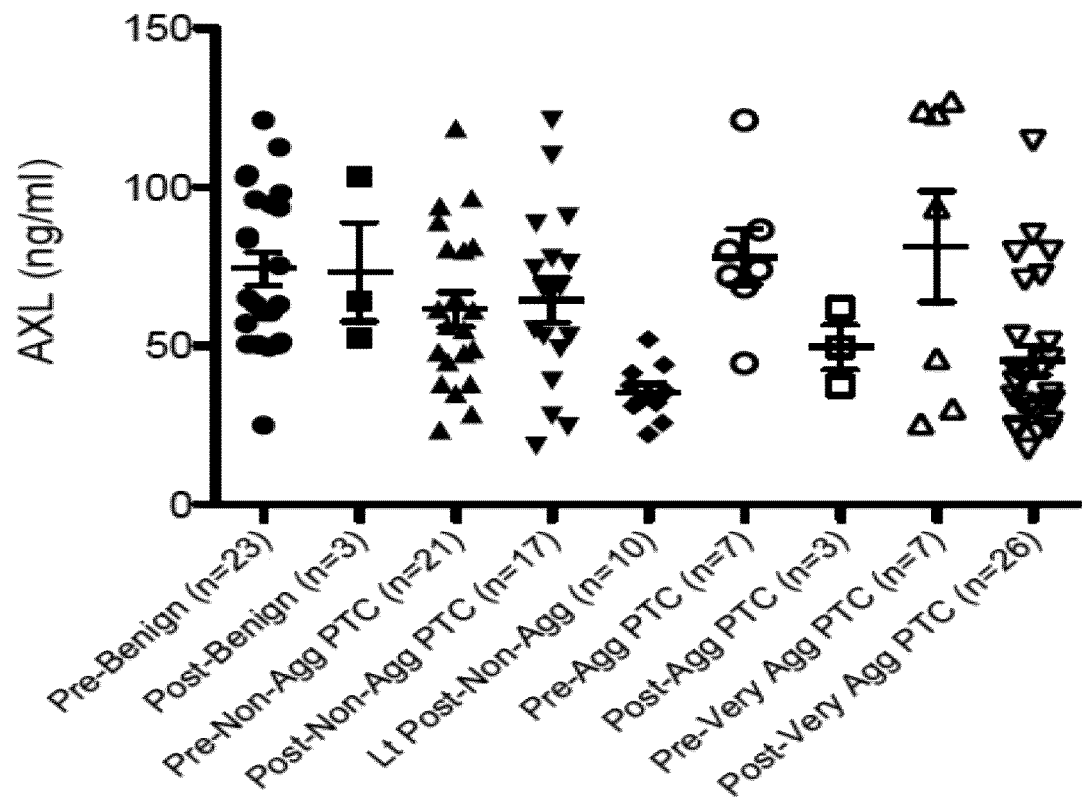


Fig.9

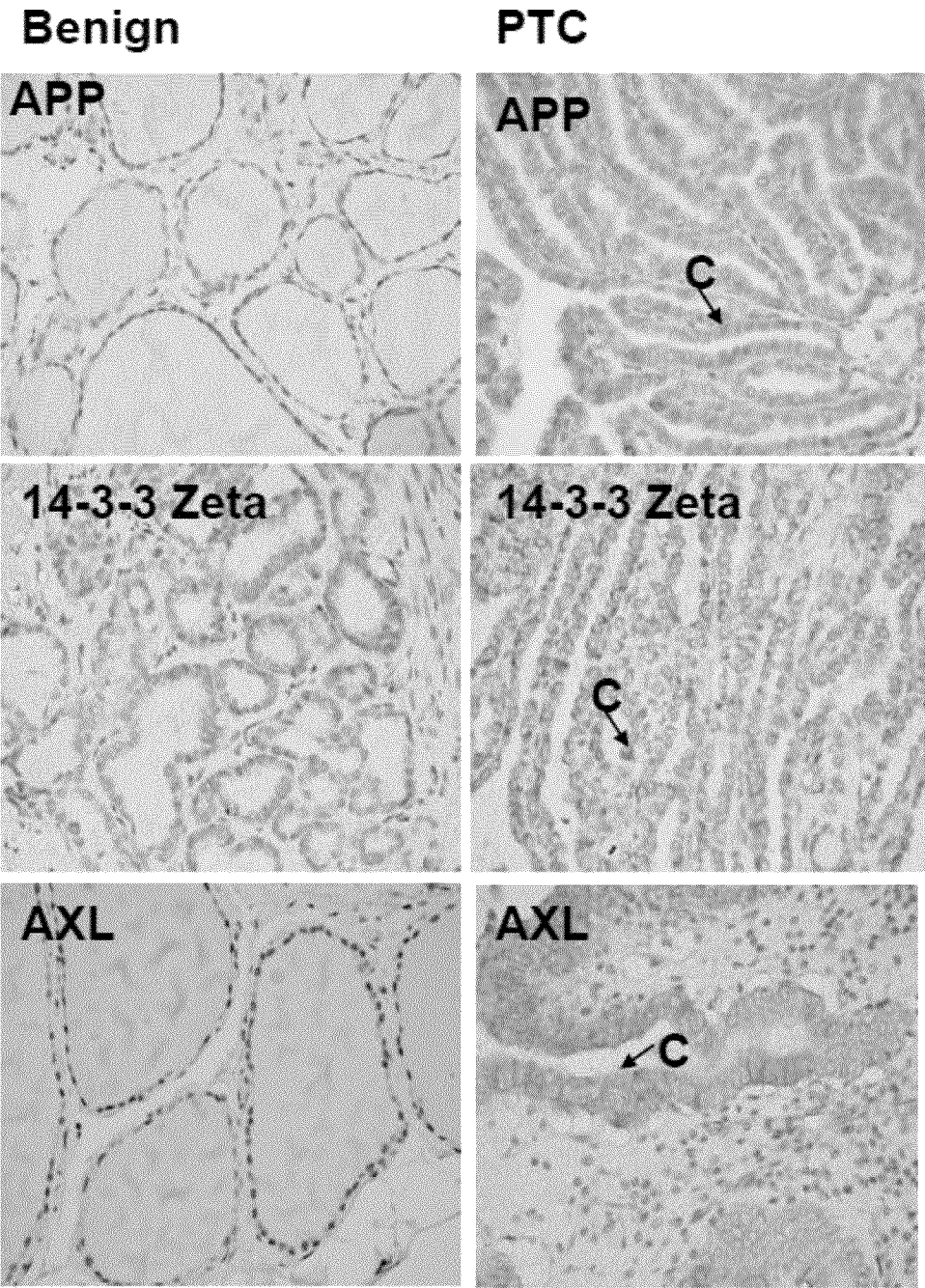


Fig. 9 (Cont'd)

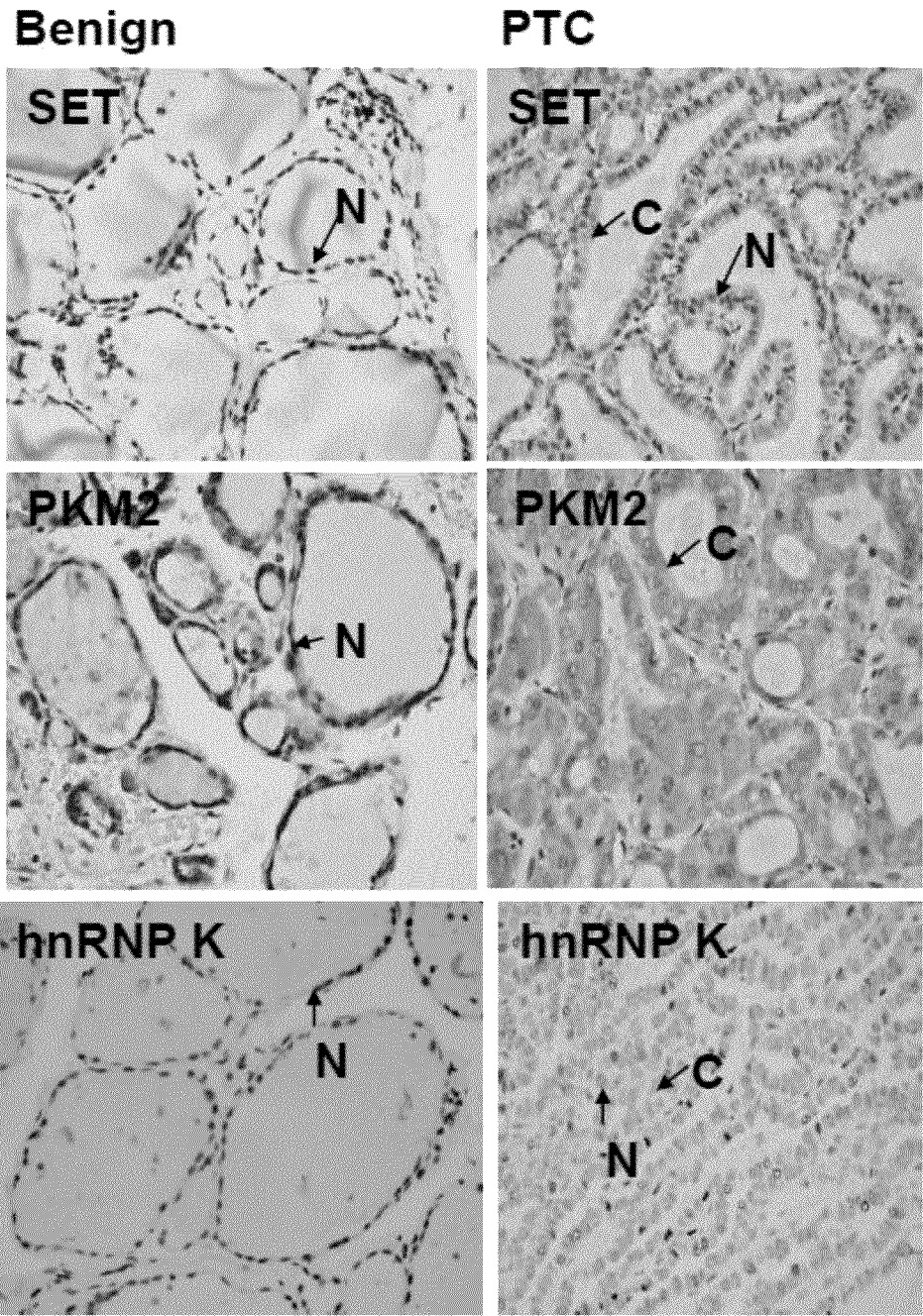


Fig. 10 (Cont'd)

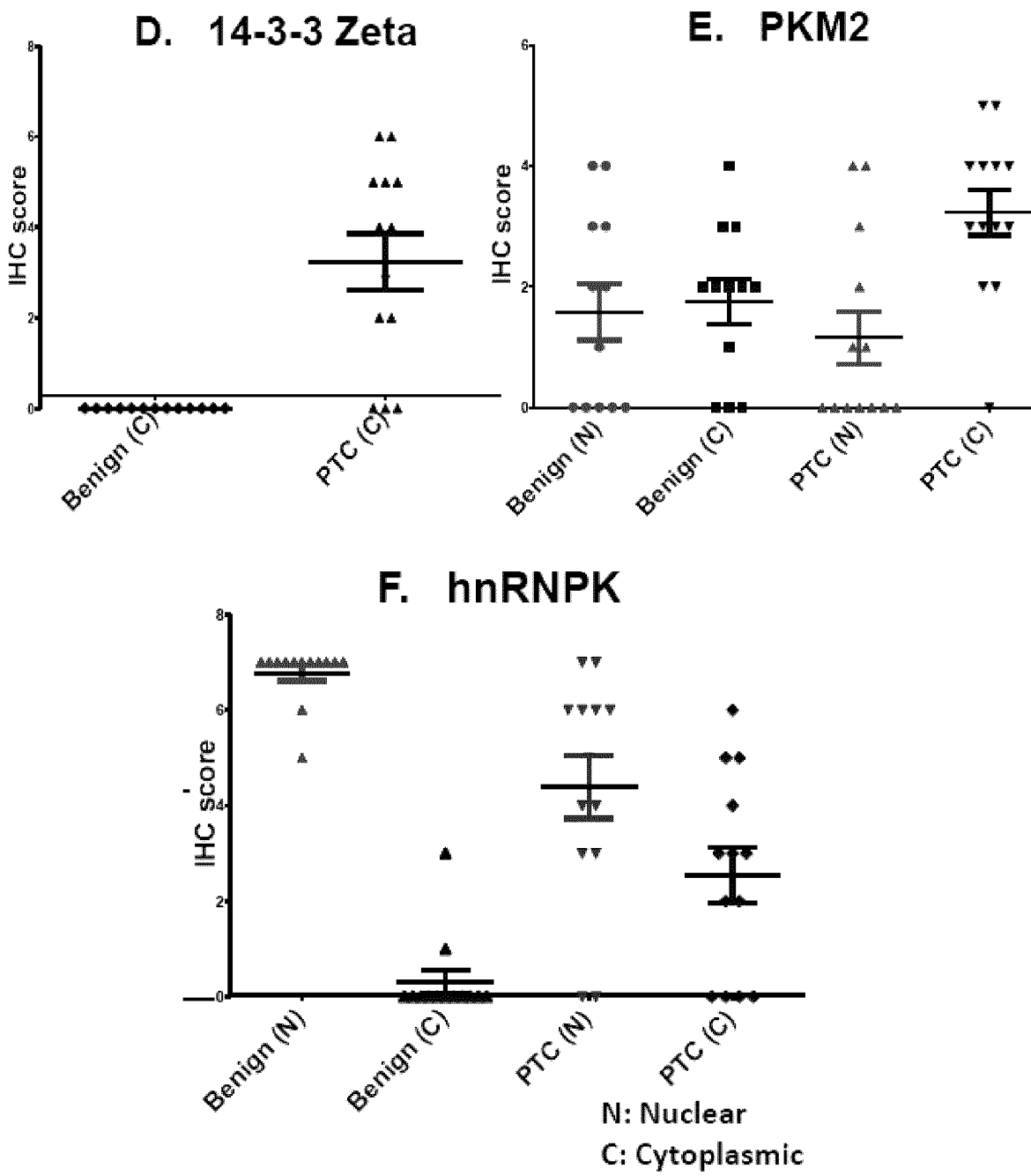


Fig. 11

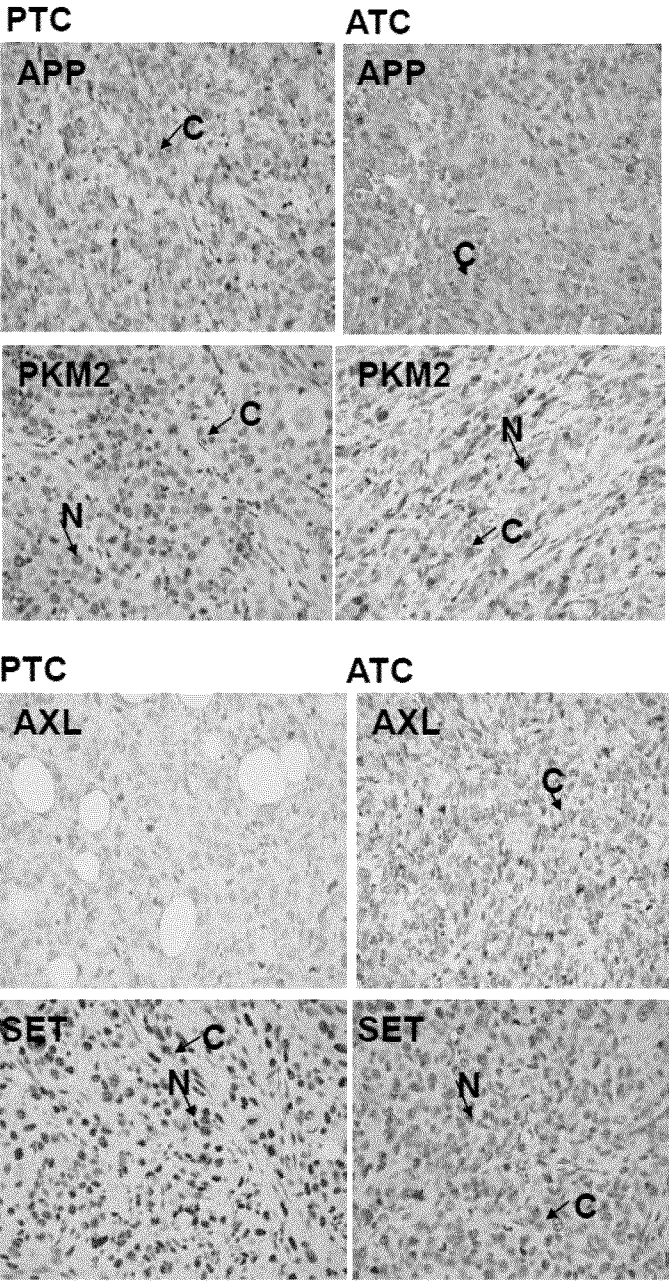


Fig. 12

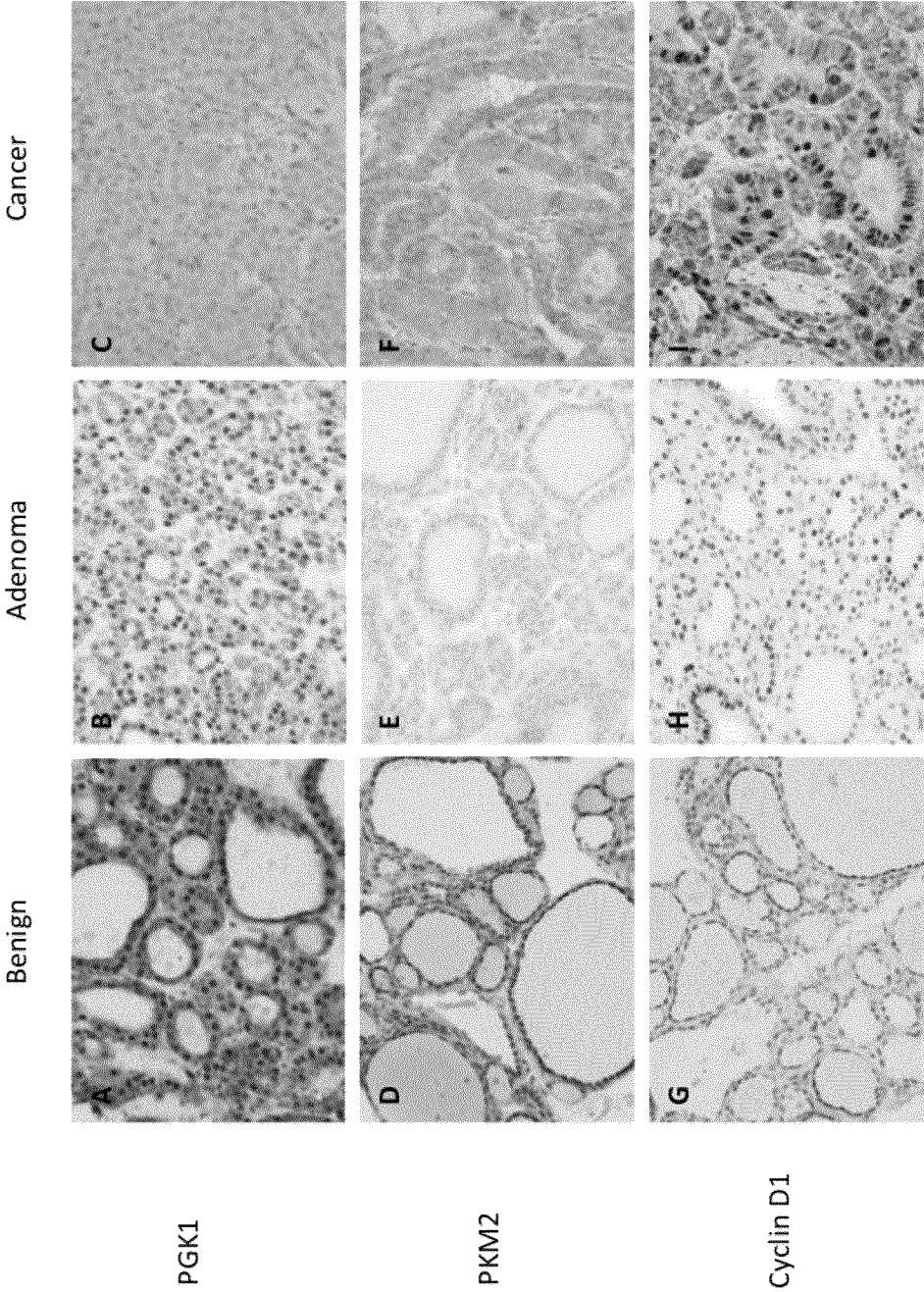


Fig. 12 (cont'd)

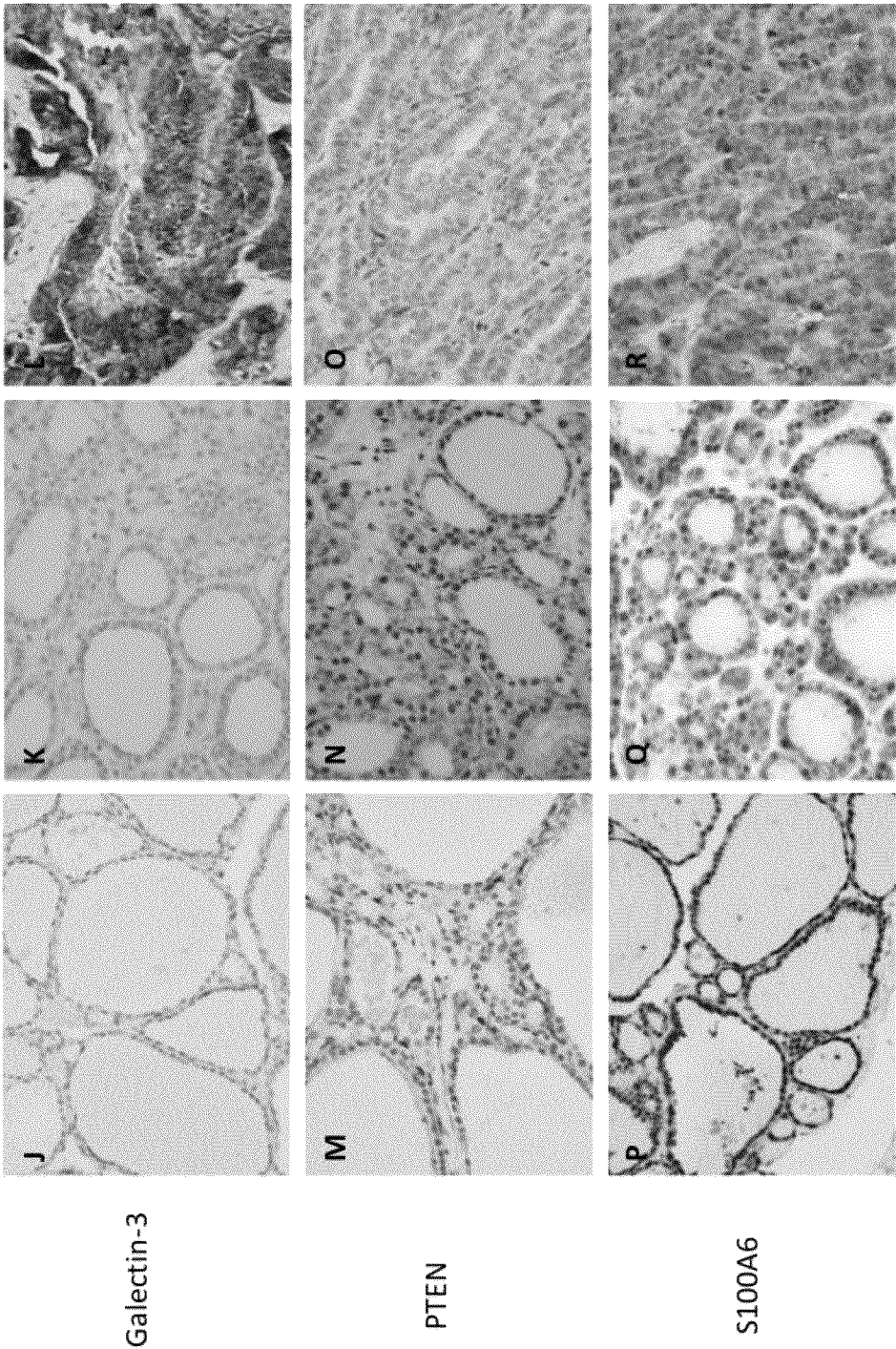
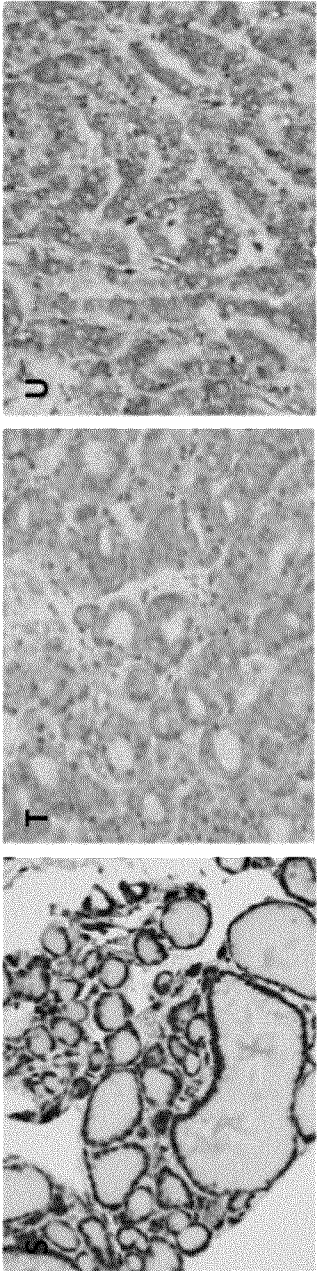


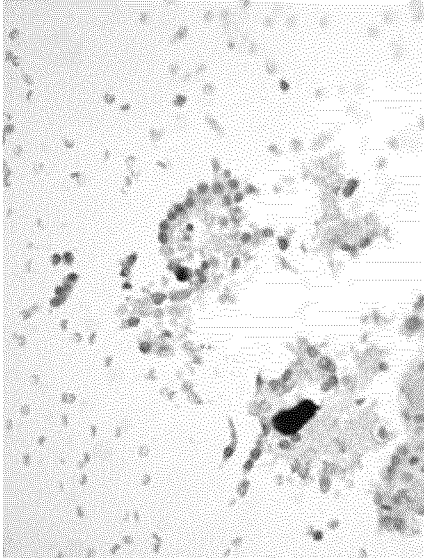
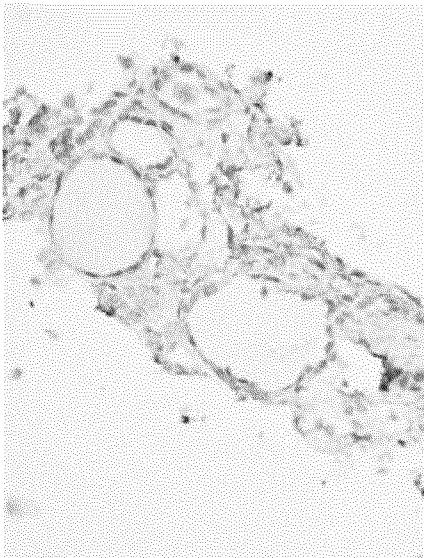
Fig. 12 (cont'd)



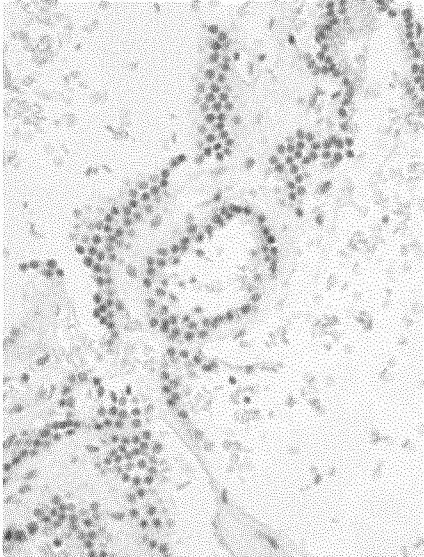
All images were captured at 40X magnification.

Figure 13

Cancer



Benign

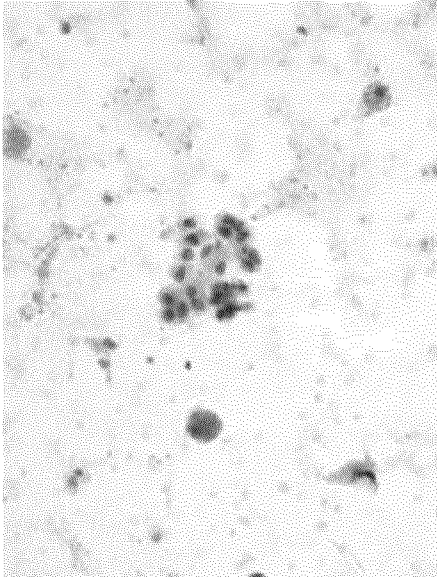
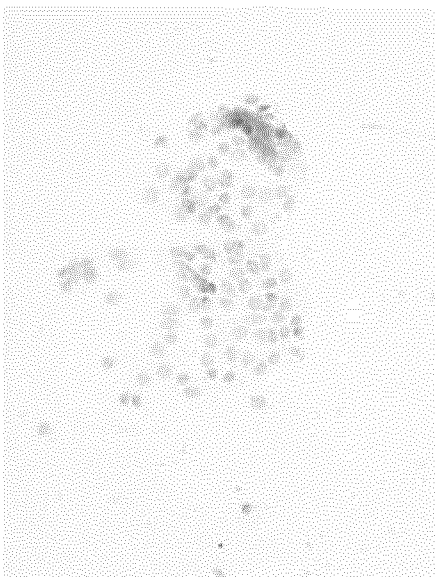


i) PGK-1

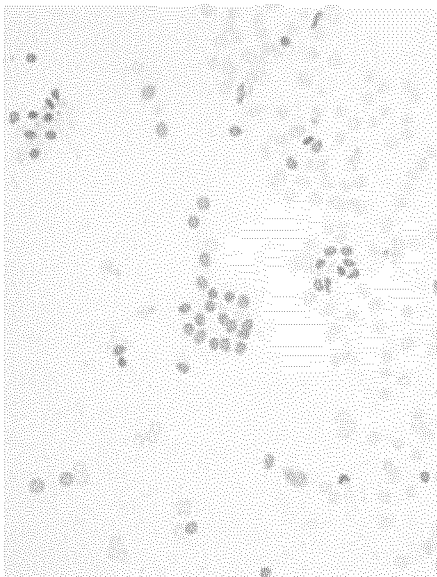
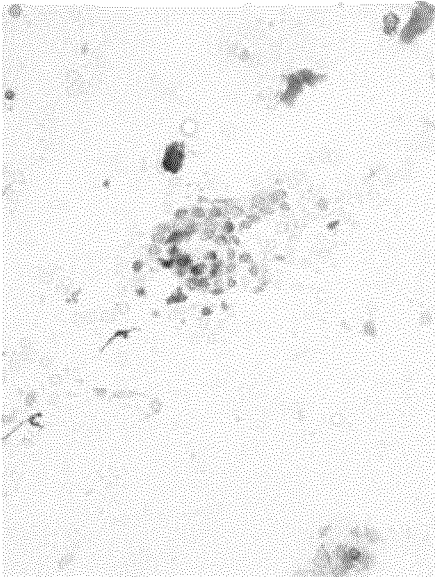
ii) Galectin 3

Figure 14

Cancer



Benign



i) PGK-1

ii) Galectin 3

Galectin-3

A: Benign

B: PTC

Figure 15A

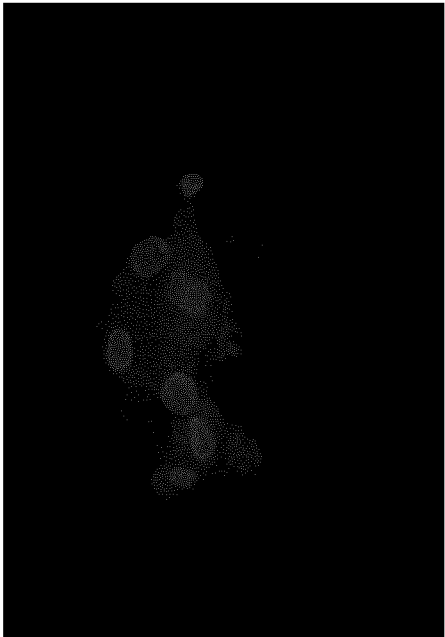


Figure 15B

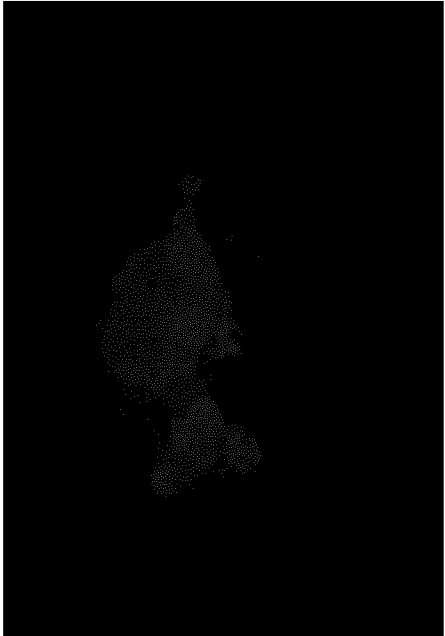


Figure 15C



Figure 15D



PGK1
A: Benign

Figure 16A

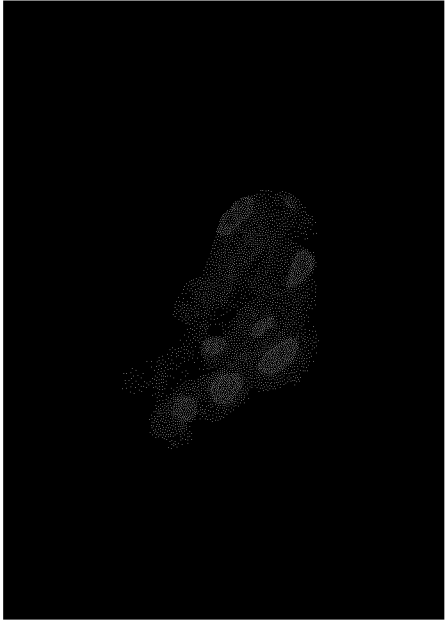


Figure 16B



B: PTC

Figure 16C

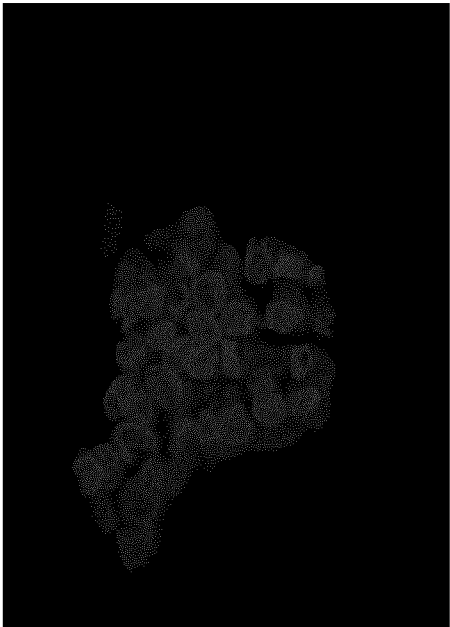


Figure 16D

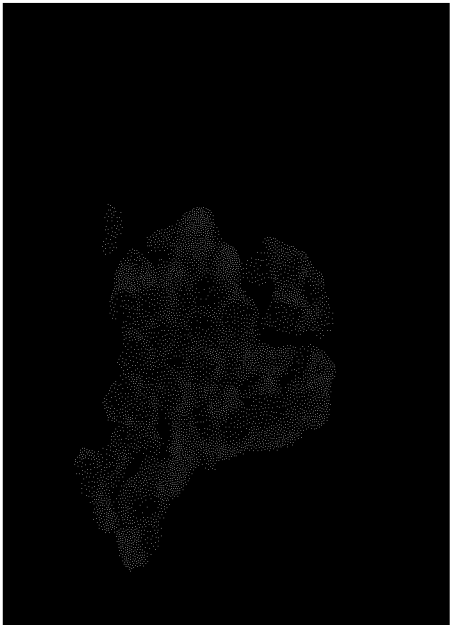




Figure 17B



Figure 17A

Figure 18B

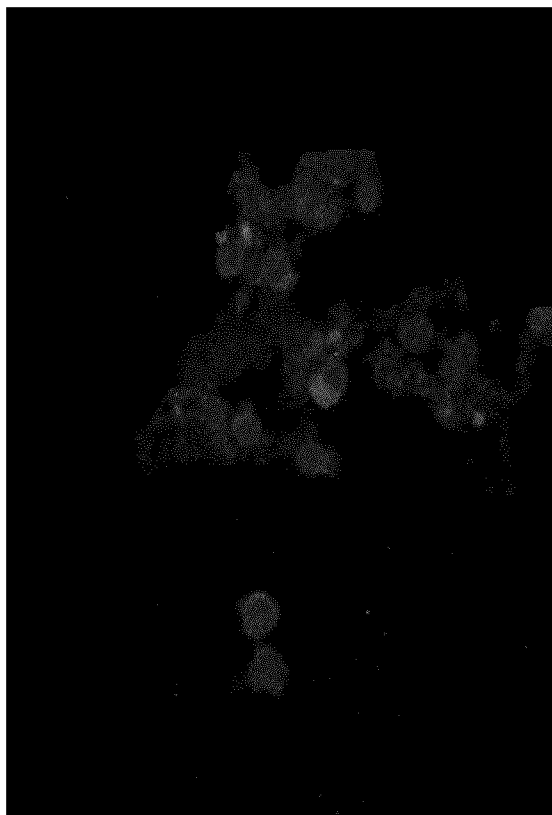
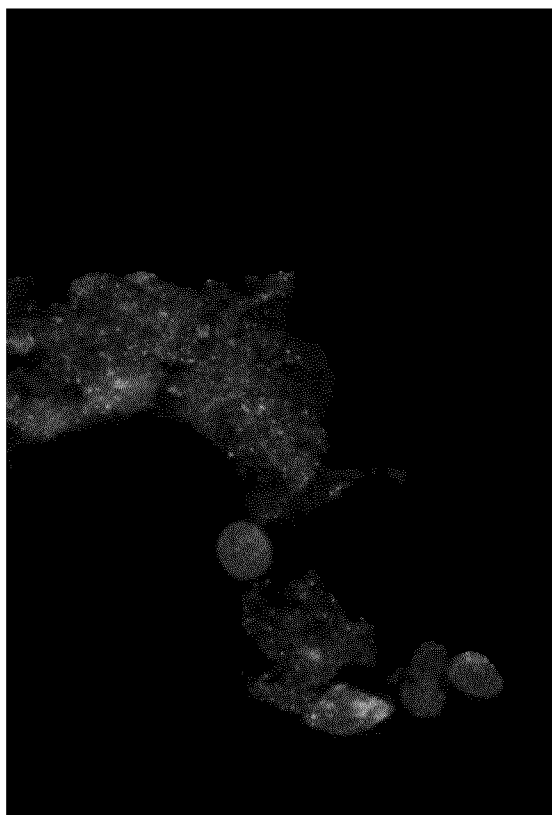


Figure 18A



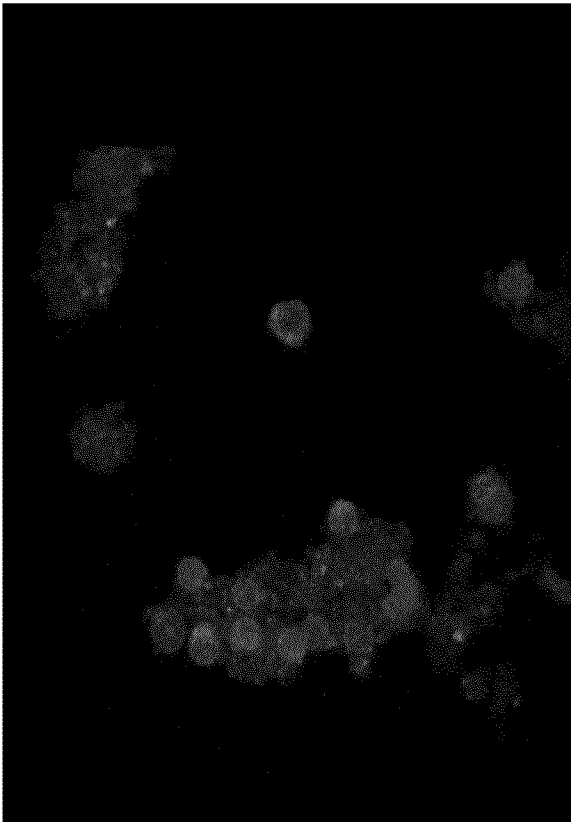


Figure 18D

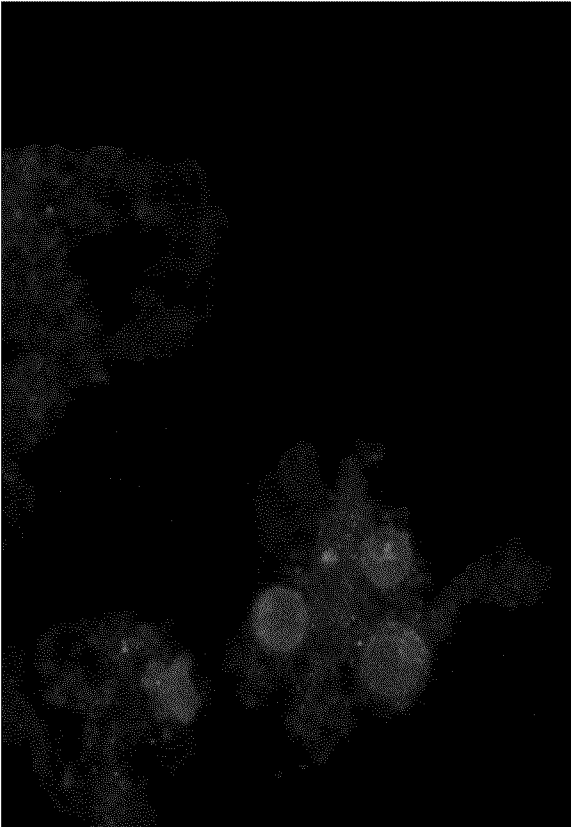


Figure 18C

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2016/050671

A. CLASSIFICATION OF SUBJECT MATTER
 IPC: **G01N 33/48** (2006.01), **G01N 33/574** (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC: **G01N 33/48** (2006.01), **G01N 33/574** (2006.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
 Questel Orbit, CIPO Intellect, CIPO Library Discovery Tool, Google Scholar

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US2012/0214165A1 (WALFISH et AL) 23 August 2012 (23-08-2012)	1-27
Y	WO2011137519A1 (WALFISH et AL) 10 November 2011 (10-11-2011)	1-27
X	GIUSTI et AL, " <i>Proteomic analysis of human thyroid fine needle aspiration fluid</i> ". Journal of Endocrinological Investigation, 2007, Vol. 30, pp. 865-869, [online] [retrieved on 16 August 2016 (16-08-2016)].	1-27

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* "A" "E" "L" "O" "P"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance earlier application or patent but published on or after the international filing date document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) document referring to an oral disclosure, use, exhibition or other means document published prior to the international filing date but later than the priority date claimed	"T" "X" "Y" "&"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art document member of the same patent family
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Date of the actual completion of the international search
 16 August 2016 (16-08-2016)

Date of mailing of the international search report
 23 September 2016 (23-09-2016)

Name and mailing address of the ISA/CA
 Canadian Intellectual Property Office
 Place du Portage I, C114 - 1st Floor, Box PCT
 50 Victoria Street
 Gatineau, Quebec K1A 0C9
 Facsimile No.: 819-953-2476

Authorized officer

Cecilia Alperin (819) 639-8408

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2016/050671**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

The claims are directed to a plurality of inventive concepts as follows:

Group A - Claims 1-21 are directed to a method of diagnosing thyroid cancer comprising comparing an amount of PGK1 in a thyroid tissue to a control amount; and

Group B - Claims 22-27 are directed to comprising comparing an amount of Profilin-1 in a thyroid tissue to a control amount.

The claims must be limited to one inventive concept as set out in PCT Rule 13.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2016/050671

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHAKER et AL, " <i>Secretome proteins as candidate biomarkers for aggressive thyroid carcinomas</i> ". Proteomics, 2013, Vol. 13, pp. 771-787, [online] [retrieved on 16 August 2016 (16-08-2016)].	1-27
P, X	RALHAN et AL, " <i>Immunohistochemical subcellular localization of protein biomarkers distinguishes benign from malignant thyroid nodules: potential for fine-needle aspiration biopsy clinical application</i> ". Thyroid, 1 November 2015 (01-11-2015), Vol. 25(11), pp. 1224-1234, [online] [retrieved on 16 August 2016 (16-08-2016)].	1-27

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2016/050671

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Date
US2010214165A1	26 August 2010 (26-08-2010)	US2010214165A1 US7932856B2 CN101813781A CN101813781B EP2228664A1 EP2228664B1 JP2010197073A JP5402073B2	26 August 2010 (26-08-2010) 26 April 2011 (26-04-2011) 25 August 2010 (25-08-2010) 03 April 2013 (03-04-2013) 15 September 2010 (15-09-2010) 09 October 2013 (09-10-2013) 09 September 2010 (09-09-2010) 29 January 2014 (29-01-2014)
WO2011137519A1	10 November 2011 (10-11-2011)	WO2011137519A1 CA2798562A1 US2011275065A1 US2015057179A1 US2016011197A1	10 November 2011 (10-11-2011) 10 November 2011 (10-11-2011) 10 November 2011 (10-11-2011) 26 February 2015 (26-02-2015) 14 January 2016 (14-01-2016)