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(54) Title: METHODS AND COMPOSITIONS FOR THE DIAGNOSIS AND TREATMENT OF THYROID CANCER

Figure 1
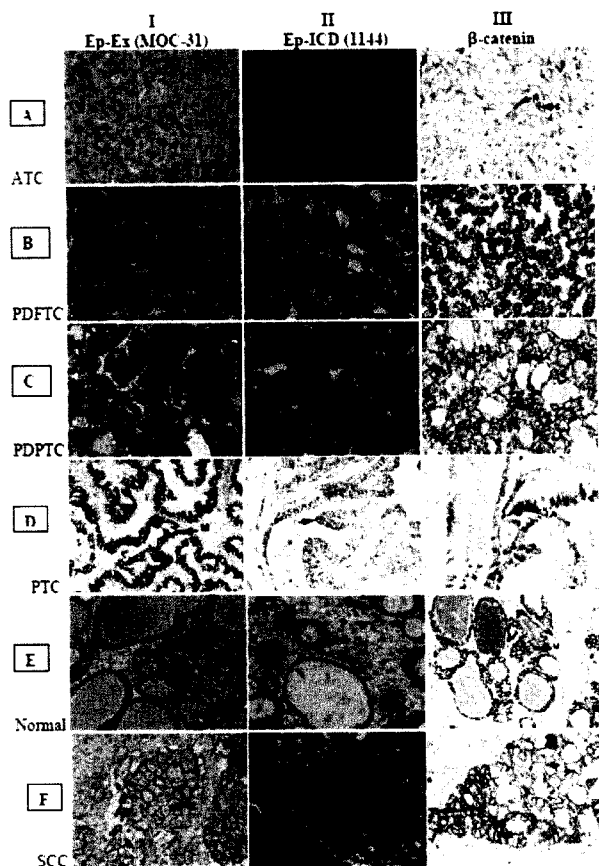
(57) **Abstract**: Methods for detecting, diagnosing and monitoring thyroid cancer in a subject are described comprising measuring in a sample from the subject markers including Ep-ICD and β-catenin. The invention also provides kits and compositions for carrying out the methods of the invention.

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TITLE: Methods and Compositions for the Diagnosis and Treatment of Thyroid Cancer

FIELD OF THE INVENTION

The invention relates to markers associated with thyroid cancer, in particular aggressive thyroid cancer, compositions, kits, and methods for detecting, diagnosing, predicting, monitoring, and characterizing thyroid cancer, and treatment of thyroid cancer.

BACKGROUND OF THE INVENTION

Epithelial cell adhesion molecule (EpCAM) is a 40kDa transmembrane glycoprotein showing frequent overexpression in several human malignancies [Spizzo et al., 2004; Went P et al., 2006; Wenqi D et al, 2009]. EpCAM was originally identified as a cancer marker, attributable to its high expression on rapidly proliferating epithelial tumors [reviewed in Trzpis M et al., 2007]. The normal epithelia express EpCAM at a variable though generally lower level than cancer cells. It is also overexpressed in normal stem and progenitor cells [Stingl J et al., 2001; Schmelzer E et al., 2007; Trzpis M et al., 2008] and in cancer-initiating cells in breast, colon, pancreas and prostate carcinomas [Al-Hajj M et al., 2003; O'Brien CA et al., 2007; Ricci-Vitiani L et al., 2007]. Recently, EpCAM has been detected in circulating tumor cells expressing E6/E7-HPV oncogenes in peripheral blood in cervical cancer patients after radical hysterectomy [Weismann P et al., 2009]. There is a large database on EpCAM staining for many cancers and normal tissues. However, all these studies used antibodies directed against the extracellular domain of EpCAM that may detect the EpCAM precursor or cell-bound EpEx, or both [Wenqi D et al., 2009].

EpCAM is a pleiotropic molecule that serves important roles in cell adhesion, cell proliferation, differentiation, migration, cell cycle regulation and is implicated in cancer and stem cell signaling [Munz et al., 2009]. The molecular mechanisms that regulate EpCAM expression are not well understood. Recently, regulated intramembrane proteolysis (RIP) has been shown to act as its mitogenic signal transducer in vitro and in vivo [Maetzel et al., 2009]. The cleavage and shedding of EpCAM ectodomain, EpEx, by proteases-TACE and Presenilin-2, releases its intracellular domain (Ep-ICD) that translocates to the nucleus. The association of Ep-ICD with FHL2 and Wnt pathway components - β-catenin and Lef-1 forms a nuclear complex that binds DNA at Lef-1 consensus sites and induces gene transcription, leading to increased cell proliferation and has been shown to be oncogenic in immunodeficient mice [Maetzel, 2009]. In view of the multiple roles of EpCAM as an

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oncogenic signal transducer, cell adhesion molecule and cancer stem cell marker [Litvinov SV et al., 1997; Munz et al., 2009], it is important to establish the clinical significance of nuclear Ep-ICD in human cancers.

Nuclear Ep-ICD was recently reported in a preliminary study in human colon cancer, but not in the normal colonic epithelium [Maetzel, 2009]. In view of the tremendous heterogeneity in solid tumors, the clinical significance of nuclear Ep-ICD in other human cancers remains to be established. Further, EpCAM has been shown to increase cell proliferation by upregulation of c-myc, cyclins A and E [Munz et al., 2004].

Thyroid cancer (TC) represents 90% of all endocrine malignancies with an estimated annual incidence of 122,800 cases worldwide and approximately 33,000 newly diagnosed cases in the USA [Reis et al., 2005; Jemal et al., 2008]. Anaplastic thyroid cancer (ATC) is a rare but very aggressive form of this malignancy, accounting for less than 2% of all thyroid cancers. ATC commonly presents as a rapidly increasing neck mass that spreads locally, compresses the adjacent structures, with a tendency to disseminate to regional lymph nodes and distant sites [Pasieka JL et al., 2003; Are C & Shaha 2006]. Most well differentiated thyroid cancers have an excellent prognosis, with relative 5-year survival rates above 95%, despite their tendency for early metastasis. However, the less-differentiated thyroid tumors - anaplastic and other aggressive metastatic thyroid cancers can be fatal with median survival time ranging from 4 months to 5 years [Are C & Shaha, 2006]. This variation in clinical outcomes may be attributed to the differences in genetic damage acquired by the aggressive and non-aggressive thyroid tumors during their malignant evolution.

The pathogenesis of ATC is linked to mutations in BRAF, RAS, β-Catenin, PIK3CA, TP53, AXIN1, PTEN and APC genes [reviewed in Smallridge RC et al., 2009]. The gene expression signatures in ATC have been identified showing the upregulation of the serine/threonine kinase Polo-like kinase 1 (PLK1) and its potential as a therapeutic target in ATC has been investigated [Salvatore G et al., 2007 CR; Nappi TC et al., 2009]. However, there are no proven predictive molecular markers to identify aggressive TCs.

SUMMARY OF THE INVENTION

The present invention relates to markers of thyroid cancer. In particular, EpCAM polypeptides and domains thereof (in particular, the ectodomain EpEx and the intracellular domain EP-ICD) and β-catenin (collectively referred to herein as "Polypeptide Thyroid Cancer Markers"), and polynucleotides encoding such polypeptides and domains thereof

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(collectively referred to herein as "Polynucleotide Thyroid Cancer Markers") constitute biomarkers for thyroid cancer, in particular aggressive thyroid cancer, more particularly anaplastic thyroid cancer (ATC). Polypeptide Thyroid Cancer Markers and Polynucleotide Thyroid Cancer Markers, and portions or fragments thereof, are sometimes collectively referred to herein as "Thyroid Cancer Markers".

The term "Thyroid Cancer Markers" in some aspects of the invention may include Wnt Proteins and polynucleotides encoding Wnt Proteins; and thus "Polypeptide Thyroid Cancer Markers" in some aspects includes Wnt Proteins, and "Polynucleotide Thyroid Cancer Markers" in some aspects includes polynucleotides encoding Wnt Proteins.

Thus, Thyroid Cancer Markers and agents that interact with the Thyroid Cancer Markers, may be used in detecting, diagnosing, characterizing, classifying, and monitoring thyroid cancer (i.e., monitoring progression of the cancer or the effectiveness of a therapeutic treatment), in the identification of subjects with a predisposition to thyroid cancer, and in determining prognosis or patient survival. In aspects of the invention, the Thyroid Cancer Markers, in particular Ep-ICD, β-catenin, and EpEx, are used in characterizing the aggressiveness of a thyroid cancer. In some aspects of the invention, the Thyroid Cancer Markers are used to determine metastatic potential or patient survival. The invention also contemplates methods for assessing the status of a thyroid tissue, and methods for the diagnosis and therapy of thyroid cancer.

A method of the invention 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 of a method of the invention, Thyroid Cancer Marker(s) detected in tumor samples provide a thyroid cancer clinical sensitivity of at least about 80 to 99%, in particular 90 to 95%, more particularly 91%, 92%, 93%, 94%, 95% or 98% thyroid cancer clinical sensitivity. In embodiments of the invention where one or more of nuclear Ep-ICD, nuclear β-catenin and cytoplasmic β-catenin are detected in a tumor sample the clinical sensitivity can be greater than about 80 to 90%, more particularly greater than about 80 to 85%, most particularly greater than about 83%, 84%, 85%, 90%, 95% or 98%. Clinical sensitivity and specificity may be determined using methods known to persons skilled in the art.

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In accordance with methods of the invention, 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

5   transcribed nucleic acid or fragment thereof, wherein the nucleic acid hybridizes with the marker.

In an aspect of the invention, a method is provided for detecting Thyroid Cancer Markers associated with thyroid cancer, in particular aggressive thyroid cancer, more particularly anaplastic thyroid carcinoma, in a patient comprising or consisting essentially of:

10   (a)   obtaining a sample from a patient;

           (b)   detecting or identifying in the sample one or more Thyroid Cancer Markers
                    and

           (c)   comparing the detected amount with an amount detected for a standard.

In accordance with methods of the invention, a thyroid tissue can be assessed or

15   characterized, for example, by detecting the presence in the sample of (a) a Thyroid Cancer Marker; (b) a transcribed nucleic acid or fragment thereof having at least a portion with which a Polynucleotide Thyroid Cancer Marker is substantially identical; and/or (c) a transcribed nucleic acid or fragment thereof, wherein the nucleic acid hybridizes with a Polynucleotide Thyroid Cancer Marker. Thyroid Cancer Markers in a sample may be

20   determined by methods as described herein and generally known in the art.

In an aspect, the invention provides a method for characterizing or classifying a thyroid sample comprising detecting a difference in the expression of a first plurality of Thyroid Cancer Marker relative to a control, the first plurality of markers consisting of Ep-ICD, β-catenin, and optionally EpEx.

25   One aspect of the invention provides a method for detecting thyroid cancer in a patient comprising determining the status of Thyroid Cancer Markers in a sample obtained from the patient, wherein an abnormal status in the sample indicates the presence of thyroid cancer. Thyroid Cancer Markers may be correlated with specific disease stages. Thus, another aspect of the invention provides a method of diagnosing a specific disease stage of thyroid

30   cancer in a patient comprising determining the status of a Thyroid Cancer Marker in a sample obtained from the patient, wherein an abnormal status of the marker indicates the presence of a specific disease stage.

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Another aspect of the invention provides a method of screening for thyroid cancer in a patient comprising identifying a patient at risk of having thyroid cancer or in need of screening and determining the status of Thyroid Cancer Markers in a sample obtained from the patient, wherein an abnormal status of the markers indicates the presence of thyroid cancer or a particular stage thereof.

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

In aspects of the invention, the abnormal status can be an elevated status, low status or negative status. In an embodiment of the invention for detecting or diagnosing thyroid cancer the abnormal status is an elevated status.

In an aspect, the invention provides a method for diagnosing ATC in a subject, the method comprising:

(a)   contacting a sample from a subject with a reagent capable of measuring a level of a target Thyroid Cancer Marker, in particular at least one Thyroid Cancer Marker selected from Ep-ICD, β-catenin, and optionally EpEx and c-myc; and

(b)   providing a diagnosis of ATC in said subject based on an increase in the level of at least one of Ep-ICD and β-catenin and optionally c-myc, and optionally a decrease in EpEx, in the sample from the subject over a control level obtained from similar samples taken from subjects who do not have ATC or from the subject at a different time.

In embodiments of this aspect of the invention, the Thyroid Cancer Markers measured are nuclear Ep-ICD, nuclear β-catenin, cytoplasmic β-catenin, and optionally EpEx.

In an embodiment of the invention, a method is provided for detecting one or more of Ep-ICD, β-catenin, EpEx, and EpCAM, associated with thyroid cancer, in particular aggressive thyroid cancer, more particularly anaplastic thyroid cancer, in a patient comprising or consisting essentially of:

(a)   obtaining a sample from a patient;

(b)   detecting or identifying in the sample one or more of Ep-ICD, β-catenin, EpEx, and EpCAM; and

(c)   comparing the detected amounts with amounts detected for a standard.

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In a particular embodiment of the invention, a method is provided for diagnosing ATC in a patient comprising or consisting essentially of:

(a)   detecting or identifying in the sample one or more of nuclear Ep-ICD, nuclear β-catenin and cytoplasmic β-catenin; and

5   (b)   comparing the detected amount with an amount detected for a standard, wherein an increase in one or more of nuclear Ep-ICD, nuclear β-catenin and cytoplasmic β-catenin is indicative of ATC.

In a particular embodiment of the invention, a method is provided for diagnosing ATC in a patient comprising or consisting essentially of:

10   (a)   detecting or identifying in the sample one or more of nuclear Ep-ICD, nuclear β-catenin, cytoplasmic β-catenin, and EpEx (e.g. membranous EpEx); and

(b)   comparing the detected amount with an amount detected for a standard, wherein an increase in one or more of nuclear Ep-ICD, nuclear β-catenin and cytoplasmic β-catenin and a decrease or absence of EpEx is indicative of

15   ATC.

In a particular aspect of the invention, a method is provided for detecting Thyroid Cancer Markers, preferably Ep-ICD and/or β-catenin, associated with aggressive or metastatic thyroid cancer, in a patient comprising or consisting essentially of:

(a)   obtaining a sample (e.g. tumor sample) from a patient;

20   (b)   detecting in the sample Thyroid Cancer Markers, preferably Ep-ICD and/or β-catenin; and

(c)   comparing the detected amount with an amount detected for a standard or cut-off value.

The term "detect" or "detecting" includes assaying, or otherwise establishing the

25   presence or absence of the target marker(s), subunits, or combinations of reagent bound targets, and the like, or assaying for ascertaining, establishing, or otherwise determining one or more factual characteristics of a thyroid cancer such as aggressiveness, metastatic potential or patient survival. 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

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

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The invention provides a method of assessing whether a patient is afflicted with or has a pre-disposition for thyroid cancer, in particular aggressive or metastatic thyroid cancer, more particularly ATC, the method comprising comparing:

(a)   levels of Thyroid Cancer Markers from the patient; and

5   (b)   standard levels of Thyroid Cancer Markers in samples of the same type obtained from control patients not afflicted with thyroid cancer or with a lower grade of thyroid cancer, wherein altered levels of Thyroid Cancer Markers relative to the corresponding standard levels of Thyroid Cancer Markers is an indication that the patient is afflicted with thyroid cancer, in particular

10   aggressive or metastatic thyroid cancer, more particularly ATC.

In an aspect of a method of the invention for assessing whether a patient is afflicted with aggressive or metastatic thyroid cancer, in particular ATC, higher levels of nuclear Ep-ICD, nuclear β-catenin, or cytoplasmic β-catenin, and lower levels or the absence of EpEx (e.g., membranous EpEx), in a sample relative to corresponding normal levels or levels from

15   a patient with a lower grade of thyroid cancer, is an indication that the patient is afflicted with aggressive or metastatic thyroid cancer, in particular ATC.

In an embodiment of a method of the invention for assessing whether a patient is afflicted with anaplastic thyroid cancer, levels of nuclear Ep-ICD in a sample from the patient are compared to a standard, and higher levels of nuclear Ep-ICD compared to a standard are

20   indicative of anaplastic thyroid cancer.

In an embodiment of a method of the invention for assessing whether a patient is afflicted with anaplastic thyroid cancer, levels of nuclear β-catenin in a sample from the patient are compared to a standard, and higher levels of nuclear β-catenin compared to a standard are indicative of anaplastic thyroid cancer.

25   In an embodiment of a method of the invention for assessing whether a patient is afflicted with anaplastic thyroid cancer, levels of cytoplasmic β-catenin in a sample from the patient are compared to a standard, and higher levels of cytoplasmic β-catenin compared to a standard are indicative of anaplastic thyroid cancer.

In an embodiment of a method of the invention for assessing whether a patient is

30   afflicted with anaplastic thyroid cancer, levels of membranous EpEx in a sample from the patient are compared to a standard, and lower levels or absence of membranous EpEx compared to a standard are indicative of anaplastic thyroid cancer.

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In an embodiment of a method of the invention for assessing whether a patient is afflicted with follicular thyroid cancer (FTC), levels of membranous EpEx, nuclear Ep-ICD, cytoplasmic Ep-ICD and β-catenin in a sample from the patient are compared to a standard.

In an embodiment of a method of the invention for assessing whether a patient is
5   afflicted with follicular thyroid cancers (FTC), levels of membranous EpEx, nuclear Ep-ICD, and cytoplasmic Ep-ICD in a sample from the patient are compared to a standard. In an embodiment, there is an absence or low levels of nuclear Ep-ICD and optionally higher levels of cytoplasmic β-catenin.

In an embodiment of a method of the invention for assessing whether a patient is
10   afflicted with papillary thyroid cancers (PTC), levels of membranous EpEx, nuclear Ep-ICD, cytoplasmic Ep-ICD and β-catenin in a sample from the patient are compared to a standard. In an embodiment, there is an absence or low levels of nuclear Ep-ICD and β-catenin.

In an embodiment of a method of the invention for assessing whether a patient is afflicted with squamous cell carcinoma of the thyroid, levels of membranous EpEx, nuclear
15   Ep-ICD, cytoplasmic Ep-ICD and β-catenin in a sample from the patient are compared to a standard.

In particular aspects, methods of the invention are used to diagnose the stage of thyroid cancer in a subject or characterizing thyroid cancer in a subject. In an embodiment, the method comprises comparing

20   (a)   levels of Thyroid Cancer Markers (e.g. biopsy sample) from a sample from the patient; and

            (b)   levels of Thyroid Cancer Markers in control samples of the same type obtained from patients without thyroid cancer or control patients with a different stage of thyroid cancer (e.g., low grade thyroid cancer) or from a
25   sample from the patient taken at a different time, wherein altered levels of Thyroid Cancer Markers, relative to the corresponding levels in the control samples is an indication that the patient is afflicted with a more aggressive or metastatic thyroid cancer.

In embodiments, the aggressive thyroid cancer is ATC and the Thyroid Cancer
30   Markers are one or more of nuclear Ep-ICD, nuclear β-catenin, and cytoplasmic β-catenin. In particular embodiments, the Thyroid Cancer Marker is nuclear Ep-ICD.

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The invention further provides a non-invasive non-surgical method for detection or diagnosis of thyroid cancer, in particular aggressive or metastatic thyroid cancer, more particularly ATC, in a subject comprising: obtaining a sample (e.g., biopsy sample) from the subject; subjecting the sample to a procedure to detect Thyroid Cancer Marker(s); detecting

5   or diagnosing thyroid cancer by comparing the levels of Thyroid Cancer Marker(s) to the levels of Thyroid Cancer Marker(s) obtained from a control subject with no thyroid cancer or a lower grade of thyroid cancer or from a sample from the patient taken at a different time. In embodiments of this method of the invention, the Thyroid Cancer Marker(s) are one or more of nuclear Ep-ICD, nuclear β-catenin, cytoplasmic β-catenin. In particular embodiments, the

10   Thyroid Cancer Marker is nuclear Ep-ICD.

In aspects of the invention, aggressive thyroid cancer, in particular ATC, is detected, diagnosed or characterized by determination of increased levels of one or more of nuclear Ep-ICD, nuclear β-catenin, cytoplasmic β-catenin, when compared to such levels obtained from a control or from a sample from the patient taken at a different time.

15   In a particular embodiment the invention provides a method for diagnosing the aggressiveness of thyroid cancer in a subject comprising:

(a)   determining the amount of nuclear Ep-ICD in a sample (e.g., tumor sample) from the subject;

(b)   determining the amount of one or both of nuclear β-catenin and β-catenin in

20   the sample;

(c)   determining the amount of EpEx in the sample;

(d)   mathematically combining the results of step (a) and step (b), and optionally step (c) to provide a mathematical combination; and

(e)   comparing or correlating the mathematical combination to the aggressiveness

25   of the thyroid cancer.

The combination is preferably compared to a mathematical combination for a predetermined standard.

In an aspect, the invention provides a method for monitoring the progression of thyroid cancer in a patient the method comprising:

30   (a)   detecting Thyroid Cancer Marker(s) in a patient sample (e.g. biopsy sample) at a first time point;

(b)   repeating step (a) at a subsequent point in time; and

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(c)   comparing the levels detected in (a) and (b), and thereby monitoring the progression of thyroid cancer in the patient.

The invention provides a method for classifying a patient having thyroid cancer, the method comprising measuring Thyroid Cancer Marker(s) in a sample (e.g. tumor sample) from the patient and correlating the values measured to values measured for the Thyroid Cancer Markers from thyroid cancer patients stratified in classification groups. The method can be used to predict patient survival, wherein the Thyroid Cancer Marker(s) are predictive of survival and wherein the classification groups comprise groups of known overall survival. In aspects of this method of the invention, the Thyroid Cancer Marker(s) are selected from Ep-ICD and β-catenin, in particular nuclear Ep-ICD, nuclear β-catenin, and cytoplasmic β-catenin. In various embodiments the values measured can be normalized to provide more accurate quantification and to correct for experimental variations.

In particularly useful aspects of the invention, Polynucleotide Thyroid Cancer Markers, preferably polynucleotides encoding Ep-ICD and/or β-catenin, are detected and levels of Polynucleotide Thyroid Cancer Markers in a sample (e.g., biopsy sample) from a patient are compared with Polynucleotide Thyroid Cancer Marker levels from samples of patients without thyroid cancer, with a lower grade of thyroid cancer, or from levels from samples of the same patient. A method of the invention may employ one or more polynucleotides, oligonucleotides, or nucleic acids capable of hybridizing to Polynucleotide Thyroid Cancer Markers and preferably polynucleotides encoding Ep-ICD. In an aspect of the invention, Ep-ICD mRNA is detected.

The present invention relates to a method for diagnosing and characterizing thyroid cancer, more particularly the stage of thyroid cancer, in a sample from a subject comprising isolating nucleic acids, preferably mRNA, from the sample, and detecting Polynucleotide Thyroid Cancer Markers in the sample. In an embodiment, the presence of increased levels of polynucleotides encoding Ep-ICD and/or β-catenin, in the sample compared to a standard or control is indicative of the aggressiveness or metastatic potential of a thyroid cancer, in particular is indicative of ATC.

The invention also provides methods for determining the presence or absence of thyroid cancer or the aggressiveness or metastatic potential of a thyroid cancer in a subject, in particular determining ATC, in the subject comprising detecting in the sample a level of nucleic acids that hybridize to a Polynucleotide Thyroid Cancer Marker(s), and comparing

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the level(s) with a predetermined standard or cut-off value, and therefrom determining the presence or absence of thyroid cancer or the aggressiveness or metastatic potential of a thyroid cancer in the subject, in particular determining ATC in the subject. In an embodiment a method is provided for determining the aggressiveness or metastatic potential of thyroid

5   cancer in a subject comprising (a) contacting a sample taken from the subject with oligonucleotides that hybridize to polynucleotides encoding Ep-ICD and/or β-catenin; and (b) detecting in the sample a level of nucleic acids that hybridize to the oligonucleotides relative to a predetermined standard or cut-off value, and therefrom determining the aggressiveness or metastatic potential of the cancer in the subject.

10 In an aspect, the invention provides a method of assessing the aggressiveness or metastatic potential of a thyroid cancer in a patient, the method comprising comparing:

(a)   levels of Polynucleotide Thyroid Cancer Marker(s) in a sample from the patient; and

(b)   control levels of Polynucleotide Thyroid Cancer Marker(s) in samples of the

15   same type obtained from control patients not afflicted with thyroid cancer or a lower grade of thyroid cancer or from a sample from the patient taken at a different time, wherein altered levels of Polynucleotide Thyroid Cancer Marker(s) relative to the corresponding control levels of Polynucleotide Thyroid Cancer Marker(s) is an indication of the aggressiveness or metastatic

20   potential of the thyroid cancer.

In a particular method of the invention for assessing whether a patient is afflicted with an aggressive or metastatic thyroid cancer, and in particular ATC, higher levels of Ep-ICD and/or β-catenin, in a sample relative to the corresponding control levels is an indication that the patient is afflicted with an aggressive or metastatic thyroid cancer.

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

30 to a Polynucleotide Thyroid Cancer Marker(s), or a complement thereof.

When using mRNA detection, the method may be carried out by combining isolated mRNA with reagents to convert to cDNA according to standard methods; treating the

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converted cDNA with amplification reaction reagents along with an appropriate mixture of primers to produce amplification products; and analyzing the amplification products to detect the presence of Polynucleotide Thyroid Cancer Marker(s) in the sample. For mRNA the analyzing step may be accomplished using RT-PCR analysis to detect the presence of Polynucleotide Thyroid Cancer Marker(s). The analysis step may be accomplished by quantitatively detecting the presence of Polynucleotide Thyroid Cancer Marker(s) in the amplification product, and comparing the quantity of Polynucleotide Thyroid Cancer Marker(s), detected against a panel of expected values for known presence or absence in normal and malignant samples (e.g. tissue sample, in particular a tissue sample from patients with a different stage of thyroid cancer), derived using similar primers.

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

Protein based methods can also be used for diagnosing and monitoring thyroid cancer, in particular the aggressiveness or metastatic potential of thyroid cancer, more particularly ATC, in a subject comprising detecting Thyroid Cancer Markers in a sample from the subject. Thyroid Cancer Markers may be detected using a binding agent for Thyroid Cancer Markers, preferably antibodies specifically reactive with Thyroid Cancer Markers, or parts thereof.

The invention provides a method of assessing whether a patient is afflicted with thyroid cancer, in particular aggressive or metastatic thyroid cancer, more particularly ATC, which comprises comparing:

(a)   levels of Polypeptide Thyroid Cancer Markers in a sample from the patient; and

(b)   control levels of Polypeptide Thyroid Cancer Markers in a non-cancer sample or sample from a patient with a lower grade of thyroid cancer or from a

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sample from the patient taken at a different time, wherein significantly different levels of Polypeptide Thyroid Cancer Markers in the sample from the patient compared with the control levels (e.g. higher in the patient samples) is an indication that the patient is afflicted with thyroid cancer or an aggressive or metastatic thyroid cancer, in particular ATC.

In another aspect the invention provides methods for determining the presence or absence of thyroid cancer or the aggressiveness or metastatic potential of a thyroid cancer in a patient, in particular ATC, comprising the steps of (a) contacting a biological sample obtained from a patient with a binding agent that specifically binds to a Polypeptide Thyroid Cancer Marker(s); and (b) detecting in the sample an amount of Polypeptide Thyroid Cancer Marker(s) that binds to the binding agent(s), relative to a predetermined standard or cut-off value, and therefrom determining the presence or absence of aggressiveness or metastatic potential of thyroid cancer in the patient.

In an embodiment, the invention relates to a method for detecting, diagnosing, staging and monitoring thyroid cancer in a subject by quantitating Polypeptide Thyroid Cancer Marker(s) in a biological sample from the subject comprising (a) reacting the biological sample with an antibody specific for Polypeptide Thyroid Cancer Marker(s) which is directly or indirectly labelled with a detectable substance; and (b) detecting the detectable substance.

In another embodiment the invention provides a method of using antibodies to detect expression of Polypeptide Thyroid Cancer Marker(s) in a sample, the method comprising: (a) combining antibodies specific for a Polypeptide Thyroid Cancer Marker(s) with a sample under conditions which allow the formation of antibody:protein complexes; and (b) detecting complex formation, wherein complex formation indicates expression of a Polypeptide Thyroid Cancer Marker(s) in the sample. Expression may be compared with standards and is diagnostic of thyroid cancer or the aggressiveness or metastatic potential of the thyroid cancer, in particular ATC.

In an aspect, the invention provides a method for monitoring the progression of thyroid cancer in a patient, the method comprising:

(a)   detecting Polypeptide Thyroid Cancer Marker(s) in a patient sample at a first time point; and

(b)   repeating step (a) at a subsequent point in time; and

(c)   comparing the levels detected in (a) and (b), and thereby monitoring the

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progression of thyroid cancer in the patient.

The invention further relates to a method of assessing the efficacy of a therapy for thyroid cancer, more particularly aggressive or metastatic thyroid cancer in a patient. This method comprises comparing:

5   (a)   levels of Thyroid Cancer Markers in a first sample obtained from the patient prior to providing at least a portion of the therapy to the patient; and

(b)   levels of Thyroid Cancer Markers in a second sample obtained from the patient following therapy.

Significantly different levels of Thyroid Cancer Markers in the second sample, relative to the first sample, is an indication that the therapy is efficacious for inhibiting thyroid cancer, more particularly anaplastic thyroid carcinoma. In an embodiment, the method is used to assess the efficacy of a therapy for inhibiting thyroid cancer, more particularly aggressive or metastatic thyroid cancer, and lower levels of nuclear Ep-ICD, nuclear β-catenin or cytoplasmic β-catenin, 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.

The invention contemplates a method for determining the effect of an environmental factor on thyroid tissue or thyroid cancer comprising comparing Thyroid Cancer Markers in the presence and absence of the environmental factor.

The invention also provides a method for assessing the potential efficacy of a test agent for treating thyroid cancer, and a method of selecting an agent for treating thyroid cancer.

The invention contemplates a method of assessing the potential of a test compound to contribute to thyroid cancer comprising:

(a)   maintaining separate aliquots of diseased cells in the presence and absence of the test compound; and

(b)   comparing the levels of Thyroid Cancer Markers in each of the aliquots.

A significant difference between the levels of markers in an aliquot maintained in the

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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.

The invention also provides a pharmaceutical composition or diagnostic composition comprising Thyroid Cancer Markers or agents that interact with Thyroid Cancer Markers. In particular, the invention provides a pharmaceutical composition or 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. 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 pharmaceutical composition or diagnostic composition of the invention comprises antibodies specific for Ep-ICD, β-catenin and/or EpEx. In an embodiment, a pharmaceutical composition or diagnostic composition of the invention comprises nucleotides (e.g. probes) that hybridize to polynucleotides encoding Ep-ICD, β-catenin and/or EpEx. In an embodiment, a diagnostic composition of the invention comprises primers that amplify polynucleotides encoding Ep-ICD, β-catenin and/or EpEx.

In another aspect, the invention 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, more particularly ATC.

The methods of the invention may comprise detecting Wnt Proteins and polynucleotides encoding the Wnt Proteins. The methods of the invention may also comprise detecting additional markers associated with thyroid cancer such as galectin-3, thyroglobulin, E-cadherin, beta–actin, FHL2 and Lef-1. Further, the amount of Thyroid Cancer Markers may be mathematically combined with other markers of thyroid cancer. In an embodiment the invention provides a method for detecting or diagnosing thyroid cancer in a subject comprising:

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(a)   determining the amount of Thyroid Cancer Markers in a sample from the subject;

(b)   determining the amount of other markers associated with thyroid cancer in particular markers selected from the group consisting of galectin-3, thyroglobulin, E-cadherin, c-Myc, beta–actin, FHL2 and Lef-1 in the sample;

(c)   mathematically combining the results of step (a) and step (b) to provide a mathematical combination; and

(d)   comparing or correlating the mathematical combination to the presence of thyroid cancer or aggressiveness or metastatic potential of thyroid cancer.

The combination is preferably compared to a mathematical combination for a predetermined standard. In particular aspects, the invention provides a method for detecting, characterizing or diagnosing thyroid cancer by determining the combination of Thyroid Cancer Markers and one or both of galectin-3 and thyroglobulin in a sample from a subject.

The invention also includes kits for carrying out methods of the invention. In an aspect the invention provides a kit for detecting, diagnosing or characterizing thyroid cancer comprising Thyroid Cancer Markers. In a particular aspect, the invention provides a test kit for diagnosing or characterizing thyroid cancer in a subject which comprises an agent that interacts with a Thyroid Cancer Marker(s). In an embodiment, the kit is for assessing whether a patient is afflicted with aggressive or metastatic thyroid cancer, in particular ATC, and it comprises reagents for identifying and/or assessing levels of Ep-ICD, β-catenin and optionally EpEx.

The invention therefore contemplates an *in vivo* method comprising administering to a mammal one or more agent that carries a label for imaging and binds to a Thyroid Cancer Marker(s), and then imaging the mammal. According to a preferred aspect of the invention, an in vivo method for imaging thyroid cancer is provided comprising:

(a)   injecting a patient with an agent that binds to a 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.

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In an embodiment of the invention the agent is an antibody which recognizes the Thyroid Cancer Marker(s). In another embodiment of the invention 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 5 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.

10   The invention also contemplates the localization or imaging methods described herein using multiple markers for thyroid cancer.

The invention provides methods of treating thyroid cancer, in particular ATC, comprising administering to a subject or using a pharmaceutical composition of the invention. In an aspect, the invention provides antagonists (e.g. antibodies) specific for Ep-15 ICD or β-catenin that can be used therapeutically to destroy or inhibit the growth of thyroid cancer cells, (e.g. ATC cells), or to block Ep-ICD or β-catenin activity. In addition, Ep-ICD or β-catenin may be used in various immunotherapeutic methods to promote immune-mediated destruction or growth inhibition of tumors expressing Ep-ICD or β-catenin.

Other objects, features and advantages of the present invention will become apparent 20 from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

25 **DESCRIPTION OF THE DRAWINGS**

The invention will now be described in relation to the drawings in which:

Figure 1. Immunohistochemical analysis of EpEx, Ep-ICD and β-catenin in thyroid cancers. The anaplastic thyroid cancers did not show detectable membranous EpEx staining (IA); all the other subtypes of thyroid cancers analyzed and normal thyroid tissues showed 30 plasma membranous EpEX staining (IB-IF). Nuclear Ep-ICD staining was only observed in undifferentiated and poorly differentiated thyroid cancers (IIA-IIC, and IIF), but not in well differentiated thyroid cancer and normal thyroid tissue (IID, IIE). Correlated with nuclear

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Ep-ICD staining, nuclear or cytoplasmic β-catenin staining was observed in aggressive thyroid cancers (IIIA-IIIC, and IIIF), while membranous staining was observed in less aggressive thyroid cancers and normal thyroid tissue (IIID, IIIE).

Figure 2. Immunohistochemical analysis of EpEx, Ep-ICD and β-catenin in the same thyroid cancer patient. No membranous EpEx staining was observed in the anaplastic thyroid cancer section (IA), faint membranous EpEx staining in squamous cell section (IB), strong membranous EpEx staining in both poorly differentiated section and normal section (IC, ID). Nuclear and cytoplasmic Ep-ICD staining in undifferentiated and poorly differentiated sections (IIA-IIC), membranous and cytoplasmic staining in normal tissue (IID). Nuclear and cytoplasmic β catenin staining in anaplastic thyroid cancer section (IIIA), membranous β-catenin staining in the other subsets of this specimen (IIIB-IIID).

Figure 3. Box-Plot analysis of EpEx, Ep-ICD and β-catenin expression in thyroid cancers. Box plots showing distribution of total immunostaining scores determined by immunohistochemistry in paraffin-embedded sections of normal thyroid tissues and different types of thyroid cancers. The vertical axis gives the total immunostaining score, obtained as described in Example 1. Panel A shows box plots for EpEx staining – I depicts membranous localization in normal tissues and PTCs, no detectable expression in ATCs and varying reduced expressions in FTC and SCC (with a median score of 3, bold horizontal line). Panel AII depicts cytoplasmic localization of EpEx in normal tissues, PTCs, PDPTC, PDFTC and FTCs, no detectable expression in ATCs and varying reduced expression in SCCs. Panel AIII depicts no detectable EpEx nuclear localization in normal tissues and all subtypes of thyroid cancers. Panel B shows box plots for Ep-ICD staining. Panel BI shows variable Ep-ICD membrane localization in normal tissues, PTCs, ATCs, FTCs and SCCs, PDPTC and PDFTC. Panel BII depicts cytoplasmic Ep-ICD localization in normal tissues, PTCs, ATCs, FTCs and SCCs, PDPTC and PDFTC. Panel BIII depicts nuclear localization in ATCs and varying expression in SCCs, (with a median score of 3, bold horizontal line, range 0–4, as shown by vertical bars), as compared to PTCs, FTCs, PDPTC, PDFTC and normal thyroid tissues with a median score of 0. Panel C shows box plots for β-catenin staining- CI depicts nuclear staining in ATCs only. Panel CII shows cytoplasmic β-catenin in all the subtypes of thyroid cancers analysed. Panel CIII shows membranous β-catenin in normal tissues and all the subtypes of thyroid cancers analyzed except most of the ATCs. Panel D shows Ep-ICD nuclear staining in different subtypes of thyroid cancers using the Visiopharm Integrator

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System. All the ATCs and one PDPTC and one PDFTC analyzed showed nuclear Ep-ICD expression.

Figure 4. Quantitative Real Time PCR analysis of EpCAM in human thyroid primary tumors. The histogram shows the levels of EpCAM transcripts in different subsets of thyroid cancers.

Figure 5. Kaplan-Meier estimation of cumulative proportion of overall survival: (A) loss of membranous EpEx expression. (B) Nuclear Ep-ICD accumulation. (C) Nuclear β-catenin accumulation. (D) concomitant nuclear Ep-ICD and β-catenin expression in thyroid cancers.

Figure 6. EpCAM expression in human thyroid cancer derived cell lines. (A) Panel I- Immunocytochemistry- EpEx staining was localized to the plasma membrane in ARO (colon cancer cells, previously considered as ATC cells), WRO (colon cancer cells, previously considered aggressive follicular thyroid cancer cells), and TT (medullary thyroid cancer cells); cytoplasmic Ep-Ex was detected in CAL-62, while no EpEx staining was observed in TPC-1 (low-grade papillary thyroid cancer cells) (Original magnification x 200). Panel II- Immunocytochemistry with Ep-ICD (1144). Ep-ICD staining was localized to the plasma membrane and cytoplasm in ARO (colon cancer cells, previously considered as ATC cells), WRO (colon cancer cells, previously considered aggressive follicular thyroid cancer cells), and TT (medullary thyroid cancer cells); cytoplasmic Ep-ICD was detected in CAL-62, while no Ep-ICD staining was observed in TPC-1 (low-grade papillary thyroid cancer cells) (Original magnification x 200). Panel III. Immunofluorescence-EpEx staining was localized to the plasma membrane of ARO, WRO, and TT (middle panel) and in cytoplasm in CAL-62 (Original magnification x 400). Panel IV- To define the nuclear localization, 4'-6-Diamidino-2-phenylindole (DAPI) nucleic acid staining (Original magnification x 400) is shown. (B) Immunofluorescence analysis. Intense EpEx staining with MOC-31 was localized to the plasma membrane in ARO and WRO cells while Ep-ICD staining was cytoplasmic and nuclear in CAL 62 cells (Original magnification x 400). (C) Western Blot analysis of EpCAM expression in the same panel of thyroid cancer cell lines. The cell lysates were separated by SDS-PAGE, and were probed for EpCAM using antibody to EpCAM (B302) (upper panel). To ensure the equal loading, the same lysates were probed for beta-actin (lower panel). A 40kDa band is observed in ARO, WRO and TT cells, but no band was detected in TPC-1 cells. (D) Quantitative Real Time PCR analysis of EpCAM in the same

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panel of thyroid cancer cell lines. The ratio of EpCAM to GAPDH in ARO, WRO, TT cells is shown, while no transcripts could be quantitated in TPC-1 cells. (E) Immunofluorescence EpEx staining with MOC-31 and β-catenin in the same panel of thyroid cancer cell lines. (F) Immunofluorescence EpEx staining with MOC-31 and c-myc in the same panel of thyroid cancer cell lines.

Figure 7 shows inhibition of EpCAM-positive thyroid cancer cell proliferation upon treatment of cancer cell lines and a positive control colon cancer cell line with the immunotoxin VB4-845/VB6-845.

Figure 8 shows the effects of VB4-845 on EpCAM expression in cell lines determined by Western blotting before and after treatment with different concentrations of VB4-845.

Figure 9 shows tumor size variation in SCID mice treated with Thyroid papillary carcinoma-1 (TPC-1) cells and VB4 (A) and PBS (B).

Figure 10 is a scatter plot showing EpEx Membrane Staining in Thyroid Cancers.

Figure 11 is a scatter plot showing EpEx Cytoplasmic Staining in Thyroid Cancers.

Figure 12 is a scatter plot showing Ep-ICD Membrane Staining in Thyroid Cancers.

Figure 13 is a scatter plot showing Ep-ICD Cytoplasmic Staining in Thyroid Cancers.

Figure 14 is a scatter plot showing Ep-ICD nuclear Staining in Thyroid Cancers.

Figure 15 is an ROC curve analysis of EpICD nuclear staining to distinguish ATC from PTC.

Figure 16 is an ROC analysis of EpEx Membrane staining to distinguish ATC from PTC.

Figure 17 shows an immunohistochemical analysis of EpEx and Ep-ICD expression in Thyroid Tumors. The photomicrographs show membrane expression of EpEx staining in thyroid benign tumor (A), thyroid non-aggressive malignant tumor (C), thyroid aggressive malignant tumor (E) and (G); Ep-ICD nuclear expression is observed in thyroid benign tumor (B), thyroid non-aggressive malignant tumor (D), thyroid aggressive malignant tumor (F) and (H). M, Membrane staining; N, nuclear staining. All the photomicrographs are at original magnification x 400.

Figure 18 is a Scatter Plot Analysis of Membrane EpEx Expression in Filipino patients. Scatter plot showing distribution of total immunostaining scores in thyroid benign tumors, clinically non-aggressive and aggressive thyroid malignant tumors. The vertical axis gives the total immunohistochemical staining score as described in the examples. A cutoff of

$\geq$ 4 was used to determine positivity. Decreased membrane expression of EpEx was observed in most of the aggressive Filipino malignant tumor cases analyzed; high membrane EpEx expression was observed in all of the benign tumor cases and non-aggressive malignant tumor cases. A cutoff score of $\leq$ 4 was used to determine positivity (Loss of Membrane expression).

Figure 19 is a Scatter Plot Analysis of Nuclear Ep-ICD Expression in Filipino patients. Scatter plots showing distribution of total immunostaining scores determined in thyroid benign tumors, clinically non-aggressive and aggressive thyroid malignant tumors. The vertical axis gives the total immunohistochemical staining score as described in the examples. A cutoff of $\geq$4 was used to determine positivity. Increased nuclear expression of Ep-ICD was observed in almost all aggressive Filipino thyroid malignant tumors analyzed, but not in benign tumors and nonaggressive malignant tumor cases.

Figure 20 shows Receiver operating characteristic (ROC) curves of membrane EpEX (A,C) and nuclear Ep-ICD (B,D) in Filipino thyroid benign tumors, non-aggressive and aggressive cancers. ROC curves were generated based on the sensitivities and 1-specificities of membrane EpEx and nuclear Ep-ICD expression. The vertical axis indicates the sensitivity and the horizontal axis indicates the 1-specificity. The sensitivity, specificity, and area under the curve (AUC) values for the cancers are summarized in Table 8.

Figure 21 shows a Box Plot Analysis of Nuclear Ep-ICD (B) and loss of Membrane EpEx Expression (A).

Figure 22 shows a Box Plot Analysis of Membrane EpEx Expression and Nuclear Ep-ICD Expression. Box plots showing distribution of total immunostaining scores determined by immunohistochemistry of tissue sections of thyroid benign tumors, clinically nonaggressive and aggressive thyroid malignant tumors. The vertical axis gives the total immunohistochemical staining score as described in the examples. A. Decreased membrane expression of EpEx was observed in most of the aggressive Filipino malignant tumor cases analyzed; high membrane EpEx expression was observed in all of the benign tumor cases and nonaggressive malignant tumor cases. A cutoff of $\leq$ 4 was used to determine positivity (Loss of Membrane expression). B. Increased nuclear expression of Ep-ICD was observed in almost all aggressive Filipino thyroid malignant tumors analyzed, but not in benign tumors and nonaggressive malignant tumor cases. A cutoff of $\geq$ 4 was used to determine positivity.

DETAILED DESCRIPTION OF THE INVENTION

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The invention relates to newly discovered correlations between expression of Thyroid Cancer Markers and thyroid cancer, in particular aggressiveness or metastatic potential of a thyroid cancer, more particularly ATC. The Thyroid Cancer Markers described herein provide methods for diagnosing, detecting or characterizing thyroid cancer, in particular

5   aggressiveness or metastatic potential of a thyroid cancer, more particularly ATC. Methods are provided for diagnosing or detecting the presence or absence of 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.

10   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 invention 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

15   invention, particular materials and methods are described herein.

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

20   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.

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,

25   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 invention, the thyroid cancer is medullary thyroid cancer. In aspects of the invention, the thyroid cancer is an aggressive cancer or has metastatic potential, in particular an aggressive medullary or

30   follicular thyroid cancer or a medullary or follicular thyroid cancer with metastatic potential. In aspects of the invention, the thyroid cancer is anaplastic thyroid carcinoma (ATC).

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"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.

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
5   Polypeptide Thyroid Cancer Markers. The sample may be derived from a biological source ("biological sample"), such as tissues (e.g., biopsy samples), 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
10 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 invention, the sample is a human physiological fluid, such as human serum. In certain aspects of the invention, the sample is a biopsy sample. In certain aspects of the invention the sample is a benign, malignant, or normal tissue sample.

15   The samples that may be analyzed in accordance with the invention 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,
20 poly(A)$^+$ messenger RNA (mRNA) or fraction thereof, cytoplasmic mRNA, or RNA transcribed from cDNA (i.e., cRNA).

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
25 label can be, without limitation, a luminescent label, fluorescent label, bio-luminescent label, chemi-luminescent label, radiolabel, and colorimetric label.

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
30 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,

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or lack thereof, during and after the course of treatment.

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, or suspected of having, being pre-disposed to, or being screened for thyroid cancer, in particular actual or

5   suspected aggressive thyroid cancer or metastatic potential, more particularly ATC. The term includes but is not limited to domestic animals, sports animals, primates and humans. Preferably, the terms refer to a human.

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

10   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

15   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 cancer, in particular aggressive or metastatic thyroid cancer, more particularly ATC. Risk factors include, but are not limited to, gender, age, genetic predisposition, environmental exposure, previous incidents of cancer, preexisting

20   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

25   Thyroid Cancer Markers disclosed herein.

As used herein, the term "treat" or "treating" refers to any method used to partially or completely alleviate, ameliorate, relieve, inhibit, prevent, delay onset of, reduce severity of and/or reduce incidence of one or more symptoms or features of a particular condition. Treatment may be administered to a subject who does not exhibit signs of a condition and/or

30   exhibits only early signs of the condition for the purpose of decreasing the risk of developing pathology associated with the condition. Thus, depending on the state of the subject, the term in some aspects of the invention may refer to preventing a condition, and includes preventing

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the onset, or preventing the symptoms associated with a condition. The term also includes maintaining the condition and/or symptom such that the condition and/or symptom do not progress in severity. A treatment may be either performed in an acute or chronic way. The term also refers to reducing the severity of a condition or symptoms associated with such
5   condition prior to affliction with the condition. Such prevention or reduction of the severity of a condition prior to affliction refers to administration of a therapy to a subject that is not at the time of administration afflicted with the condition. Preventing also includes preventing the recurrence of a condition, or of one or more symptoms associated with such condition. The terms "treatment" and "therapeutically" refer to the act of treating, as "treating" is
10   defined above. The purpose of intervention is to combat the condition and includes the administration of therapy to prevent or delay the onset of the symptoms or complications, or alleviate the symptoms or complications, or eliminate the condition.

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

The term "EpCAM" refers to a type I membrane protein comprising an epidermal
20   growth factor (EGF)-like domain and a thyroglobulin repeat domain. In particular, it is composed of a large extracellular domain (265 amino acids) (EpEx), a single transmembrane part of 23 amino acids (amino acids 266-288 in SEQ ID NO. 1), and a short cytoplasmic domain of 26 amino acids (Ep-ICD – amino acids 289-413 in SEQ ID NO. 1). Two EGF-like repeats are located within the extracellular domain (Balzar et al., 2001). The mature enzyme
25   consists of 314 amino acids. [See Baeuerie PA and O Gires, British Journal of Cancer (2007) 96, pages 417-423 for a review of EpCAM (CD326).] The term includes native-sequence polypeptides, isoforms, precursors, and chimeric or fusion proteins of EpCAM, in particular human EpCAM. EpCAM polypeptides that can be employed in the present invention include, without limitation, polypeptides comprising the sequences found in Accession No.
30   NP_002345 and SEQ ID NO. 1. In particular aspects of the invention, domains of EpCAM are utilized in the methods of the present invention, in particular Ep-ICD and EpEx.

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The term "β-catenin" refers to an adherens junction protein which contains several armadillo repeats, i.e. sequences of approximately 50 amino acids involved in protein-protein interactions. Each repeat consists of three helices, with helix 1 and 3 antiparallel to each other and perpendicular to helix 2, and a conserved glycine residue that allows the sharp turn between helices 1 and 2 (see Aberle H, et al, J Cell Sci. 1994 Dec;107 (Pt 12):3655-63; van Hengel, J., et al, Cytogenet. Cell Genet. 70 (1-2), 68-70 (1995)). The term includes native-sequence polypeptides, isoforms, precursors, and chimeric or fusion proteins of β-catenin, in particular human β-catenin. β-catenin polypeptides that can be employed in the present invention include, without limitation, polypeptides comprising the sequences found in Swiss-Prot Accession No: P35222.1, Genbank NP_001091679 and SEQ ID NO. 7.

"Wnt Proteins" refers to a family of highly conserved secreted signaling molecules that regulate cell-to-cell interactions during embryogenesis. Wnt Proteins include proteins that regulate the production of Wnt signaling molecules, their interactions with receptors on target cells and the physiological responses of target cells that result from contact of cells with Wnt ligands, includes target proteins. Wnt Proteins include without limitation Wnt proteins (e.g., Wnt1, Wnt3, Wnt4, Wnt5B, Wnt7A, Wnt10A, Wnt10B), cell-surface receptors of the Frizzled (FRZ) family, Dishevelled family proteins, axin proteins (e.g. Axin1, Axin2), WTX, PORC1, RSPO4, VANGL1, GSK-3, APC, TCF/LEF family transcription factors (e.g. TCF4), the transmembrane protein LRP, sclerostin, trimeric G proteins, CK1, GSK3, Norrin, WTX, PORC1, RSPO4, VANGL1, and target proteins such as C-myc. (See MacDonald BT, et al, Dev Cell. 2009 Jul;17(1):9-26; Cadigan KM Curr Biol. 2008 Oct 28;18(20):R943-7.)

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

The term "polypeptide variant" means a polypeptide having at least about 10%, 20%, 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% amino acid sequence identity, particularly at least about 70-80%, more particularly at least about 85%, still more particularly at least about 90%, most particularly at least about

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95%, 97%, or 99% amino acid sequence identity with a native-sequence polypeptide. Particular polypeptide variants have at least 70-80%, 85%, 90%, 95%, 97% or 99% amino acid sequence identity to the sequences identified in Accession No. NP_002345 and SEQ ID NO: 1 or Swiss-Prot Accession No: P35222.1, Genbank NP_001091679 and SEQ ID NO. 7.

5   Such variants include, for instance, polypeptides wherein one or more amino acid residues are added to, or deleted from, the N- or C-terminus of the full-length or mature sequences of the polypeptide, including variants from other species, but exclude a native-sequence polypeptide. In aspects of the invention variants retain the immunogenic activity of the corresponding native-sequence polypeptide.

10   Sequence identity of two amino acid sequences or of two nucleic acid sequences is defined as the percentage of amino acid residues or nucleotides in a candidate sequence that are identical with the amino acid residues in a polypeptide or nucleic acid sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence

15   identity. Alignment for purposes of determining percent amino acid or nucleic acid sequence identity can be achieved in various conventional ways, for instance, using publicly available computer software including the GCG program package (Devereux J. et al., Nucleic Acids Research 12(1): 387, 1984); BLASTP, BLASTN, and FASTA (Atschul, S.F. et al. J. Molec. Biol. 215: 403-410, 1990). The BLAST X program is publicly available from NCBI and

20   other sources (BLAST Manual, Altschul, S. et al. NCBI NLM NIH Bethesda, Md. 20894; Altschul, S. et al. J. Mol. Biol. 215: 403-410, 1990). Skilled artisans can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. Methods to determine identity and similarity are codified in publicly available computer programs.

25   Polypeptide variants include polypeptides comprising amino acid sequences sufficiently identical to or derived from the amino acid sequence of a native polypeptide which includes fewer amino acids than the full-length polypeptides. A portion or fragment of a polypeptide can be a polypeptide which is for example, 3-5, 8-10, 10, 15, 15-20, 20, 25, 30, 35, 40, 45, 50,60, 70, 80, 90, 100 or more amino acids in length. Portions or fragments in

30   which regions of a polypeptide are deleted can be prepared by recombinant techniques and can be evaluated for one or more functional activities such as the ability to form antibodies

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specific for a polypeptide. A portion or fragment of a polypeptide may comprise a domain of the polypeptide, in particular an extracellular domain or intracellular domain.

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

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

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

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

"Polynucleotide" refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. The term includes double- and single-stranded DNA and RNA, modifications such as methylation or capping and unmodified forms of the polynucleotide. The terms "polynucleotide" and "oligonucleotide" are used interchangeably

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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. Polynucleotides for use in the methods of the invention may be of any length suitable for a particular method. In certain applications the term refers to antisense nucleic

5   acid molecules (e.g. an mRNA or DNA strand in the reverse orientation to a sense Polynucleotide Thyroid Cancer Markers).

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,

10   and a chimeric polypeptide. A polynucleotide encoding an EpCAM polypeptide that can be employed in the present invention includes, without limitation, nucleic acids comprising a sequence of Accession No. UniProtKB/TrEMBL Q6FG26, UniProtKB/Swiss-Prot 16422, Genbank NM_002354 and BC014785 or SEQ ID NO. 2 or fragments thereof. A polynucleotide encoding a β-catenin polypeptide that can be employed in the present

15   invention includes, without limitation, nucleic acids comprising a sequence of GenBank Accession Nos. NM_001904.3, NM_001098209, or NM_001098210, or SEQ ID NO. 8, 9 or 10.

Polynucleotides used in the methods of the invention include complementary nucleic acid sequences, and nucleic acids that are substantially identical to these sequences (e.g. at

20   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).

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

25   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.

Polynucleotides which may be used in the methods disclosed herein also include

30   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

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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 (Tm) for the specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength, pH,

5   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,

10   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 6xSCC, 0.5% SDS and 50% formamide followed by washing in a solution of 0.1x SCC and 0.5% SDS at 68°C.

Polynucleotide Thyroid Cancer Markers also include truncated nucleic acids or

15   nucleic acid fragments and variant forms of the nucleic acids disclosed or referenced herein that arise by alternative splicing of an mRNA corresponding to a DNA. A fragment of a polynucleotide includes a polynucleotide sequence that comprises a contiguous sequence of approximately at least about 6 nucleotides, in particular at least about 8 nucleotides, more particularly at least about 10-12 nucleotides, and even more particularly 15-20 nucleotides

20   that correspond to (i.e. identical or complementary to), a region of the specified nucleotide sequence.

"Significantly different" levels of markers or a "significant difference" in marker levels in a patient sample compared to a control or standard (e.g. normal levels, levels from a different disease stage, or levels in other samples from a patient) may represent levels that are

25   higher or lower than the standard error of the detection assay, preferably the levels are at least about 1.5, 2, 3, 4, 5, 6, 7, 8, 9 or 10 times higher or lower, respectively, than the control or standard.

"Microarray" and "array," refer to nucleic acid or nucleotide arrays or protein or peptide arrays that can be used to detect biomolecules associated with thyroid cancer, for

30   instance to measure gene expression. A variety of arrays are available commercially, such as, for example, as the *in situ* synthesized oligonucleotide array GeneChip™ made by Affymetrix, Inc. or the spotted cDNA array, LifeArray™ made by Incyte Genomics Inc.. The

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preparation, use, and analysis of microarrays are well known to those skilled in the art. (See, for example, Brennan, T. M. et al. (1995) U.S. Pat. No. 5,474,796; Schena, et al. (1996) Proc. Natl. Acad. Sci. 93:10614-10619; Baldeschweiler et al. (1995), PCT Application WO95/251116; Shalon, D. et al. (I 995) PCT application WO95/35505; Heller, R. A. et al.

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

"Binding agent" refers to a substance such as a polypeptide, antibody, ribosome, or aptamer that specifically binds to a Polypeptide Thyroid Cancer Marker. A substance "specifically binds" to a Polypeptide Thyroid Cancer Marker if it reacts at a detectable level

10   with the polypeptide, and does not react detectably with peptides containing unrelated sequences or sequences of different polypeptides. Binding properties may be assessed using an ELISA, which may be readily performed by those skilled in the art.

A binding agent may be a ribosome, with or without a peptide component, an RNA or DNA molecule, or a polypeptide. A binding agent may be a polypeptide that comprises a

15   Polypeptide Thyroid Cancer Marker sequence, a peptide variant thereof, or a non-peptide mimetic of such a sequence. By way of example a Polypeptide Thyroid Cancer Marker sequence may be a peptide portion of the polypeptide that is capable of modulating a function mediated by the polypeptide.

An aptamer includes a DNA or RNA molecule that binds to nucleic acids and

20   proteins. An aptamer that binds to a Thyroid Cancer Marker can be produced using conventional techniques, without undue experimentation. [For example, see the following publications describing *in vitro* 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);

25   and Uphoff et al., Curr. Opin. Struct. Biol. 6:281-287 (1996)].

Antibodies for use in the present invention 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,

30   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

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antibodies, but in which the remaining portions are of human origin), derivatives, such as enzyme conjugates or labelled 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

5   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 invention the antibodies are IgG antibodies or a class or subclass thereof. An antibody may be from any

10   animal origin including birds and mammals (e.g. human, murine, donkey, sheep, rabbit, goat, guinea pig, camel, horse, or chicken).

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

15   antibody libraries, antibodies isolated from an animal (e.g. a mouse or cow) that is transgenic and/or transchromosomal for human immunoglobin genes, or antibodies prepared, expressed, created or isolated by any other means that involves slicing of immunoglobulin gene sequences to other DNA sequences.

A "monoclonal antibody" refers to an antibody obtained from a population of

20   homogenous or substantially homogenous antibodies. Generally each monoclonal antibody recognizes a single epitope on an antigen. In aspects of the invention, 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

25   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.

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 Polypeptide Thyroid Cancer Markers may be utilized to prepare antibodies. See,

30   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.

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(1989) Science 246:1275-1281 for the preparation of monoclonal Fab fragments; and, Pound (1998) Immunochemical Protocols, Humana Press, Totowa, N.J for the preparation of phagemid or B-lymphocyte immunoglobulin libraries to identify antibodies. Antibodies specific for Polypeptide Thyroid Cancer Markers may also be obtained from scientific or

5   commercial sources. In an embodiment of the invention, antibodies are reactive against Polypeptide Thyroid Cancer Markers if they bind with a K_a of greater than or equal to 10^{-7} M.

Examples of antibodies specific for EpCAM polypeptides are shown in Table 1.

The "status" of a marker refers to the presence, absence or extent/level of the marker or some physical, chemical or genetic characteristic of the marker. Such characteristics

10   include without limitation, expression level, activity level, structure (sequence information), copy number, post-translational modification etc. The status of a marker may be directly or indirectly determined. In some embodiments status is determined by determining the level of a marker in the sample. The "level" of an element in a sample has its conventional meaning in the art, and includes quantitative determinations (e.g. mg/mL, fold change, etc) and

15   qualitative determinations (e.g. determining the presence or absence of a marker or determining whether the level of the marker is high, low or even present relative to a standard).

The term "abnormal status" means that a marker's status in a sample is different from a reference status for the marker. A reference status may be the status of the marker in

20   samples from normal subjects, averaged samples from subjects with the condition or sample(s) from the same subject taken at different times. An abnormal status includes an elevated, decreased, present or absent marker(s). Determining the level of a marker in a sample may include determining the level of the marker in a sample and abnormal status could be either lower levels (including undetectable levels) or higher levels (including any

25   amount over zero) compared to a standard. A subject may have an increased likelihood of a condition disclosed herein if the status of a marker in the subject's sample is correlated with the condition (e.g. a level of the marker is closer to a standard or reference or is present in levels that exceed some threshold value where exceeding that value is correlated with the condition). A subject with an increased likelihood of a condition disclosed herein includes a

30   subject with an abnormal status for a marker and as such the subject has a higher likelihood of the condition than if the subject did not have that status.

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An "elevated status" means one or more characteristics of a marker are higher than a standard. In aspects of the invention, the term refers to an increase in a characteristic as compared to a standard. A "low status" means one or more characteristics of a marker are lower than a standard. In aspects of the invention, the term refers to a decrease in a

5   characteristic as compared to a standard. A "negative status" means that one or more characteristic of a marker is absent or undetectable.

General Methods

A variety of methods can be employed for the diagnostic and prognostic evaluation of thyroid cancer involving Thyroid Cancer Markers and the identification of subjects with a

10   predisposition to such disorders. Such methods may, for example, utilize Polynucleotide Thyroid Cancer Markers and fragments thereof, and binding agents (e.g. antibodies) directed against Polypeptide Thyroid Cancer 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

15   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

20   progression toward a disorder state.

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, higher levels of nuclear Ep-ICD, nuclear β-

25   catenin or cytoplasmic β-catenin are indicative of aggressive thyroid cancer or metastatic thyroid cancer, in particular ATC.

In an aspect, the invention contemplates a method for determining the aggressiveness or stage of thyroid cancer, more particularly ATC, comprising producing a profile of levels of Polypeptide Thyroid Cancer Markers, and other markers associated with thyroid cancer, in

30   cells from a patient, and comparing the profile with a reference to identify a profile for the test cells indicative of aggressiveness or stage of disease.

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The methods of the invention 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

5   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

10   quantitated for the same subject.

The invention also contemplates the methods described herein using multiple markers for thyroid cancer. Therefore, the invention 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

15   reagents to detect the other markers or polynucleotides encoding the markers. Examples of other markers include without limitation galectin-3, thyroglobulin, E-cadherin, beta-catenin, FHL2 and Lef-1, c-Myc, and beta–actin, in particular galectin-3.

Nucleic Acid Methods

As noted herein thyroid cancer, in particular aggressive thyroid cancer or a thyroid

20   cancer with metastatic potential, more particularly ATC, 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.

Probes may be used in hybridization techniques to detect polynucleotides. The

25   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

30   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

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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

5   sequences. Computer programs known in the art can be used to design primers with the required specificity and optimal amplification properties.

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, ^{3}H, ^{14}C or the like. Other detectable substances that may be used include antigens that are recognized by

10   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

15   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.

20   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

25   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.

In order to maximize hybridization under assay conditions, primers and probes employed in the methods of the invention generally have at least about 60%, preferably at

30   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

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preferably at least 20 nucleotides in length. In an embodiment the primers and probes are at least about 10-40 nucleotides in length. Examples of primers are SEQ ID NOs. 3-6.

Hybridization and amplification reactions may also be conducted under stringent conditions as discussed herein.

5   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.

The primers and probes may be used *in situ* i.e., directly on tissue sections (fixed
10   and/or frozen) of patient tissue obtained from biopsies or resections.

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

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

Oligonucleotides or longer fragments derived from Polynucleotide Thyroid Cancer Markers may be used as targets in a microarray. The microarray can be used to monitor the
30   expression levels of the polynucleotides and to identify genetic variants, mutations, and polymorphisms. The information from the microarray may be used to determine gene function, to understand the genetic basis of a disorder, to diagnose a disorder, and to develop

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and monitor the activities of therapeutic agents. Thus, the invention also includes an array comprising Polynucleotide Thyroid Cancer Markers, and optionally other thyroid cancer markers. The array can be used to assay expression of Polynucleotide Thyroid Cancer Markers in the array. The invention allows the quantitation of expression of the polynucleotides.

The invention provides microarrays comprising Polynucleotide Thyroid Cancer Markers. In one embodiment, the invention provides a microarray for distinguishing samples associated with thyroid cancer, in particular aggressive thyroid cancer or thyroid cancer with metastatic potential, in particular ATC, comprising a positionally-addressable array of polynucleotide probes bound to a support, the polynucleotide probes comprising sequences complementary and hybridizable to Polynucleotide Thyroid Cancer Markers.

In an embodiment, the array can be used to monitor the time course of expression of Polynucleotide Thyroid Cancer Markers in the array. This can occur in various biological contexts such as tumor progression.

An array can also be useful for ascertaining differential expression patterns of Polynucleotide Thyroid Cancer Markers, and optionally other thyroid cancer markers in normal and abnormal cells. This may provide a battery of nucleic acids that could serve as molecular targets for diagnosis or therapeutic intervention.

Protein Methods

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, the presence or absence of an aggressive thyroid cancer or a thyroid cancer with metastatic potential, in particular ATC, in a subject may be determined by (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 invention, the binding agent is an antibody.

In an aspect, the invention 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

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for Polypeptide Thyroid Cancer Markers which are directly or indirectly labeled with detectable substances and detecting the detectable substances.

In an aspect of the invention, a method for detecting or diagnosing aggressiveness or metastatic potential of a thyroid cancer, in particular ATC, 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 aggressive thyroid cancer or a thyroid cancer with metastatic potential, in particular ATC.

In an embodiment, the invention 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.

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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 or from the same individual at a different point in time.

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.

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-cantilivers. 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.

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

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detectable substance. Examples of detectable substances include, but are not limited to, the following: radioisotopes (e.g., ^{3}H, ^{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.

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-steriod isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate, triose phosphate isomerase, asparaginase, glucose oxidase, and acetylcholine esterase.

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.

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.

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

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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

5   substance.

In the context of the methods of the invention, 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,

10   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)

15   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.

Where a radioactive label is used as a detectable substance, a Polypeptide Thyroid

20   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 the grains.

Time-resolved fluorometry may be used to detect a fluorescent signal, label, or detectable substance. For example, the method described in Christopoulos TK and Diamandis

25   EP Anal. Chem., 1992:64:342-346 may be used with a conventional time-resolved fluorometer.

According to an embodiment of the invention, an immunoassay for detecting Polypeptide Thyroid Cancer Markers in a biological sample comprises contacting an amount of a binding agent that specifically binds to Polypeptide Thyroid Cancer Markers in the

30   sample under conditions that allow the formation of a complex(es) comprising the binding agent and Polypeptide Thyroid Cancer Markers and determining the presence or amount of the complex(es) as a measure of the amount of the Polypeptide Thyroid Cancer Markers

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contained in the sample.

In accordance with an embodiment of the invention, a method is provided wherein Polypeptide Thyroid Cancer Marker antibodies are directly or indirectly labelled with enzymes, substrates for the enzymes are added wherein the substrates are selected so that the substrates, or a reaction product of an enzyme and substrate, form fluorescent complexes with lanthanide metals, preferably europium and terbium. A lanthanide metal(s) is added and Polypeptide Thyroid Cancer Markers are quantitated in the sample by measuring fluorescence of the fluorescent complexes. Enzymes are selected based on the ability of a substrate of the enzyme, or a reaction product of the enzyme and substrate, to complex with lanthanide metals.

Examples of enzymes and substrates for enzymes that provide such fluorescent complexes are described in U.S. Patent No. 5,312,922 to Diamandis. By way of example, when the antibody is directly or indirectly labelled with alkaline phosphatase the substrate employed in the method may be 4-methylumbelliferyl phosphate, 5-fluorosalicyl phosphate, or diflunisal phosphate. The fluorescence intensity of the complexes is typically measured using a time-resolved fluorometer.

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

Aspects of the methods of the invention involve (a) reacting a biological sample from a subject with antibodies specific for Polypeptide Thyroid Cancer Markers wherein the antibodies are directly or indirectly labelled with enzymes; (b) adding substrates for the enzymes wherein the substrates are selected so that the substrates, or reaction products of the enzymes and substrates form fluorescent complexes; (c) quantitating Polypeptide Thyroid Cancer Markers in the sample by measuring fluorescence of the fluorescent complexes; and (d) comparing the quantitated levels to levels obtained for other samples from the subject patient, or control subjects. In an embodiment, the Polypeptide Thyroid Cancer Markers are Ep-ICD and β-catenin and the quantitated levels are compared to levels quantitated for normal subjects, subjects with an early stage of disease or the same subject at a different

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point in time, wherein an increase in the levels of the markers compared with the control subjects is indicative of ATC and/or poor prognosis or survival.

A particular embodiment of the invention 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. Increased levels of Ep-ICD and/or β-catenin as compared to the standard may be indicative of anaplastic thyroid carcinoma.

In accordance with an embodiment, the present invention 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.

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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"),

5   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

10   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,

15   the label may be measured without separating the capture antibody and liquid test mixture.

In particular sandwich immunoassays of the invention mouse polyclonal/monoclonal antibodies   specific   for   Polypeptide   Thyroid   Cancer   Markers   and   rabbit polyclonal/monoclonal antibodies specific for Polypeptide Thyroid Cancer Markers are utilized.

20   In a typical two-site immunometric assay for Polypeptide Thyroid Cancer Markers one or both of the capture and detection antibodies are polyclonal antibodies or one or both of the capture and detection antibodies are monoclonal antibodies (i.e. polyclonal/polyclonal, monoclonal/monoclonal, or monoclonal/polyclonal). The label used in the detection antibody can be selected from any of those known conventionally in the art. The label may be an

25   enzyme or a chemiluminescent moiety, but it can also be a radioactive isotope, a fluorophor, a detectable ligand (e.g., detectable by a secondary binding by a labeled binding partner for the ligand), and the like. In an aspect, the antibody is labelled with an enzyme which is detected by adding a substrate that is selected so that a reaction product of the enzyme and substrate forms fluorescent complexes. The capture antibody may be selected so that it

30   provides a means for being separated from the remainder of the test mixture. Accordingly, the capture antibody can be introduced to the assay in an already immobilized or insoluble form, or can be in an immobilizable form, that is, a form which enables immobilization to be

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accomplished subsequent to introduction of the capture antibody to the assay. An immobilized capture antibody may comprise an antibody covalently or noncovalently attached to a solid phase such as a magnetic particle, a latex particle, a microtiter plate well, a bead, a cuvette, or other reaction vessel. An example of an immobilizable capture antibody is

5   antibody which has been chemically modified with a ligand moiety, e.g., a hapten, biotin, or the like, and which can be subsequently immobilized by contact with an immobilized form of a binding partner for the ligand, e.g., an antibody, avidin, or the like. In an embodiment, the capture antibody may be immobilized using a species specific antibody for the capture antibody that is bound to the solid phase.

10   **Screening Methods**

The invention also contemplates methods for evaluating test agents or compounds for their potential efficacy in treating thyroid cancer, in particular aggressive thyroid cancer, more particularly ATC. Test agents and compounds include but are not limited to peptides such as soluble peptides including Ig-tailed fusion peptides, members of random peptide

15   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

20   (e.g. antisense, siRNA), and small organic or inorganic molecules. The agents or compounds may be endogenous physiological compounds or natural or synthetic compounds.

The invention provides a method for assessing a test agent for potential efficacy in treating thyroid cancer, in particular aggressive thyroid cancer, more particularly ATC, the method comprising comparing:

25   (a)   levels of one or more Thyroid Cancer Markers, and optionally other markers 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

30   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

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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.

In an aspect, the invention provides a method of selecting an agent for treating thyroid
5   cancer, in particular aggressive thyroid cancer, more particularly ATC, 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;

10   (c)   comparing one or more Thyroid Cancer Markers, and optionally other markers, in each of the aliquots; and

(d)   selecting one of the test agents which alters the levels of one or more Thyroid Cancer Markers, and optionally other markers in the aliquot containing that test agent, relative to other test agents.

15   In an aspect, the invention provides a method of selecting an agent for inhibiting thyroid cancer in a subject the method comprising (a) obtaining a sample comprising cancer cells from the subject; (b) separately exposing aliquots of the sample in the presence of a plurality of test agents; (c) comparing levels of one or more Thyroid Cancer Markers in each of the aliquots; and (d) selecting one of the test agents which alters the levels of Thyroid
20   Cancer Markers in the aliquot containing that test agent, relative to other test agents, wherein the thyroid cancer markers are Ep-ICD and/or β-catenin. This method may further comprise administering to a subject at least one of the test agents which alters the levels of Thyroid Cancer Markers in the aliquot containing that test agent, relative to other test agents.

In an aspect the invention provides a method of assessing the thyroid cancer cell
25   carcinogenic potential of a test compound, the method comprising: (a) maintaining separate aliquots of thyroid cancer cells in the presence and absence of the test compound; and (b) comparing expression of one or more Thyroid Cancer Markers, in each of the aliquots, and wherein a significant difference in levels of Thyroid Cancer Markers in the aliquot maintained in the presence of the test compound, relative to the aliquot maintained in the
30   absence of the test compound, is an indication that the test compound possesses thyroid cancer cell carcinogenic potential, wherein the Thyroid Cancer Markers are Ep-ICD and/or β-catenin.

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Kits

The invention contemplates kits for carrying out the methods of the invention to diagnose thyroid cancer, and in particular to detect the aggressiveness or metastatic potential of a thyroid cancer, more particularly ATC. Such kits typically comprise two or more components required for performing a diagnostic assay. Components include but are not limited to compounds, reagents, containers, and/or equipment. Accordingly, the methods described herein may be performed by utilizing pre-packaged diagnostic kits comprising at least agents (e.g. antibodies, probes, primers, etc) described herein, which may be conveniently used, e.g., in clinical settings to diagnose patients afflicted with thyroid cancer, or exhibiting a predisposition to developing thyroid cancer and in particular to determine the aggressiveness or metastatic potential of a thyroid cancer, more particularly ATC.

The invention contemplates a container with a kit comprising a binding agent(s) as described herein for diagnosing thyroid cancer, in particular determining the aggressiveness or metastatic potential of a thyroid cancer, more particularly ATC. By way of example, the kit may contain antibodies specific for Polypeptide Thyroid Cancer Markers, antibodies against the antibodies labelled with an enzyme(s), and a substrate for the enzyme(s). The kit may also contain microtiter plate wells, standards, assay diluent, wash buffer, adhesive plate covers, and/or instructions for carrying out a method of the invention using the kit.

In an aspect, the invention provides a test kit for diagnosing thyroid cancer in a subject, in particular the aggressiveness or metastatic potential of a thyroid cancer, more particularly ATC, which comprises an antibody that binds to Polypeptide Thyroid Cancer Markers and/or polynucleotides that hybridize to or amplify Polynucleotide Thyroid Cancer Markers. In another aspect the invention relates to use of an antibody that binds to a Polypeptide Thyroid Cancer Marker and/or a polynucleotide that hybridize to or amplifies a Polynucleotide Thyroid Cancer Marker, in the manufacture of a composition for diagnosing or detecting a thyroid cancer, in particular diagnosing or detecting the aggressiveness or metastatic potential of a thyroid cancer.

In a further aspect of the invention, the kit includes antibodies or antibody fragments which bind specifically to epitopes of Polypeptide Thyroid Cancer Markers and means for detecting binding of the antibodies to their epitopes associated with thyroid cancer cells, either as concentrates (including lyophilized compositions), which may be further diluted prior to testing. In particular, the invention provides a kit for diagnosing the aggressiveness or

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metastatic potential of a thyroid cancer, in particular ATC, comprising a known amount of a first binding agent that specifically binds to a Polypeptide Thyroid Cancer Marker wherein the first binding agent comprises a detectable substance, or it binds directly or indirectly to a detectable substance.

5   A kit may be designed to detect the levels of Polynucleotide Thyroid Cancer Markers in a sample. Such kits generally comprise oligonucleotide probes or primers, as described herein, which hybridize to or amplify Polynucleotide Thyroid Cancer Markers. Oligonucleotides may be used, for example, within PCR or hybridization procedures. Test kits useful for detecting target Polynucleotide Thyroid Cancer Markers are also provided 10   which comprise a container containing a Polynucleotide Thyroid Cancer Marker, and fragments or complements thereof. A kit can comprise one or more of the primers of SEQ ID NOs. 3 to 6.

The kits of the invention can further comprise containers with tools useful for collecting test samples (e.g. serum) including lancets and absorbent paper or cloth for 15   collecting and stabilizing blood.

Computer Systems

Analytic methods contemplated herein can be implemented by use of computer systems and methods described below and known in the art. Thus, the invention provides computer readable media comprising one or more Thyroid Cancer Markers. "Computer 20   readable media" refers to any medium that can be read and accessed directly by a computer, including but not limited to magnetic storage media, such as floppy discs, hard disc storage medium, and magnetic tape; optical storage media such as CD-ROM; electrical storage media such as RAM and ROM; and hybrids of these categories such as magnetic/optical storage media. Thus, the invention contemplates computer readable medium having recorded thereon 25   markers identified for patients and controls.

"Recorded" refers to a process for storing information on computer readable medium. The skilled artisan can readily adopt any of the presently known methods for recording information on computer readable medium to generate manufactures comprising information on one or more markers disclosed herein.

30   A variety of data processor programs and formats can be used to store information on one or more Thyroid Cancer Markers. For example, the information can be represented in a word processing text file, formatted in commercially-available software such as WordPerfect

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and MicroSoft Word, or represented in the form of an ASCII file, stored in a database application, such as DB2, Sybase, Oracle, or the like. Any number of dataprocessor structuring formats (e.g., text file or database) may be adapted in order to obtain computer readable medium having recorded thereon the marker information.

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

The invention provides a medium for holding instructions for performing a method 10   for determining whether a patient has thyroid cancer, in particular aggressive thyroid cancer, more particularly ATC, or a pre-disposition to such condition, comprising determining the presence or absence of one or more Thyroid Cancer Markers, and based on the presence or absence of the markers, determining the condition or a pre-disposition to the condition, optionally recommending a procedure or treatment.

15   The invention also provides in an electronic system and/or in a network, a method for determining whether a subject has a condition disclosed herein, or a pre-disposition to a condition disclosed herein, comprising determining the presence or absence of one or more markers, and based on the presence or absence of the markers, determining whether the subject has the condition or a pre-disposition to the condition, and optionally recommending 20   a procedure or treatment.

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

The invention still further provides a system for identifying selected records that 30   identify a diseased cell or tissue. A system of the invention generally comprises a digital computer; a database server coupled to the computer; a database coupled to the database server having data stored therein, the data comprising records of data comprising one or more

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markers disclosed herein, and a code mechanism for applying queries based upon a desired selection criteria to the data file in the database to produce reports of records which match the desired selection criteria.

The invention contemplates a business method for determining whether a subject has a condition disclosed herein or a pre-disposition to a condition disclosed herein, in particular ATC, comprising: (a) receiving phenotypic information on the subject and information on one or more markers disclosed herein associated with samples from the subject; (b) acquiring information from a network corresponding to the markers; and (c) based on the phenotypic information, information on the markers and acquired information, determining whether the subject has the condition or a pre-disposition to the condition, and optionally recommending a procedure or treatment.

In an aspect of the invention, the computer systems, components, and methods described herein are used to monitor a condition or determine the stage of a condition.

Therapeutic Applications

The invention contemplates therapeutic applications associated with the Thyroid Cancer Markers disclosed herein including thyroid cancer, in particular aggressive thyroid cancer, more particularly ATC. Thyroid Cancer Markers may be a target for therapy. For example, nuclear Ep-ICD can be a target for treatment of aggressive thyroid cancers and ATC. Therapeutic methods include immunotherapeutic methods including the use of antibody therapy. In one aspect, the invention provides one or more antibodies that may be used to prevent thyroid cancer, in particular aggressive thyroid cancer, more particularly ATC. In another aspect, the invention provides a method of preventing, inhibiting or reducing thyroid cancer, in particular aggressive thyroid cancer, more particularly ATC, comprising administering to a patient an antibody which binds to a Thyroid Cancer Marker (e.g. Ep-ICD), in an amount effective to prevent, inhibit, or reduce the condition or the onset of the condition.

An antibody which binds to a Thyroid Cancer Marker, in particular Ep-ICD, may be in combination with a label, drug or cytotoxic agent, a target-binding region of a receptor, an adhesion molecule, a ligand, an enzyme, a cytokine, or a chemokine. In aspects of the invention, the Thyroid Cancer Marker, in particular Ep-ICD, may be conjugated to cytotoxic agents (e.g., chemotherapeutic agents) or toxins or active fragments thereof. Examples of toxins and corresponding fragments thereof include diptheria A chain, exotoxin A chain, ricin

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A chain, abrin A chain, curcin, crotin, phenomycin, enomycin and the like. A cytotoxic agent may be a radiochemical prepared by conjugating radioisotopes to antibodies, or binding of a radionuclide to a chelating agent that has been covalently attached to the antibody. An antibody may also be conjugated to one or more small molecule toxins, such as a calicheamicin, a maytansine, a trichothene, and CC1065 (see U.S. Pat. No. 5,208,020).

The methods of the invention contemplate the administration of single antibodies as well as combinations, or "cocktails", of different individual antibodies such as those recognizing different epitopes of other markers. Such cocktails may have certain advantages inasmuch as they contain antibodies that bind to different epitopes of Thyroid Cancer Markers and/or exploit different effector mechanisms. Such antibodies in combination may exhibit synergistic therapeutic effects. In addition, the administration of one or more marker specific antibodies may be combined with other therapeutic agents. The specific antibodies may be administered in their "naked" or unconjugated form, or may have therapeutic agents conjugated to them.

The invention also contemplates a method of treating thyroid cancer in a subject, comprising delivering to the subject in need thereof, an antibody specific for Ep-CAM, in particular EpEx or Ep-ICD. In an aspect of the invention, the antibody is conjugated to a cytotoxic agent or toxin (see above). The antibody may be a therapeutic antibody disclosed for example in US Patent Nos. 7557190 and US7459538, US Published Application Nos. 20050163785 and 20070122406, and 20070196366 and McDonald et al.(Drug Design, Development and Therapy 2008;2:105-114). In a particular embodiment, the antibody is an antibody conjugated to a toxin, more particularly VB4-845 immunotoxin (Viventia Biotechnologies Inc., Ontario, Canada).

More particularly, and according to one aspect of the invention, there is provided a method of treating a subject having thyroid cancer wherein an antibody specific for Ep-CAM, in particular EpEx or Ep-ICD, is administered in a therapeutically effective amount. In a further aspect, the antibody is provided in a pharmaceutically acceptable form.

In an aspect, the invention provides a pharmaceutical composition for the treatment of thyroid cancer characterized in that the composition comprises an antibody specific for Ep-CAM, in particular EpEx or Ep-ICD, together with a pharmaceutically acceptable carrier, excipient or vehicle.

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Antibodies used in the methods of the invention may be formulated into pharmaceutical compositions comprising a carrier suitable for the desired delivery method. Suitable carriers include any material which when combined with the antibodies retains the function of the antibody and is non-reactive with the subject's immune systems. Examples

5   include any of a number of standard pharmaceutical carriers such as sterile phosphate buffered saline solutions, bacteriostatic water, and the like (see, generally, Remington's Pharmaceutical Sciences 16th Edition, A. Osal., Ed., 1980).

One or more marker specific antibody formulations may be administered via any route capable of delivering the antibodies to the site or injury. Routes of administration

10 include, but are not limited to, intravenous, intraperitoneal, intramuscular, intradermal, and the like. Antibody preparations may be lyophilized and stored as a sterile powder, preferably under vacuum, and then reconstituted in bacteriostatic water containing, for example, benzyl alcohol preservative, or in sterile water prior to injection.

Treatment will generally involve the repeated administration of the antibody

15 preparation via an acceptable route of administration at an effective dose. Dosages will depend upon various factors generally appreciated by those of skill in the art, including the etiology of the condition, stage of the condition, the binding affinity and half life of the antibodies used, the degree of marker expression in the patient, the desired steady-state antibody concentration level, frequency of treatment, and the influence of any therapeutic

20 agents used in combination with a treatment method of the invention. A determining factor in defining the appropriate dose is the amount of a particular antibody necessary to be therapeutically effective in a particular context. Repeated administrations may be required to achieve a desired effect. Direct administration of one or more marker antibodies is also possible and may have advantages in certain situations.

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

30 as levels of markers.

Polynucleotide Thyroid Cancer Markers disclosed herein can be turned off by transfecting a cell or tissue with vectors that express high levels of the polynucleotides. Such

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constructs can inundate cells with untranslatable sense or antisense sequences. Even in the absence of integration into the DNA, such vectors may continue to transcribe RNA molecules until all copies are disabled by endogenous nucleases. Vectors derived from retroviruses, adenovirus, herpes or vaccinia viruses, or from various bacterial plasmids, may be used to

5   deliver polynucleotides to a targeted organ, tissue, or cell population. Methods well known to those skilled in the art may be used to construct recombinant vectors that will express polynucleotides such as antisense. (See, for example, the techniques described in Sambrook et al (supra) and Ausubel et al (supra).)

Methods for introducing vectors into cells or tissues which are suitable for *in vivo*, *in*

10   *vitro* and *ex vivo* therapy are well known in the art. For example, delivery by transfection, or by liposome are well known in the art.

Modifications of gene expression can be obtained by designing antisense molecules, DNA, RNA or PNA, to the regulatory regions of a Polynucleotide Thyroid Cancer Marker, i.e., the promoters, enhancers, and introns. Preferably, oligonucleotides are derived from the

15   transcription initiation site, e.g. between -10 and +10 regions of the leader sequence. The antisense molecules may also be designed so that they block translation of mRNA by preventing the transcript from binding to ribosomes. Inhibition may also be achieved using "triple helix" base-pairing methodology. Triple helix pairing compromises the ability of the double helix to open sufficiently for the binding of polymerases, transcription factors, or

20   regulatory molecules. Therapeutic advances using triplex DNA are reviewed by Gee J E et al (In: Huber B E and B I Carr (1994) Molecular and Immunologic Approaches, Futura Publishing Co, Mt Kisco N.Y.).

Ribozymes are enzymatic RNA molecules that catalyze the specific cleavage of RNA. Ribozymes act by sequence-specific hybridization of the ribozyme molecule to

25   complementary target RNA, followed by endonucleolytic cleavage. The invention therefore contemplates engineered hammerhead motif ribozyme molecules that can specifically and efficiently catalyze endonucleolytic cleavage of a polynucleotide marker.

Specific ribozyme cleavage sites within any potential RNA target may initially be identified by scanning the target molecule for ribozyme cleavage sites which include the

30   following sequences, GUA, GUU and GUC. Once the sites are identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for secondary structural features which

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may render the oligonucleotide inoperable. The suitability of candidate targets may also be determined by testing accessibility to hybridization with complementary oligonucleotides using ribonuclease protection assays.

The invention provides a method of preventing, inhibiting, or reducing thyroid cancer,
5   in particular aggressive thyroid cancer, more particularly ATC, in a patient comprising:

(a)   obtaining a tumor sample from the patient;

(b)   separately maintaining aliquots of the sample in the presence of a plurality of test agents;

(c)   comparing levels of Thyroid Cancer Markers and optionally one or more other
10   markers disclosed herein, in each aliquot;

(d)   administering to the patient at least one test agent which alters the levels of Thyroid Cancer Markers and optionally other markers in the aliquot containing that test agent, relative to the other test agents.

An active therapeutic substance described herein may be administered in a convenient
15   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
20   appropriate solvent with a suitable surfactant. Dispersions may be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof, or in oils.

A composition described herein can be prepared by per se 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
25   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
30   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.

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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 invention may be administered concurrently, separately, or sequentially with other therapeutic agents or therapies.

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

The following non-limiting examples are illustrative of the present invention:

Example 1

EpEx and Ep-ICD protein expression in primary human thyroid cancers as well as in a panel of aggressive and non-aggressive thyroid cancer cell lines and by immunohistochemistry (IHC) using antibodies directed against Ep-Ex and Ep-ICD domains of EpCAM were investigated and the findings confirmed by western blotting. To determine if EpCAM overexpression is attributed to increased transcription quantitative real time PCR (Q-PCR) was used for analysis of EpCAM transcripts in these tumors. Further, concurrent staining for nuclear Ep-ICD and β-catenin was carried out to establish the prognostic value of oncogenic Ep-ICD signaling in thyroid cancer.

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

Materials and Methods

Patients and tissue specimens:

The study was approved by Ontario Ethics Committee and Mount Sinai Hospital, Toronto, Canada. All patients were informed and signed consent was obtained. Thirty thyroid carcinoma paraffin blocks were retrieved from the archives of the Department of Pathology, Mount Sinai Hospital, Toronto, Canada. Thirty fresh frozen samples comprising of 15 thyroid tumors and 15 adjacent normal tissues were also included in the study for quantitative real time PCR analysis. The tissues were flash frozen in RNAlater TissueProtect solution (Qiagen, Mississauga, ON) and stored at -80°C till use. Each case was reviewed by the pathologist prior to further experiments.

The patient follow up data were retrieved from a data bank to correlate the protein expression in tumors with clinical outcome to evaluate the prognostic relevance of these proteins. The patients were followed up for a minimum period of 15 months and a maximum period of 199 months.

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Antibodies

Anti-EpCAM monoclonal antibody MOC-31(AbD Serotec, Oxford, UK) recognizes an extracellular component (EGF1 domain- aa 27-59) in the amino-terminal region of EpCAM [Chaudry Ma et al., 2007]. Intracellular domain of EpCAM, α- EpICD antibody 1144 (Epitomics) recognizes the cytoplasmic domain of EpCAM. β-catenin antibody raised against aa 571-781 of β-catenin (Cat.# 610154, B D Sciences, San Jose, CA) and c-myc antibody (C19, sc-788, affinity purified rabbit polyclonal antibody, Santa Cruz Biotechnology Inc.).

Cell lines

The colon cancer cell line WRO (previously considered a thyroid cancer cell line) from M. Ringel, The Ohio State University, OH) and ARO - colon cancer cell line (previously considered as ATC cell line) were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2mmol/L L-glutamine, 1 mmol/L sodium pyruvate and 1x non-essential amino acids. TPC-1, a well differentiated papillary thyroid carcinoma cell line, was maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% FBS and 2 mmol/L L-glutamine. The medullary thyroid cancer cell line, TT, (from J. Fagin, University of Cincinnati, Cincinnati, OH) was grown in F-12K medium (Invitrogen Life Technologies, Grand Island, NY) supplemented with 10% FBS. The anaplastic thyroid cancer cell line, CAL62 (from J. Fagin) was grown in DMEM supplemented with 10% FBS. All cell lines were cultured in a humidified, 5% CO_2 incubator at 37°C; 70-80% confluent cells were used for the experiments described below.

Immunohistochemistry for EpEx and Ep-ICD expression in thyroid cancers

Serial sections in 4μm thickness were cut from the paraffin blocks and mounted on glass slides. The sections were deparaffined and hydrated through xylene and graded alcohol series. The slides were treated with 0.3% H_2O_2 at room temperature for 30 minutes to block the endogenous peroxidase activity. After blocking non-specific binding with normal horse or goat serum, the sections were incubated with anti-EpEx mouse monoclonal antibody MOC-31 (dilution 1:200), or α- EpICD antibody 1144 (dilution 1:200), or mouse monoclonal β-catenin antibody. (dilution 1:200) for 30 minutes and biotinylated secondary antibody (horse anti-mouse or goat anti-rabbit) for 30 minutes. The sections were finally incubated with VECTASTAIN Elite ABC Reagent (Vector labs, Burlingame, CA) and diaminobenzedine was used as the chromogen.

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Evaluation of immunohistochemical staining.

Immunopositive staining was evaluated in five areas of the tissue sections as described [Ralhan et al., 2008]. Sections were scored as positive if epithelial cells showed immunopositivity in the cytoplasm, plasma membrane, and/or nucleus when observed by two evaluators who were blinded to the clinical outcome. These sections were scored as follows: 0, < 10% cells; 1, 10-30% cells; 2, 30-50% cells; 3, 50-70% cells; and 4, >70% cells showed immunoreactivity. Sections were also scored semi-quantitatively on the basis of intensity as follows: 0, none; 1, mild; 2, moderate; and 3, intense. Finally, a total score (ranging from 0 to 7) was obtained by adding the scores of percentage positivity and intensity for each of the thyroid cancer and normal thyroid tissue sections. The immunohistochemical data were subjected to statistical analysis as described previously [Ralhan et al., 2008].

The immunohistochemical scoring data were verified using the Visiopharm Integrator System (Visiopharm, Horsholm, Denmark). Only the nuclear staining was quantitated, as the software did not permit simultaneous quantitation of membranous, cytoplasmic and nuclear staining based on differences in intensity of positive brown staining.

Statistical analysis

The immunohistochemical data were subjected to statistical analysis using SPSS 10.0 software (Chicago). Box plots were used to determine the distribution of total score of membranous EpEx, nuclear Ep-ICD and nuclear or cytoplasmic β-catenin expression in normal thyroid tissues and thyroid cancers. A cut-off = or> 2 was defined as positive criterion for nuclear β-catenin immunopositivity for statistical examination. For membranous β-catenin, score of 6 was defined as loss of expression.

The correlation between expression of EpEx, Ep-ICD and/or β-catenin staining with overall patient survival was evaluated using life tables constructed from survival data with Kaplan- Meier plots.

RNA isolation from cell lines, frozen specimens, paraffin sections and first strand cDNA synthesis

All RNA isolations were performed according to the manufacturer's instructions. Total RNA were extracted from cell lines using RNeasy Mini Kit (Qiagen, Maryland, MA). High Pure RNA Tissue Kit and High Pure RNA Paraffin Kit (Roche, Mannheim, Germany) were used to isolate RNA from fresh frozen thyroid tissue specimens and FFPE samples, respectively. The quantity of RNA was measured using ND-1000 spectrophotometer

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(Nanodrop Technologies, Wilmington, DE). First strand cDNA synthesis was performed using Transciptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). Five µl of the reaction product was used as a template for real-time PCR.

Quantitative Real-time RT-PCR

Quantitative Real-time RT-PCR (Q-PCR) analyses were performed using LightCycler480 (Roche, Mannheim, Germany) with SYBR Green I Master kit (Roche, Mannheim, Germany) according to manufacturer's instructions. The real-time PCR reaction initiated with incubation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 10 sec, annealing at 65°C for 15 sec, and extension at 72°C for 15 sec. The melting curve analyses were performed immediately after the completion of the PCR. All reactions were performed in triplicate and the experiments were repeated at least twice. The data were analyzed using LightCycler480 software1.5.

The primers for EpCAM and GAPDH were designed using ProbeFinder assay design software (Roche, Mannheim, Germany), were synthesized and HPLC purified by Sigma. Primer sequences were as follows: EpCAM, 5'-CCATGTGCTGGTGTGTGAA-3' [SEQ ID NO. 3] (forward) and 5'-TGTGTTTTAGTTCAATGGATGATCCA-3' [SEQ ID NO. 4] (reverse); GAPDH, 5'-AGCCACATCGCTCAGACAC-3' [SEQ ID NO. 5] (forward) and 5'-GCCCAATACGACCAAATCC-3' [SEQ ID NO. 6] (reverse).

Immunocytochemistry

The aggressive and non-aggressive thyroid carcinoma cells (WRO, CAL-62, TT and TPC-1) and control cells (ARO) were plated (1x103) on cover slips and grown overnight. Thereafter, the cells were washed with PBS thrice and fixed using 4% paraformaldehyde. For Ep-Ex, Ep-ICD and β-catenin detection by immunocytochemistry, fixed cells were stained with MOC-31, 1144 (dilution 1:200) or mouse monoclonal β-catenin antibody respectively for 30 minutes and biotinylated secondary antibody for 30 minutes. The sections were finally incubated with VECTASTAIN Elite ABC Reagent (Vector labs, Burlingame, CA) and diaminobenzedine was used as the chromogen.

For immunofluorescence detection, goat anti-mouse IgG-FITC (Sigma, St Louis, MO) or IgG-Alexa were used as the secondary antibodies. Nuclei were stained with DAPI. Immunofluorescence was detected using a fluorescent microscope (Leica DM IRBE, Houston, TX).

Western blotting

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Cell lysates were prepared in lysis buffer (0.15mM NaCl, 5mM EDTA (pH 8.0), 1% Triton, 10mM Tris-Cl (pH 7.4)), and protease inhibitors cocktail (Roche Diagnostics, Indianapolis, IN). The cell lysates (30 micrograms protein) were resolved by SDS-PAGE and transferred to a PVDF membrane (Millipore, Billerica, MA). The membrane was probed with the anti-EpCAM mouse monoclonal antibody B302 (Santa Cruz Biotechnology, Santa Cruz, CA) (dilution 1:500), followed by a horse raddish peroxidase-conjugated secondary goat anti-mouse antibody and chemiluminescence detection system according to the manufacturer's instructions (PerkinElmer Life Sciences, Boston, MA). As a control for protein loading, blots were probed for β-Actin, using a mouse monoclonal antibody, C-4 (Santa Cruz Biotechnology, Santa Cruz, CA) (dilution 1:1000). Quantitation was performed by densitometry analysis using ImageGauge software (Altura Software Inc.).

Results

Immunohistochemical Analysis of EpEx and Ep-ICD expression in Thyroid Cancers

To determine the clinical significance of Ep-Ex and Ep-ICD in thyroid cancers, their expressions were analyzed in archived tissues by immunohistochemistry using domain specific antibodies MOC-31 and 1144 respectively. No plasma membrane EpEx immunoreactivity was observed in ATC (Figure 1, panel IA). To determine if the loss of membranous EpEx resulted in its cytoplasmic/nuclear accumulation, Ep-ICD immunostaining was carried out using 1144 antibody - intense nuclear and cytoplasmic Ep-ICD immunostaining was observed in ATC (Figure 1, panel IIA). The activated Ep-ICD has been shown to bind to β-catenin and activate cell proliferation in cancer cells in vitro [Maetzel et al., 2009]. β-catenin immunostaining was carried out in serial sections to determine if there was any correlation between cytoplasmic/nuclear Ep-ICD and nuclear/ cytoplasmic β-catenin. The study showed concurrent cytoplasmic and nuclear β-catenin immunostaining in ATCs (Figure 1, panel IIIA).

In comparison, a subset of the poorly differentiated follicular thyroid cancers (PDFTC) showed intense focal EpEx membrane staining localized to the regions of cell-cell contacts (Figure 1, panel IB); moderate nuclear staining and cytoplasmic Ep-ICD immunostaining was observed in these tumors (Figure 1, panel IIB); and mild cytoplasmic staining and predominant membrane staining was observed for β-catenin (Figure 1, panel IIIB). The poorly differentiated papillary thyroid cancers (PDPTC) showed EpEx membrane staining (Figure 1, panel IC); no nuclear staining and faint cytoplasmic Ep-ICD

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immunostaining was observed in these tumors (Figure 1, panel IIC); membrane and mild cytoplasmic staining was observed for β-catenin (Figure 1, panel IIIC). The well differentiated papillary thyroid cancer (WDPTC) showed intense EpEx membrane staining (Figure 1, panel ID); no nuclear staining but mild cytoplasmic Ep-ICD immunostaining was

5   observed in these tumors (Figure 1, panel IID); and intense membrane staining was observed for β-catenin (Figure 1, panel IIID). In comparison the normal (non-malignant) thyroid tissues showed basal EpEx membrane immunoreactivity (Figure 1, panel IE) and faint or no cytoplasmic or nuclear Ep-ICD staining (Figure 1, panel IIE) and basal membrane immunoreactivity for β-catenin (Figure 1, panel IIIE). The squamous cell carcinoma variant

10   showed faint EpEx membrane immunoreactivity (Figure 1, panel IF); intense cytoplasmic and nuclear Ep-ICD staining (Figure 1, panel IIF); and membranous and cytoplasmic immunoreactivity for β-catenin (Figure 1, panel IIIF).

The nuclear Ep-ICD staining was quantified using visioform; the histogram showing percentage nuclear Ep-ICD positivity in different subtypes of thyroid cancers is given in

15   Figure 1G. All the five ATCs showed nuclear positivity; the total nuclear Ep-ICD positive area ranged from 12- 40%. Notably, one PDPTC and one PDFTC also showed nuclear Ep-ICD positivity. Overall, analysis of β-catenin expression in different subtypes of thyroid tumors showed cytoplasmic and nuclear expression in ATCs, while membrane localization was observed in PDFTC and PDPTC and in WDPTC as well as in the non-malignant thyroid

20   tissues.

Analysis of tissue sections from different tissue blocks of the same patient with different pathology demonstrated differences in expression patterns of these proteins as were observed in individual thyroid tumors. Figure 2 panel AI depicts an ATC section showing no EpEx membrane staining, while the panel AII shows intense nuclear and cytoplasmic

25   localization of Ep-ICD in serial ATC section and panel AIII shows nuclear and cytoplasmic β-catenin expression. Another tissue block from the same patient showed SCC and Panel BI shows focal faint membrane EpCAM expression, while Panel BII shows intense nuclear and cytoplasmic Ep-ICD expression and Panel BIII shows nuclear and membranous β-catenin expression. In comparison another tissue block from the same patient showing PDFTC

30   demonstrated only membranous EpEx (Panel CI), while only cytoplasmic Ep-ICD was observed (Panel CII) and membranous β-catenin with no nuclear immunostaining was observed (Panel CIII). The normal thyroid tissue from the same patient showed intense

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membranous staining for EpCAM (Panel DI), no nuclear and intense staining for Ep-ICD (Panel DII) and membranous staining for β-catenin (Panel DIII). These different staining patterns observed in the same patient support observations of differential expression of these proteins in different subsets of thyroid cancers.

5   **Box-Plot analysis.**

The distribution of total immunostaining scores of EpEx, Ep-ICD and β-catenin, determined in paraffin-embedded sections of normal thyroid tissues and different subtypes of thyroid cancers are shown in Figure 3. Panel A shows box plots for EpEx staining – AI depicts membranous EpEx localization in normal tissues and PTCs, no detectable expression

10   in ATCs and varying reduced expressions in FTC and SCC (with a median score of 3, bold horizontal line). Panel AII depicts cytoplasmic EpEx localization in normal tissues, PTCs, PDPTC, PDFTC and FTCs, no detectable expression in ATCs and varying reduced expression in SCCs. Panel AIII depicts no detectable nuclear EpEx staining in normal tissues, or any of the thyroid cancers.

15   Panel B shows box plots for Ep-ICD staining – I depicts membranous Ep-ICD localization in some PTCs, PDFTC and PDPTC, but no membranous staining in ATCs, FTCs and SCCs. Panel BII depicts cytoplasmic Ep-ICD localization in normal tissues, PTCs, ATCs, FTCs and SCCs, PDPTC and PDFTC. Panel BIII depicts nuclear Ep-ICD localization in ATCs and varying expression in SCCs, (with a median score of 3, bold horizontal line,

20   range 0–4, as shown by vertical bars), as compared to PTCs, FTCs, PDPTC, PDFTC and normal thyroid tissues with a median score of 0.

Panel C shows box plots for β-catenin staining- I depicts nuclear staining in ATCs only. Panel CII shows cytoplasmic β-catenin in all the subtypes of thyroid cancers analyzed. Panel CIII shows membranous β-catenin in normal tissues and all the subtypes of thyroid

25   cancers analyzed except most of the ATCs.

The immunohistochemical scoring data were further verified using the Visiopharm Integrator System. Figure 3D shows the Ep-ICD nuclear staining in different subtypes of thyroid cancers. All the ATCs and one PDPTC and one PDFTC analyzed showed nuclear Ep-ICD expression.

30   **Quantitative Real-time RT-PCR Analysis of EpCAM expression in Thyroid Cancers**

The differential expression of EpCAM in aggressive and non-aggressive thyroid cancers was determined at transcript level by Q-PCR. Figure 4 shows the levels of EpCAM

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transcripts in thyroid tumors and non-malignant (histologically normal) thyroid tissues. The ATCs showed very low levels of EpCAM transcripts in comparison with FTCs and PTCs. No correlation was observed between EpCAM transcripts and aggressiveness of thyroid cancers.

Association of EpEx, Ep-ICD and β-catenin expression with disease outcome

5   Kaplan-Meier Survival analysis revealed reduced overall survival for thyroid cancer patients showing nuclear Ep-ICD expression ($p<0.0001$, Figure 5A). The median overall survival was 5 months in patients showing nuclear Ep-ICD as compared to 185 months for patients who did not. Patients showing loss of membranous EpEx had shorter overall survival (median = 5months) than those showing membranous expression (median = 185 months, $p<$

10   0.0001, Figure 5B). The patients showing nuclear β-catenin had shorter overall survival (median = 5months) than those who did not (median = 185 months, $p = 0.0014$, Figure 5C). Thyroid cancer patients showing nuclear expression of both Ep-ICD and β-catenin had shorter overall survival (median = 5months) than those who did not (median = 185 months, $p = 0.0014$, Figure 5D).

15 **Subcellular localization EpEx in aggressive human thyroid cancer cell lines**

The differential subcellular localization of Ep-Ex and Ep-ICD observed in aggressive and non-aggressive human thyroid cancers is simulated in thyroid cancer cell lines propagated in vitro was determined by immunocytochemistry. Strong EpEx immunostaining localized to the plasma membrane was observed in WRO cells, medullary thyroid cancer

20   cells- TT, and the positive control colon cancer cells- ARO (previously considered as ATC cells) by immunocytochemistry, while no membraneous EpCAM staining was detected in anaplastic thyroid cancer cells (CAL-62) and in low-grade papillary thyroid cancer cells (TPC-1) (Figure 6A panel I). These findings were confirmed by immunofluorescence (Figure 6A panel III).

25   Cytoplasmic and nuclear Ep-ICD staining was observed in CAL-62 cells, in comparison, WRO cells showed cytoplasmic Ep-ICD and faint nuclear staining (Figure 6B panels II and IV). The merged images of EpEx and Ep-ICD staining are presented in Figure 6B, panel IV depicts strong membrane and faint cytoplasmic staining in WRO cells. In TT cells EpEx showed strong focal staining at cell-cell contacts in the membrane and faint

30   cytoplasmic Ep-ICD staining. In comparison, the anaplastic CAL-62 cells showed nuclear and cytoplasmic Ep-ICD staining and no or faint EpEx staining. In contrast, the non-aggressive papillary thyroid cancer cell line TPC-1 did not show detectable EpEx or Ep-ICD

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staining.

Western blot analysis corroborated EpEx marked overexpression in ARO, WRO and TT cells; in comparison reduced EpEx levels were observed in CAL-62 cells and no EpEx was detected in TPC-1 cells (Figure 6C).

5   Q-PCR analysis showed no marked difference in the levels of EpCAM transcripts in the same panel of cancer cell lines. Figure 6D shows EpCAM/GAPDH ratios in ARO, WRO, TT and CAL-62 cells; no transcripts could be quantitated in TPC-1 cells.

EpCAM as Oncogenic Signal Transducer

The oncogenic potential of EpCAM is proposed to be activated by release of its
10   intracellular domain, which can signal into the cell nucleus by activation of Wnt pathway components. To determine if there is any correlation between the loss of EpEx expression from the plasma membrane, cytoplasmic accumulation and translocation into the nucleus, with subcellular localization of the Wnt pathway component β-catenin and expression of target genes such as c-myc, the expressions of these proteins were analyzed in the above
15   panel of thyroid cancer cell lines. Figure 6E and 6F show intense EpEx expression at the cell cell contacts and cytoplasmic and nuclear localization of β catenin and c-myc in WRO and ARO cells, but not in CAL-62, TT and TPC-1 cells.

Discussion

The key findings of the study are: (i) The anaplastic thyroid tumors showed loss of
20   membrane EpEx, but increased Ep-ICD accumulation in cytoplasm and nucleus of tumor cells, that was paralleled by concurrent β-catenin expression, suggesting that Ep-ICD may be acting as an oncogenic signal transducer in these tumors and consequent activation of Wnt pathway components including β-catenin might account for the rapid growth of these tumors and their poor prognosis; (ii) EpEx membrane overexpression was observed in both well
25   differentiated- follicular and papillary thyroid cancers, while a subset of poorly differentiated-follicular and papillary thyroid cancers showed nuclear Ep-ICD; (iii) EpEx was overexpressed on the surface of cancer cells in culture, WRO and TT, but was not detected on the membrane in anaplastic thyroid cancer cells (CAL62) and in the less aggressive cells TPC-1, while nuclear Ep-ICD was detected in CAL62 cells, supporting the clinical findings.

30   The study is the first report using an antibody specific for the cytoplasmic domain of Ep-ICD that demonstrates its cytoplasmic and nuclear accumulation in ATCs. The regulated intramembrane proteolysis (RIP) of EpCAM has recently been proposed to produce Ep-ICD

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that has been shown to transduce EpCAM signaling in cancer cells and activate Wnt proteins-resulting in increased nuclear accumulation of β-catenin and the target genes - c-myc and cyclinD1 (Munz et al., 2009). It is demonstrated that concomitant expression of cytoplasmic and nuclear Ep-ICD and β-catenin in ATCs, suggesting that activation of Ep-ICD signaling and consequent Wnt pathway component activation might account for the rapid growth of ATCs.

β-catenin plays an important role as a signaling factor involved in canonical Wnt pathway [Li H et al., 2002]. Nuclear localization of β-catenin is involved in precancerous change in oral leukoplakia [Ishida K et al., 2007] and is known to associate with malignant transformation of human cancers including colorectal, gastric and esophageal tumors [Morin PJ 1997, Ogasawara N 2006, Takayama T,1996, Zhou XB 2002]. The activation of canonical Wnt signaling pathway results in nuclear translocation of β-catenin [Lustig B 2003], hence nuclear β-catenin is a marker for active cell proliferation. In contrast to membranous and cytoplasmic expression, nuclear localization of β-catenin is implicated in tumor progression. The nuclear β-catenin expression in ATCs is a reflection of the aggressive nature of these tumors.

Further, the in vitro findings in aggressive thyroid cancer cell lines CAL62 suggest colocalization of Ep-ICD, β-catenin and c-myc supporting the activation of oncogenic Ep-ICD signaling, Wnt pathway component activation and overexpression of its target protein – cmyc that might account for the aggressive behavior of ATCs and a subset of SCCs, PDPTCs and PDFTCs. The survival analysis data also demonstrate association of loss of membranous EpEx with reduced overall survival of thyroid cancer patients (p< 0.0001). Furthermore, nuclear Ep-ICD accumulation (p< 0.0001) and nuclear β-catenin expression (p = 0.0014) alone, or concomitant with Ep-ICD (p = 0.0014) were found to be associated with reduced overall survival (median =5months) as compared to those thyroid cancer patients who did not show nuclear accumulation of these proteins (median = 185 months). This is the first report underscoring the clinical significance of nuclear Ep-ICD alone, or in correlation with nuclear β-catenin, as adverse prognosticators for aggressive thyroid cancers.

The in vitro findings in thyroid cancer cell lines and primary thyroid tumors suggest EpEx overexpression in the plasma membranes of well and poorly differentiated thyroid cancers and underscore its potential as an immuontherapeutic target. It is noteworthy that in an earlier immunohistochemical study, Ensinger et al., (2006) reported EpEx overexpression

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in well and poorly differentiated thyroid cancers, but no expression was observed in the 22 ATCs analyzed. The results also confirm the loss of EpEx expression from the cell surface in ATCs using MOC-31, an antibody that recognizes the extracellular domain of Ep-CAM. EpEx overexpression on the plasma membrane of most well and poorly differentiated follicular and papillary thyroid tumor cells make it an ideal candidate as a cancer marker as well as an immunotherapeutic target. The loss of membrane EpEx and its nuclear localization in ATCs suggests that novel therapeutic approaches are needed for targeting Ep-ICD in these tumors.

Conclusions

In conclusion, loss of membranous EpEx and nuclear accumulation of Ep-ICD in aggressive thyroid cancers (anaplastic thyroid cancers and some poorly differentiated papillary thyroid cancers) was demonstrated. A concomitant increase in nuclear β-catenin in these tumors suggested activation of Wnt pathway signaling in these tumors. Further, loss of membranous EpEx, or nuclear accumulation of Ep-ICD alone, or in combination with β-catenin, was associated with poor overall survival of thyroid cancer patients. Ep-ICD may serve as a marker for aggressive thyroid cancers and is a potential target for novel therapeutics.

Example 2   *EpCAM – Potential Therapeutic Target*

Inhibition of EpCAM-positive thyroid cancer cell proliferation upon treatment with VB4-845/VB6-845

The effects of EpCAM-specific immunotoxin, VB4-845/VB6-845, on cell proliferation were examined in the panel of thyroid cancer cell lines as well as in the positive control colon cancer cell line with different levels of EpCAM expression. As shown in the Figure 7, the MTT based cell viability assay showed that VB4-845 inhibited proliferation of WRO and ARO cells, with IC_{50} of 1 pM and 0.7 pM, respectively. In comparison, the medullary thyroid cancer cell line, TT, was marginally responsive to the immunotoxin treatment, while the papillary cell line, TPC-1, and anaplastic cell line, CAL-62, with no detectable membrane EpCAM expression were non-responsive to VB4-845. Similar results were observed in the same cell lines treated with VB6-845 (data not shown).

Induction of Apoptosis by VB4-845 in thyroid cancer cell lines.

Cell cycle analysis of VB4-845 treated thyroid cancer cells by FACS showed a time dependent induction of apoptosis reflected by a marked increase in subG0 fraction in WRO

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and the positive control ARO cells as compared to TT and TPC-1 cells. The effects of VB4-845 on EpCAM expression in cell lines were also determined by Western blotting before and after treatment with different concentrations of VB4-845. Figure 8 shows a dose dependent decrease in EpCAM expression in WRO cells treated with the immunotoxin; no EpCAM expression was detected in untreated or VB4-845 treated TPC-1 cells.

VB4-845 cytotoxicity resulted from the binding of immunotoxin and EpCAM.

Immunotoxin VB4-845 is a recombinant fusion protein that combines an anti-EpCAM single chain variable fragment with the toxicity of Pseuodomonas exotoxin A. The protein is flanked by two hexahistinide tags. As determined in flow cytometry assay, the anti-His antibody was detected in the cells showing EpCAM expression, after two hours incubation with VB4-845.

TPC-1 cells (10^6) were injected into 6-week old SCID mice. Four weeks later, 7.5ug VB4 in 100ul PBS was peritumorally injected for each tumor every 2 days. Approximately two weeks later, the mice were sacrificed mainly due to the oversized tumor. The size of the tumors were measured and compared between VB4 treatment and PBS treatment. Also the EpCAM expression was screened for TPC-1 both *in vivo* and in *vitro*. Due to the tumor size variation, the tumor volumes were converted into percentage. With VB4 treatment, four out of ten tumors decreased (Figure 9(A)), while only one out of eight tumors decreased in PBS group (Figure 9(B)).

Example 3

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

Patients and tissue specimens:

The study was approved by Mount Sinai Hospital Research Ethics Board, Toronto, Canada. For IHC analysis, archived tissue blocks of normal thyroid tissues (N=9), Non-neoplastic-Hyperplastic /colloid nodules (N=1), papillary thyroid carcinoma (PTC, N=86), follicular thyroid carcinoma (FTC, N=2), poorly-differentiated PTC (N=1), poorly-differentiated FTC (N=1), medullary thyroid carcinoma(N=3), Insular carcinoma (N=6), SCC (N=4), anaplastic thyroid carcinoma (N=11) were retrieved from the tumor bank, reviewed by the pathologist and used for cutting tissue sections for immunostaining with Ep-ICD and EpEx (Moc31) antibodies as described below.

The following is a discussion of the results of the study.

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Scatter Plot Analysis

The scatter plots in Figure 10-14 illustrate the distribution of Ep-ICD and EpEx membrane /cytoplasmic/nuclear immunohistochemical staining scores in the normal thyroid tissue and nine subtypes of thyroid tumor tissues analyzed. The ATC and SCC groups showed marked reduction in membranous EpEx staining with an average score of less than 4, with insular showing moderate decrease in membranous EpEx. Notably, ATCs showed loss of EpEx with the membrane IHC score of less than 1 (Figure 10). Similar to the normal thyroid tissue group, the other less aggressive thyroid tumour subtypes showed high EpEx membrane staining with average IHC scores greater than 6 (Figure 10). In the observation on the EpEx cytoplasm staining (Figure 11), similar as the normal thyroid group, some less aggressive thyroid cancer subtypes such as PTC, FTC and showed higher EpEx staining than those more aggressive thyroid cancer subtypes including ATC, SCC and insular subtypes. The ATC group showed no detectable low EpEx staining or faint immunoreactivity.

Importantly, using an antibody specific to the intracellular cytoplasmic domain of EpCAM (Ep-ICD), the membrane staining is shown in Figure 12 and Figure 13 shows the average expression level at IHC score of 4-5 observed in all of the thyroid tumour subtypes and also the normal thyroid group. Elevated nuclear Ep-ICD staining (above the cutoff ≥ 4) was observed in 10 of the 11 ATC tissue blocks examined (Figure 14) with a mean staining score of 4.7. In the less aggressive subtype SCC group, 2 of the 4 cases showed nuclear Ep-ICD staining reaching cutoff of 4. Among all the 86 PTC tissue blocks, the majority of the tumors showed very low EpICD nuclear staining with an average score of 0.6 (Table 2), which is similar to the normal group. Other subtypes such as HP, FTC, PDPTC, PDFTC, MTC, Insular, all showed low levels of EpICD nuclear staining with Ep-ICD nuclear expression scores between 0 to 2 (Figure 14).

Immunohistochemical analysis of Ep-ICD and EpEx expression in thyroid tumors

Among the two tumor thyroid cancer subtypes (papillary thyroid carcinoma and anaplastic thyroid carcinoma) that were compared (Table 2), the ATC group demonstrated nuclear Ep-ICD positivity in 10 of 11 tissue blocks (90.9%) when choosing a cut off value of ≥ 4 to determine positivity, while all the 11tissues showed loss of EpEx membrane expression at a cutoff value of ≤ 4. Only 1 of 86 PTC tissues (1.2%) demonstrated nuclear Ep-ICD positivity. The correlation of a high nuclear EpICD score of 4.5 in PTC with clinical history revealed that the patient was a 35 year old male with evidence of lymph node

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metastasis. Another PTC patient with a nuclear Ep-ICD score of 3 had metastatic pancreatic cancer with sepsis. The Ep-ICD and EpEx IHC staining scores differed significantly between PTC and ATC groups and distinguished aggressive from non-aggressive thyroid cancer (TCs).

ROC Curve Analysis

ROC curves were generated for membrane EpEx and nuclear Ep-ICD to distinguish the most aggressive thyroid cancer subtype- ATC from the most frequently observed but non-aggressive thyroid cancer subtype- PTC (Figures 15 and 16). Results of ROC analysis are summarized in Table 4. At a cutoff of ≥ 4 nuclear Ep-ICD accumulation distinguished ATC from PTC with a sensitivity of 90.9%, specificity of 98.8% and an AUC of 0.9931(Figure 15 and Table 4). This suggests the high level of nuclear EpICD accumulation has the potential to serve as a good biomarker to distinguish aggressive from other non-aggressive thyroid cancer subtypes. As shown in Figure 15 and Table 5, when the cutoff of nuclear EpICD IHC staining score is chosen between 2.5 to 4, this biomarker (nuclear EpICD) can distinguish ATC from PTC with high sensitivity of 90-100% and high specificity of 95-98%. At a cutoff of ≤ 4, the loss of membrane EpEx expression could also distinguish all of the ATC cases from PTC with a high sensitivity of 100%, high specificity of 98.8% and an AUC value of 0.914 (Figure 16 and Table 6). The positive predictive value is 91.67% and the negative predictive value is 100% (Figure 16 and Table 6). As shown in Figure 16 and Table 7, the cutoff of EpEx membrane IHC staining score is chosen between 3.5 to 5, this biomarker (membrane EpEx) can distinguish ATC from PTC with high sensitivity of 90-100% and high specificity of 95-100%.

Example 4 *Filipino Thyroid Cancer Study*

The Filipino population has been observed to have a higher incidence of thyroid cancer and the tumors are more aggressive than in non-Filipino patients. The expression of EpEx and Ep-ICD has been analyzed in aggressive and non-aggressive thyroid cancers in Filipino patients. The results are presented below.

Immunohistochemical analysis of Ep-ICD expression in Filipino thyroid tumors

Among the three tumor groups that were compared (Table 3), the aggressive malignant tumor group exhibited nuclear Ep-ICD positivity in 7 of 10 tissues and 6 of 10 tissues showed the loss of EpEx membrane expression at an IHC score cutoff value of 4. No loss of EpEx membrane expression was observed in any of the 9 benign tumor cases and 11

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non-aggressive malignant cases analyzed. Non-aggressive thyroid cancers did not show nuclear Ep-ICD positivity and only 1 of 9 benign tumors analyzed showed nuclear Ep-ICD positivity. The photomicrographs shown in Figure 17 depict membrane EpEx expression in benign thyroid tumor (A) and non-aggressive malignant tumor (C), the loss of EpEx

5   membrane expression was observed in some areas of all of the 10 thyroid aggressive malignant tumor cases (E, G). Ep-ICD nuclear expression was observed in the thyroid aggressive malignant tumors (F, H), but not in the benign tumor group and the non-aggressive malignant tumor group (B, D).

Box Plot Analysis

10   The scatter plots in Figures 18 and 19 and the box plots in Figures 21A and 21B and 22A and 22B show the distribution of membrane EpEx and nuclear Ep-ICD staining scores in the three groups (30 cases in total) of Filipino thyroid tumor cases analyzed. Elevated nuclear Ep-ICD staining (above the cutoff ≥ 4) was found in 7 of the 10 aggressive malignant tumors examined (Figure 22B.), showing a mean staining score of 4.3. Nuclear Ep-ICD

15   staining reached cutoff ≥ 4 was observed in only 1 in the 9 benign tumors and none of the 11 non-aggressive malignant tumor tissues. All of the 9 benign thyroid tumor tissues examined and all of the 11 non-aggressive malignant tumors show high level membrane EpEx staining with a mean score of around 7 scores (Figure 21(A)). The loss of membrane EpEx expression was observed in two third of the aggressive malignant cases (Figure 22A.).

20   **ROC Curve Analysis**

ROC curves were generated for membrane EpEx and nuclear Ep-ICD to distinguish malignant thyroid tumors from benign tumors (Figure 20A, B) and also to distinguish aggressive malignant tumors from the nonaggressive tumors (Figure 20C, D). Relevant ROC analysis including results are summarized in Table 8.

25   Nuclear Ep-ICD accumulation distinguished thyroid malignant tumors from benign tumors with a 33.33% sensitivity, a specificity of 88.89% and with the AUC value of 0.703. Nuclear Ep-ICD accumulation distinguished aggressive thyroid malignant tumors from nonaggressive cancers with an 80% sensitivity, a specificity of 100% and an AUC of 1.0 (Table 8). The loss of membrane EpEx expression distinguished thyroid malignant tumors

30   from benign tumors with a 28.57% sensitivity, a specificity of 100% and with the AUC value of 0.857. Nuclear Ep-ICD accumulation distinguished aggressive thyroid malignant tumors

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from nonaggressive cancers with a 60% sensitivity, a specificity of 100% and an AUC of 0.914 (Table 8).

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Table 1

Known Antibodies Directed Against the EpCAM

Antibody	Epitope	Reference
AUA1	EGF-like domain I	Durbin et al
Ber-EP4	EGF-like domain I	Latza et al
CO 17-1A	EGF-like domain I	Herlyn et al
C215	EGF-like domain I	Bjork et al
ESA, EGP-2, EGP40	Not established	Simon et al
FU-MK-1	Not established	Watanabe et al
GA733-2	EGF-like domain I	Szala et al
HEA125	Not established	Momburg et al
K928	Not established	Quak et al
K931	EGF-like domain I	Copper MP
KSA, KS-1, KS1/4	EGF-like domain I	Varki et al
MM104	Cysteine-poor region	Schön et al
MH99	EGF-like domain I	Mattes et al
MOC31	EGF-like domain I	Myklebust et al
MT201	Not established	Naundorf et al
VU-1D9	EGF-like domain I	Tsubura et al
2G8	EGF-like domain II	Unpublished data
311-1K1	Cysteine-poor region	Helfrich et al
323/A3	EGF-like domain I	Edwards et al

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Table 2

Immunohistochemical Analysis of EpEx and Ep-ICD in Benign and Malignant Thyroid Tumors

Tumour Tissue	Number Blocks (N)	Nuclear EpICD Positive (n)	Nuclear EpICD Positivity (%)	IHC Score Mean ± Std. Deviation	t-test p Value	Membrane EpEx (n)	Membrane EpEx Percentage (%)	IHC Score Mean ± Std. Deviation	t-test P Value
PTC	86	1	1.2	0.57±0.77	P<0.001	1	1.2	6.47±0.66	P<0.001
ATC	11	10	90.9	4.73±1.01		11	100	0.61±1.36	

5   Note: A cutoff value of ≥4 was used to determine nuclear Ep-ICD positivity; a cutoff value of ≤4 scores was used to determine the loss of membrane EpEX expression.

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Table 3

Biomarker Analysis of Nuclear Ep-ICD Expression and Membrane EpEx Expression in FilipinoThyroid Tumor Cancers

Ep-ICD Nuclear Staining Scores	AUC	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Asymptotic Sig.
PTC vs. ATC	0.993	90.90	98.80	90.90	98.80	<0.001
EpEX Membrane Staining Scores	AUC	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Asymptotic Sig.
PTC vs. ATC	0.914	100	98.80	91.67	100	<0.001

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Note: A cutoff value of ≥ 4 was used to determine nuclear Ep-ICD positivity; a cutoff value of ≤ 4 scores was used to determine the loss of membrane EpEX expression.

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Table 4

ROC curve for nuclear EpICD

Area under the ROC curve	
Area	0.9931
Std. Error	0.006375
95% confidence interval	0.9806 to 1.006
P value	<0.0001
Data	
Controls (PTC)	86
Patients (ATC)	11
Missing Controls	0
Missing Patients	0

5

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Table 5

EpICD Nuclear Staining to Distinguish ATC from PTC

Cutoff	Sensitivity%	Specificity %
>2.300	100	95.35
>2.850	100	96.51
>3.150	90.91	97.67
>3.650	90.91	98.84
>4.250	54.55	98.84
>4.750	54.55	100
>5.500	27.27	100

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Table 6

ROC Curve for EpEX-Membrane

Area under the ROC curve	
Area	0.9989
Std. Error	0.001768
95% confidence interval	0.9955 to 1.002
P value	<0.0001
Data	
Controls (PTC)	86
Patients (ATC)	11
Missing Controls	0
Missing Patients	0

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Table 7

EpEx membrane staining to distinguish ATC from PTC

Cutoff	Sensitivity%	Specificity %
<3.300	90.91	100
<3.550	90.91	98.84
<4.000	100	98.84
<4.550	100	97.67
<4.750	100	96.51
<5.050	100	95.35

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Table 8

Biomarker Analysis of Nuclear Ep-ICD Expression and Membrane EpEx Expression in

Filipino Thyroid Tumor Cancers

Ep-ICD Nuclear Staining Scores	AUC	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Asymptotic Sig.
Benign vs. Malignant Tumors	0.703	33.33	88.89	87.5	36.3	0.085
Nonaggressive vs. Aggressive Cancers	1.0	80.00	100	100	83.33	0.000
Loss of EpEX Membrane Staining Scores	AUC	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Asymptotic Sig.
Benign vs. Malignant Tumors	0.857	28.57	100	100	37.50	0.002
Nonaggressive vs. Aggressive Cancers	0.914	60.00	100	100	73.33	0.001

Note: A cutoff value of 4 was used to determine positivity.

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Full Citations for Publications

1.   Spizzo G, et al, . *Breast Cancer Res Treat.* 2004;**86**:207–13

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81

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The present invention is not to be limited in scope by the specific embodiments described herein, since such embodiments are intended as but single illustrations of one aspect of the invention and any functionally equivalent embodiments are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

All publications, patents and patent applications referred to herein are incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety. All publications, patents and patent applications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing the antibodies, methodologies etc. which are reported therein which might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

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Sequence Listing

SEQ ID NO. 1

NP_002345

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TAGVRRTDKD TEITCSERVR TYWIIIELKH KAREKPYDSK SLRTALQKEI TTRYQLDPKF     180
ITSILYENNV ITIDLVQNSS QKTQNDVDIA DVAYYFEKDV KGESLFHSKK MDLTVNGEQL     240
DLDPGQTLIY YVDEKAPEFS MQGLKAGVIA VIVVVVIAVV AGIVVLVISR KKRMAKYEKA     300
EIKEMGEMHR ELNA                                                       314
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SEQ ID NO. 2

Homo sapiens epithelial cell adhesion molecule (EPCAM), mRNA.
ACCESSION   NM_002354

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 181 cggcgagcga gcaccttcga cgcggtccgg ggaccccctc gtcgctgtcc tcccgacgcg
 241 gacccgcgtg ccccaggcct cgcgctgccc ggccggctcc tcgtgtccca ctcccggcgc
 301 acgccctccc gcgagtcccg ggcccctccc gcgcccctct tctcggcgcg cgcgcagcat
 361 ggcgcccccg caggtcctcg cgttcgggct tctgcttgcc gcggcgacgg cgacttttgc
 421 cgcagctcag gaagaatgtg tctgtgaaaa ctacaagctg gccgtaaact gctttgtgaa
 481 taataatcgt caatgccagt gtacttcagt ggtgcacaa aatactgtca tttgctcaaa
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 601 agcaaaacct gaaggggccc tccagaacaa tgatgggctt tatgatcctg actgcgatga
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 781 ctactggatc atcattgaac taaaacacaa agcaagagaa aaaccttatg atagtaaaag
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1381 acatctttga aggtcatgag tttgttagtt aacatcata tatttgtaat agtgaaacct
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SEQ ID NO. 3

5'-CCATGTGCTGGTGTGTGAA-3'

SEQ ID NO. 4

5'-TGTGTTTTAGTTCAATGGATGATCCA-3'

83

SEQ ID NO. 5

5'-AGCCACATCGCTCAGACAC-3'

SEQ ID NO. 6

5'-GCCCAATACGACCAAATCC-3'

SEQ ID NO. 7

β-catenin Swiss-Prot: P35222.1 and Genbank NP_001091679

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181 keasrhaimr spqmvsaivr tmqntndvet arctagtlhn lshhreglla ifksggipal
241 vkmlgspvds vlfyaittlh nlllhqegak mavrlagglq kmvallnktn vkflaittdc
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541 vrahqdtqrr tsmggtqqqf vegvrmeeiv egctgalhil ardvhnrivi rglntiplfv
601 qllyspieni qrvaagvlce laqdkeaaea ieaegatapl tellhsrneg vatyaaavlf
661 rmsedkpqdy kkrlsvelts slfrtepmaw netadlgldi gaqgeplgyr qddpsyrsfh
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781 l
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SEQ ID NO. 8

β-catenin mRNA NM_001904.3 (homo sapiens)

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 121 acggaggaag gtctgaggag cagcttcagt ccccgccgag ccgccaccgc aggtcgagga
 181 cggtcggact cccgcggcgg gaggagcctg ttcccctgag ggtatttgaa gtataccata
 241 caactgtttt gaaaatccag cgtggacaat ggctactcaa gctgatttga tggagttgga
 301 catggccatg gaaccagaca gaaagcggc tgttagtcac tggcagcaac agtcttacct
 361 ggactctgga atccattctg gtgccactac cacagctcct tctctgagtg gtaaaggcaa
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84

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40 **SEQ ID NO. 9**

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   protein), beta 1, 88kDa

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 50   301 catggccatg gaaccagaca gaaaagcggc tgttagtcac tggcagcaac agtcttacct
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85

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     3301 gtttaccagt tgccttttat cccaaagttg ttgtaacctg ctgtgatacg atgcttcaag
     3361 agaaaatgcg gttataaaaa atggttcaga attaaacttt taattcattc gattg
```

SEQ ID NO. 10

NM_001098210 mRNA   linear homo sapiens catenin (cadherin-associated protein), beta 1,

```
        1 aggatacagc ggcttctgcg cgacttataa gagctccttg tgcggcgcca ttttaagcct
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      181 cggtcggact cccgcggcgg gaggagcctg ttcccctgag ggtatttgaa gtataccata
      241 caactgtttt gaaaatccag cgtggacaat ggctactcaa gctgatttga tggagttgga
      301 catggccatg gaaccagaca gaaaagcggc tgttagtcac tggcagcaac agtcttacct
      361 ggactctgga atccattctg tgccactac cacagctcct tctctgagtg gtaaaggcaa
      421 tcctgaggaa gaggatgtgg ataccтccca agtcctgtat gagtgggaac agggattttc
      481 tcagtccttc actcaagaac aagtagctga tattgatgga cagtatgcaa tgactcgagc
      541 tcagagggta cgagctgcta tgttccctga cacattagat gagggcatgc agatcccatc
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1021 gttgttttat gccattacaa ctctccacaa ccttttatta catcaagaag gagctaaaat
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2101 aagagtagct gcaggggtcc tctgtgaact tgctcaggac aaggaagctg cagaagctat
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2341 tgagactgct gatcttggac ttgatattgg tgcccaggga gaacccttg gatatcgcca
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3241 ttaattcatt cgattg
```

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What is Claimed Is:

1.   A method for detecting thyroid cancer markers associated with thyroid cancer in a subject comprising or consisting essentially of:

5   (a)   obtaining a sample from a patient;

         (b)   detecting or identifying in the sample one or more thyroid cancer markers; and

         (c)   comparing the detected amount with an amount detected for a standard, wherein the thyroid cancer markers are Ep-ICD and/or β-catenin.

2.   A method for diagnosing thyroid cancer comprising comparing:

10   (a)   levels of thyroid cancer markers that are extracted from a sample from a subject; and

         (b)   levels of thyroid cancer markers in a control sample, wherein a significant difference in levels of thyroid cancer markers relative to the corresponding levels in the control is indicative of thyroid cancer, wherein the thyroid cancer

15   markers are Ep-ICD, β-catenin, and optionally EpEx.

3.   A method of claim 1 or 2 wherein the subject is at risk of having thyroid cancer or in need of screening.

4.   A method for characterizing or classifying a thyroid sample comprising detecting a difference in the expression of thyroid cancer markers relative to a control, wherein

20   the thyroid cancer markers are Ep-ICD, β-catenin, and optionally EpEx.

5.   A method of diagnosing a specific disease stage of thyroid cancer in a subject comprising determining the status of a thyroid cancer marker in a sample obtained from the subject, wherein an abnormal status of the marker indicates the presence of a specific disease stage and wherein the thyroid cancer marker is Ep-ICD or β-catenin.

25   6.   A diagnostic method for identifying a subject who is a candidate for treatment for thyroid cancer comprising determining the status of thyroid cancer markers in a sample obtained from the subject, wherein an abnormal status of the thyroid cancer markers in the sample indicates that treatment is desirable or necessary, and wherein the thyroid cancer markers are Ep-ICD, β-catenin, and optionally EpEx.

30   7.   A method of claim 5 or 6 wherein the abnormal status can be an elevated status, low status or negative status.

8.   A method for diagnosing the aggressiveness or metastatic potential of a thyroid cancer in a subject comprising:

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(a)   obtaining a sample from the subject;

(b)   detecting in the sample a thyroid cancer marker; and

(c)   comparing the detected amount with an amount detected for a standard, wherein the Thyroid Cancer Marker is Ep-ICD and/or β-catenin.

9.   A method of claim 1 or 8, wherein the standard or control comprises levels or an amount detected in a subject with a lower grade of thyroid cancer.

10.   A method for diagnosing anaplastic thyroid cancer (ATC) in a subject, the method comprising:

(a)   contacting a sample from a subject with a reagent capable of measuring a level of a target thyroid cancer marker wherein the thyroid cancer marker is Ep-ICD, β-catenin, and optionally EpEx; and

(b)   providing a diagnosis of ATC in said subject based on an increase in the level of at least one of Ep-ICD and β-catenin, and optionally a decrease in EpEx, in the sample from the subject over a control level obtained from similar samples taken from subjects who do not have ATC or from the subject at a different time.

11.   A method for monitoring the progression of a thyroid cancer in a subject, the method comprising: (a) detecting in a sample from a patient at a first time point, a thyroid cancer marker; (b) repeating step (a) at a subsequent point in time; and (c) comparing levels detected in steps (a) and (b), and thereby monitoring the progression of the cancer, wherein the thyroid cancer marker is Ep-ICD or β-catenin.

12.   A method of any one of claims 1 to 11 wherein the thyroid cancer marker is a polypeptide detected by the following steps:

(a)   contacting the sample with a binding agent that specifically binds to the polypeptide or a part thereof; and

(b)   detecting in the sample an amount of polypeptide that binds to the binding agent relative to a predetermined standard.

13.   A method for detecting or diagnosing the presence of an aggressive thyroid cancer or a thyroid cancer with metastatic potential in a subject comprising the steps of:

(a)   providing a binding agent that specifically binds to a thyroid cancer marker;

(b)   contacting the binding agent with a sample from the subject under conditions that allow the formation of a complex comprising the binding agent and thyroid cancer marker;

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(c)   determining the presence or amount of the complexes; and

(d)   comparing the amount with a standard to determine the presence of an aggressive thyroid cancer or a thyroid cancer with metastatic potential in the subject, wherein the thyroid cancer marker is Ep-ICD or β-catenin.

14.   A method of claim 12 or 13 wherein the binding agent is an antibody.

15.   A method of any one of claims 1 to 11 wherein the thyroid cancer marker is a polynucleotide.

16.   A method of claim 15 wherein the polynucleotide is mRNA or fragments thereof.

17.   A method of claim 15 wherein the polynucleotide is detected by

(a)   contacting the sample with oligonucleotides that hybridize to the polynucleotides; and

(b)   detecting in the sample levels of nucleic acids that hybridize to the polynucleotides relative to a predetermined standard or cut-off value, and therefrom determining the presence or absence of thyroid cancer in the subject.

18.   A method of claim 16 wherein the mRNA is detected using an amplification reaction.

19.   A method of claim 18 wherein the amplification reaction is a polymerase chain reaction employing oligonucleotide primers that hybridize to the polynucleotides, or complements of such polynucleotides.

20.   A method of claim 19 wherein the polynucleotide is detected using RT-PCR.

21.   A method of claim 16 wherein the mRNA is detected using a hybridization technique employing oligonucleotide probes that hybridize to the polynucleotides or complements of such polynucleotides.

22.   A method for assessing the potential efficacy of a test agent for inhibiting thyroid cancer in a subject, the method comprising comparing: (a) levels of one or more thyroid cancer markers, in a first sample obtained from a subject and exposed to the test agent, wherein the thyroid cancer markers are Ep-ICD and/or β-catenin, and (b) levels of the thyroid cancer markers in a second sample obtained from the subject, wherein the sample is not exposed to the test agent, wherein a significant difference in the levels of expression of the thyroid cancer markers in the first sample, relative to the second sample, is an indication that the test agent is potentially efficacious for inhibiting thyroid cancer in the subject.

23.   A method of selecting an agent for inhibiting thyroid cancer in a subject the method

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comprising (a) obtaining a sample comprising cancer cells from the subject; (b) separately exposing aliquots of the sample in the presence of a plurality of test agents; (c) comparing levels of one or more thyroid cancer markers in each of the aliquots; and (d) selecting one of the test agents which alters the levels of thyroid cancer markers in the aliquot containing that test agent, relative to other test agents, wherein the thyroid cancer markers are Ep-ICD and/or β-catenin.

24. A method of claim 23 further comprising administering to the subject at least one of the test agents which alters the levels of thyroid cancer markers in the aliquot containing that test agent, relative to other test agents.

25. A method of assessing the thyroid cancer cell carcinogenic potential of a test compound, the method comprising: (a) maintaining separate aliquots of thyroid cancer cells in the presence and absence of the test compound; and (b) comparing expression of one or more thyroid cancer markers, in each of the aliquots, and wherein a significant difference in levels of thyroid cancer markers in the aliquot maintained in the presence of the test compound, relative to the aliquot maintained in the absence of the test compound, is an indication that the test compound possesses thyroid cancer cell carcinogenic potential, wherein the thyroid cancer markers are Ep-ICD and/or β-catenin.

26. A method as claimed in any one of claims 1 to 21 wherein the sample is obtained from tissues, extracts, cell cultures, cell lysates, lavage fluid or physiological fluids.

27. A method as claimed in claim 26 wherein the sample is obtained from a tumor tissue.

28. A kit for detecting and/or diagnosing an aggressive thyroid cancer or a thyroid cancer with metastatic potential comprising a binding agent that binds to a thyroid cancer marker or an agent that hybridizes to or amplifies a polynucleotide encoding a thyroid cancer marker wherein the thyroid cancer marker is Ep-ICD or β-catenin.

29. Use of antibodies that bind to a thyroid cancer marker or a polynucleotide that hybridizes to or amplifies a polynucleotide encoding a thyroid cancer marker for detecting and/or diagnosing aggressiveness or metastatic potential of a thyroid cancer, wherein the thyroid cancer marker is Ep-ICD or β-catenin.

30. A composition comprising antibodies that bind to a thyroid cancer marker or a polynucleotide that hybridizes to or amplifies a polynucleotide encoding a thyroid cancer marker for use in detecting and/or diagnosing aggressiveness or metastatic

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potential of a thyroid cancer, wherein the thyroid cancer marker is Ep-ICD or β-catenin.

31.   A method of treating thyroid cancer in a subject, comprising delivering to the subject in need thereof, an antibody specific for Ep-CAM, Ep-ICD or β-catenin.

5

1/40
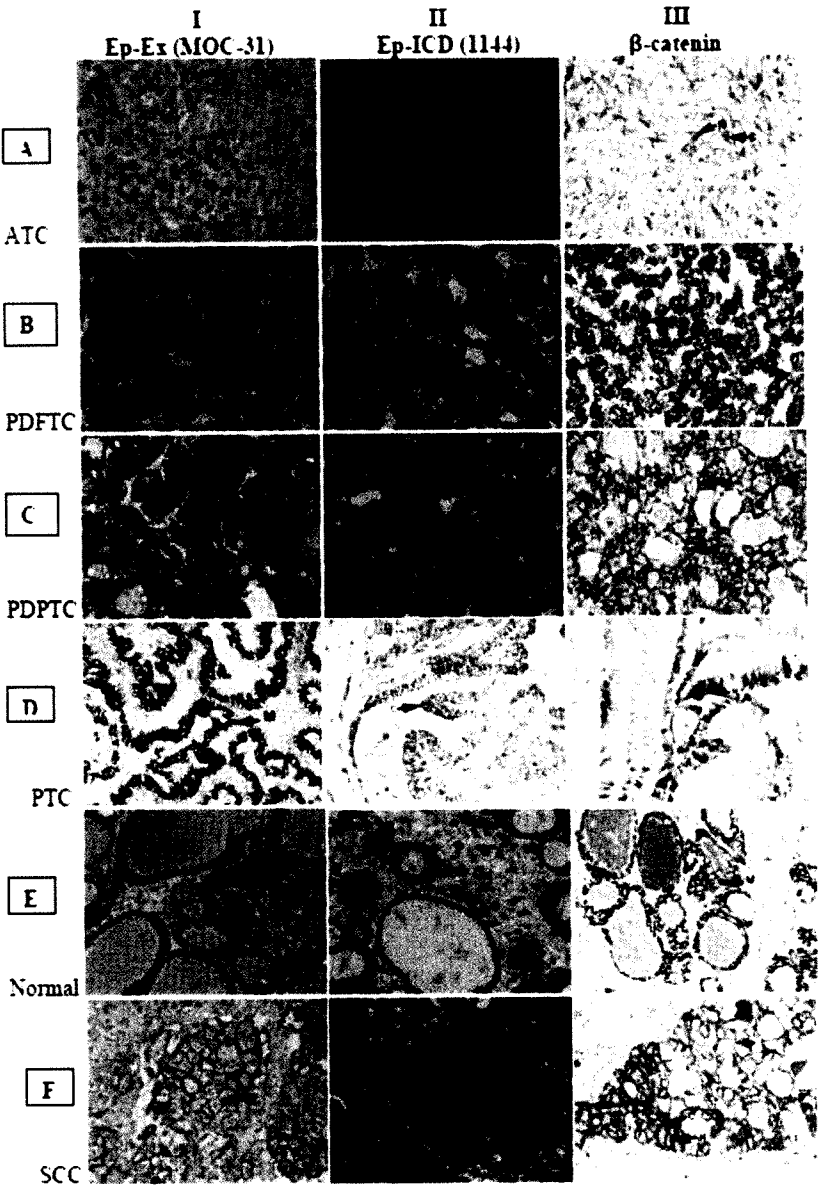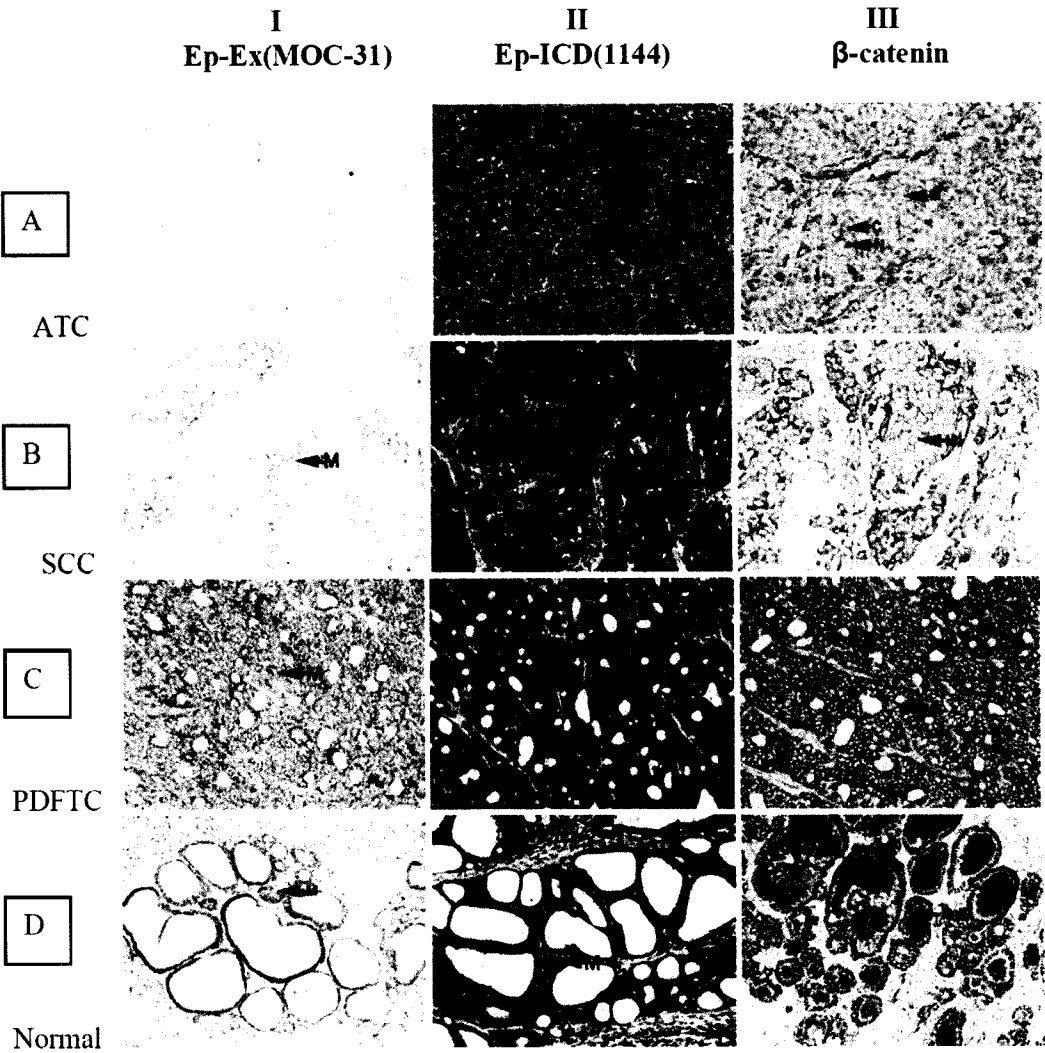
Figure 1

Figure 2

Figure 3

AI
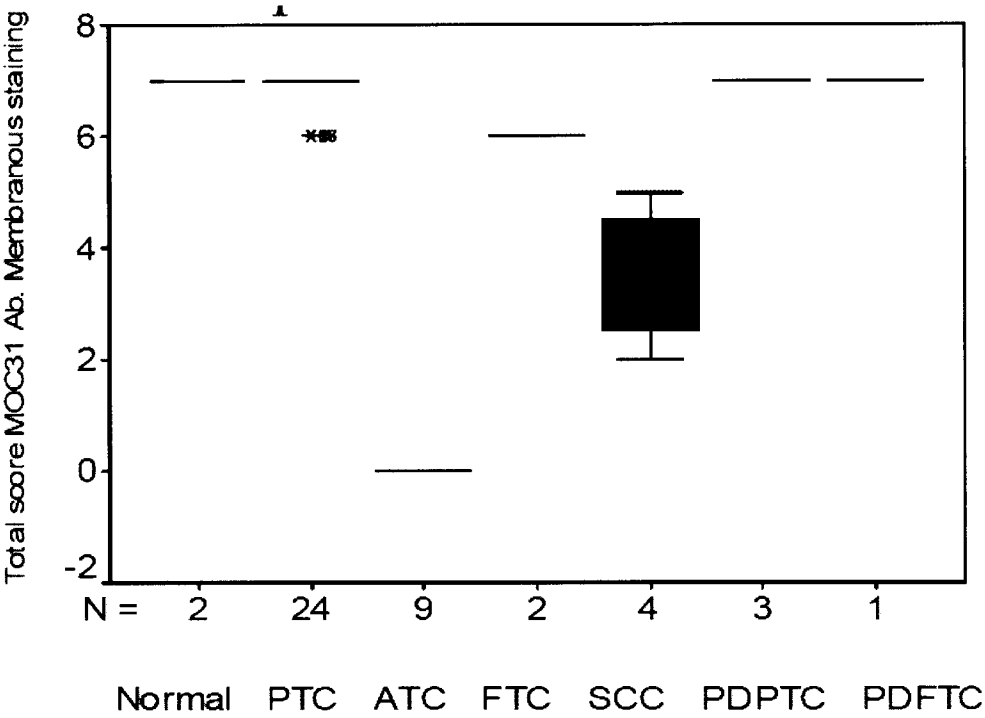
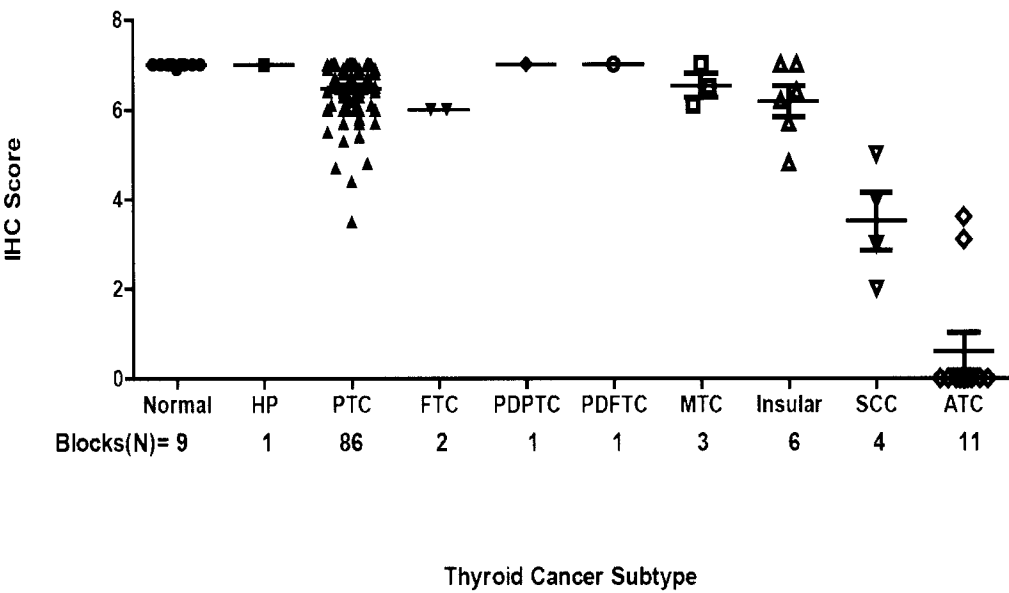Figure 3
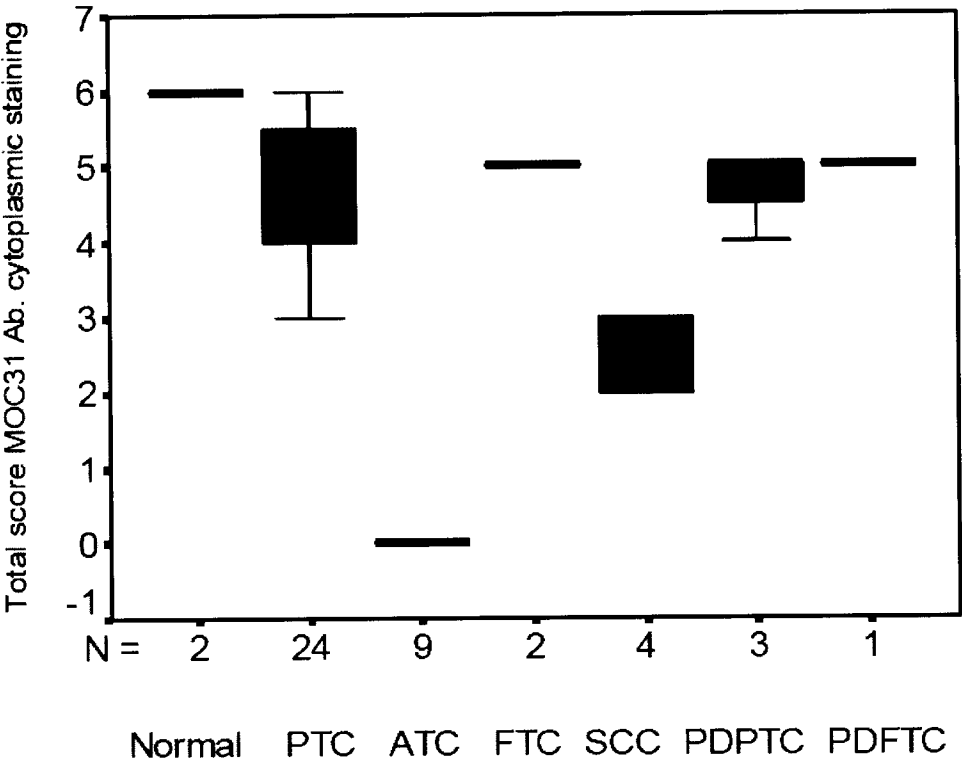
Figure 3

AIII

Figure 3

BI

Figure 3

BII
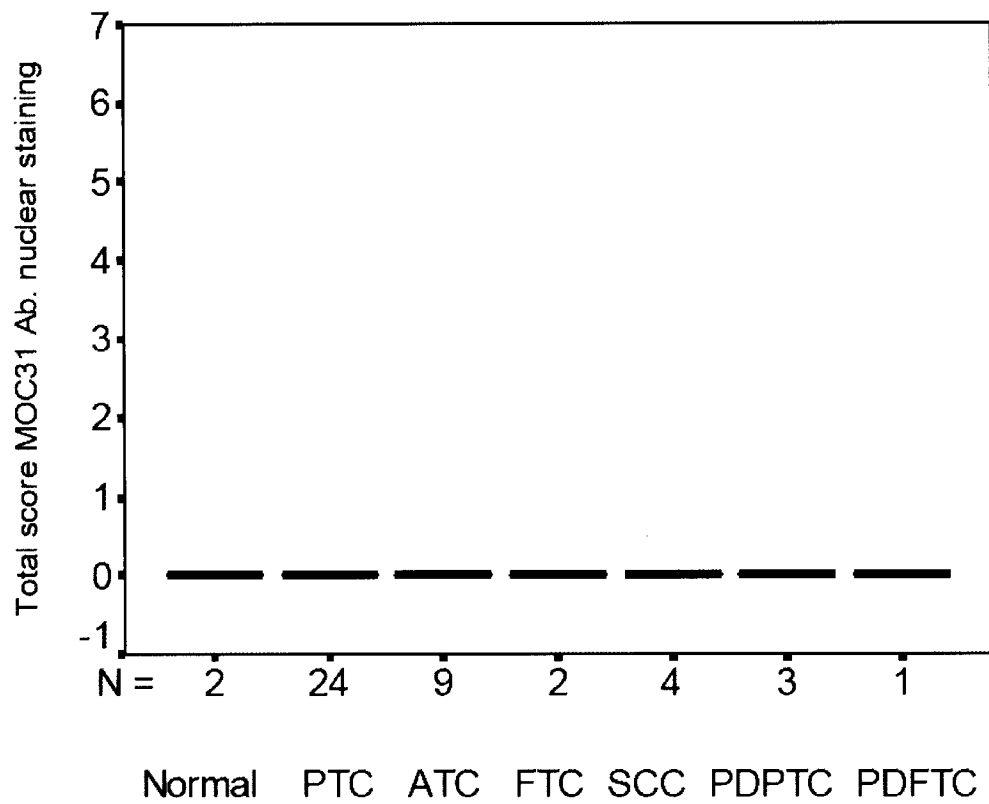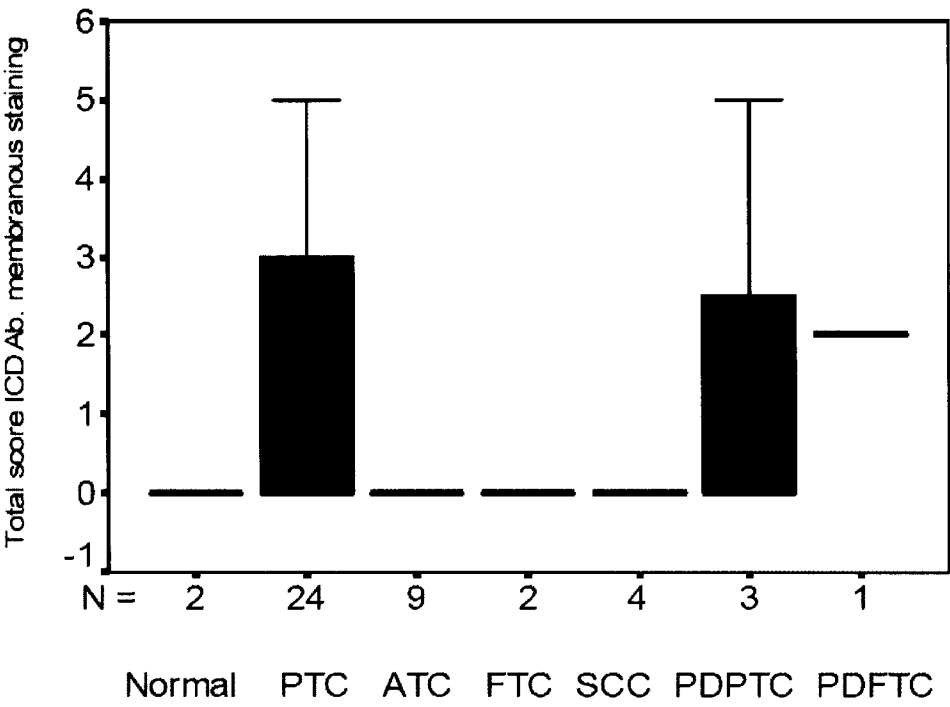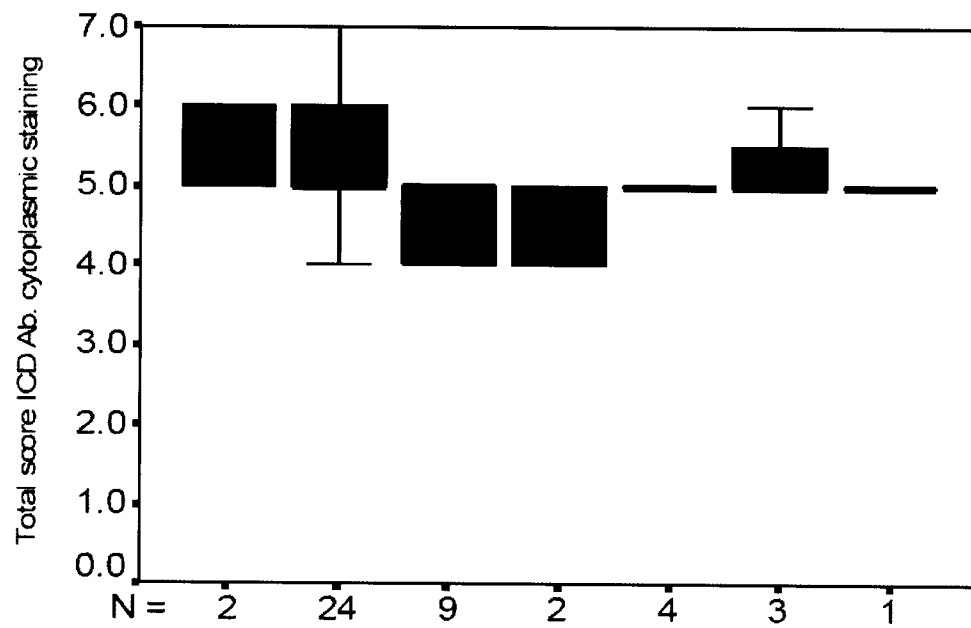
BIII
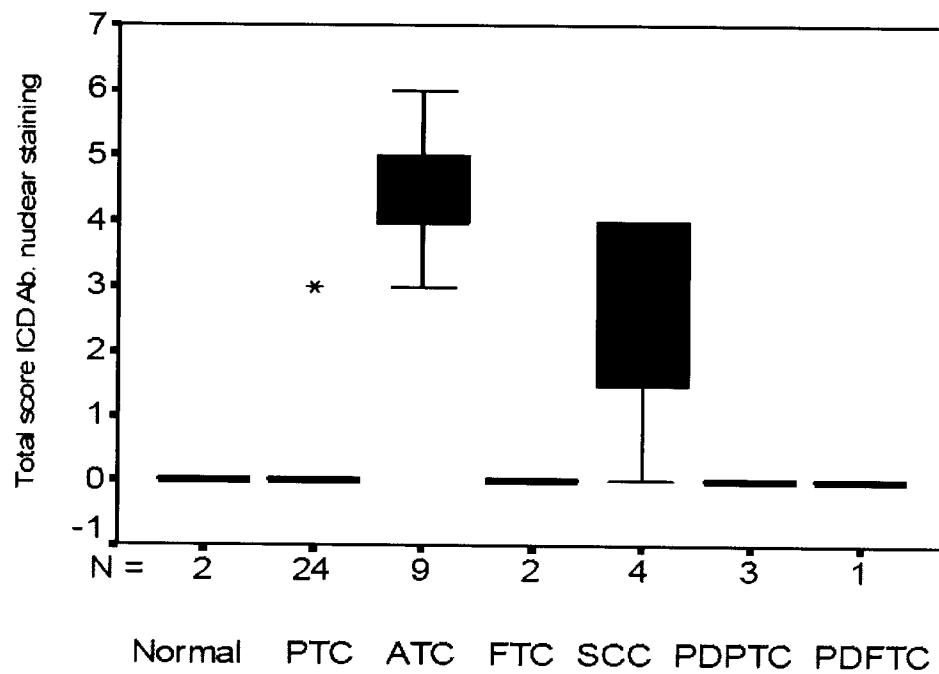
Figure 3

CI

CII

Figure 3

CIII

Figure 3

D

Figure 4

EpCAM/GAPDH
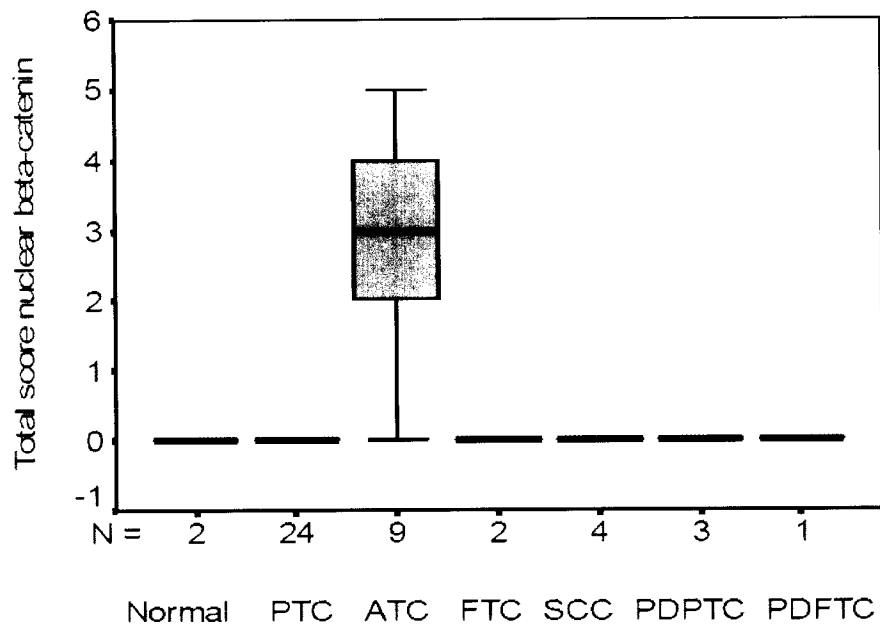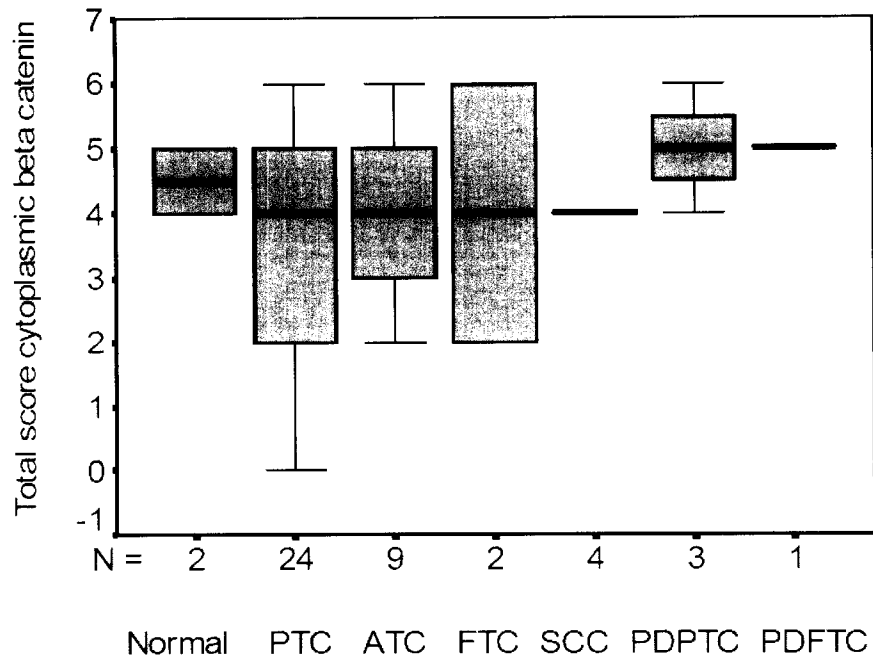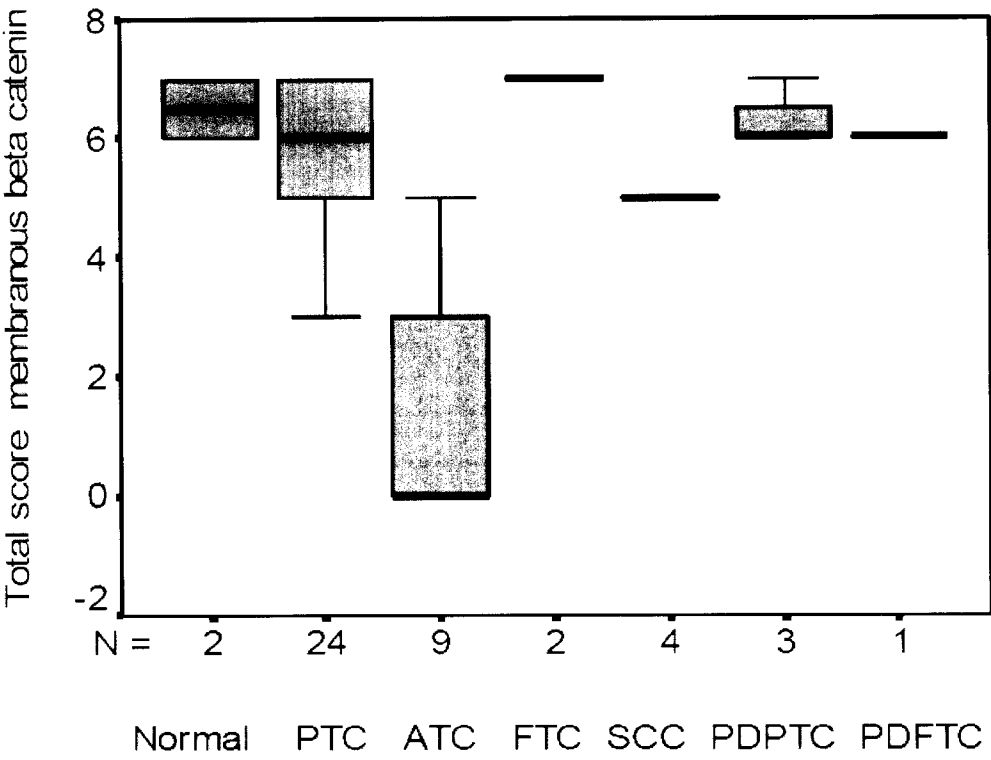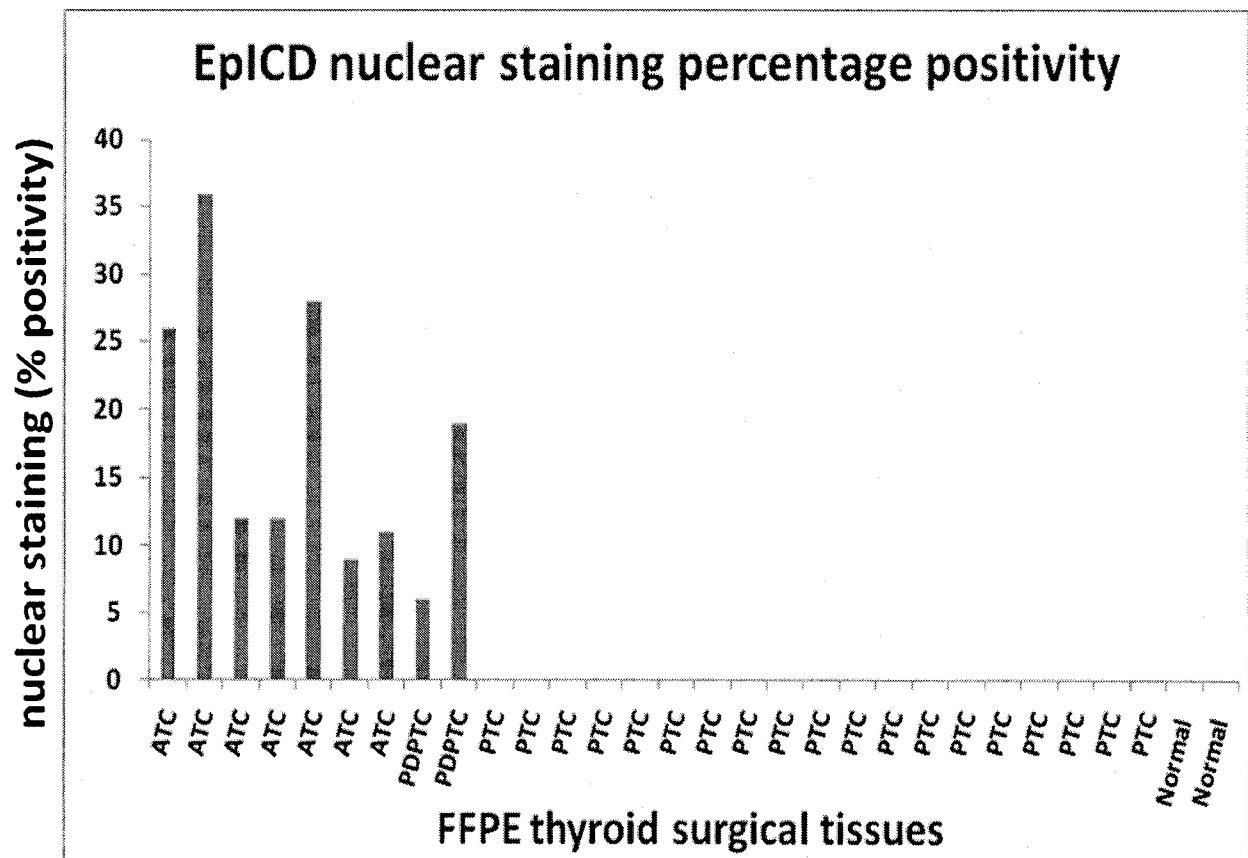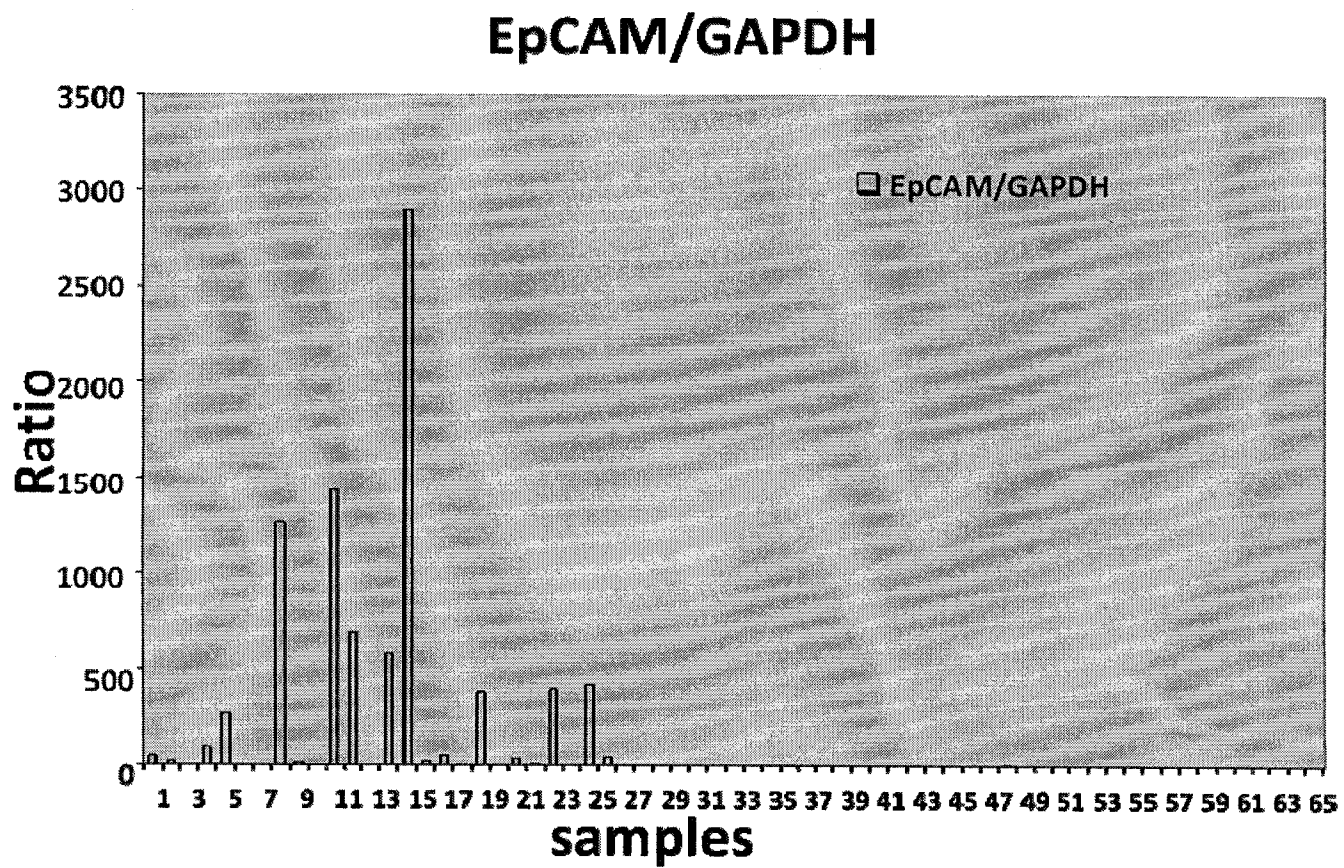
Figure 5

(A)
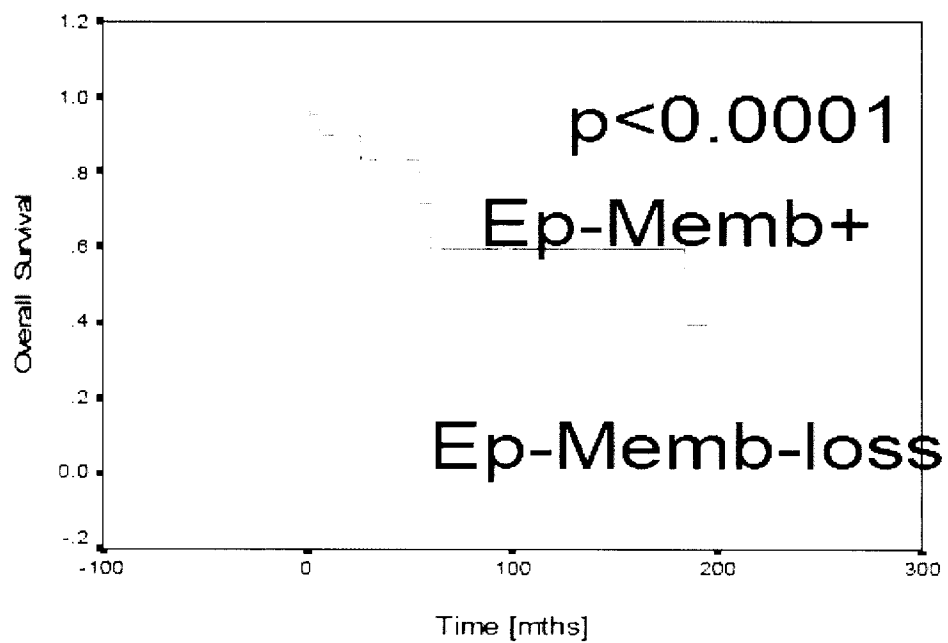
Median DFS, Ep-Memb-loss = 5 mths
Median DFS, Ep-Memb+ = 185 mths

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Figure 5

(B)
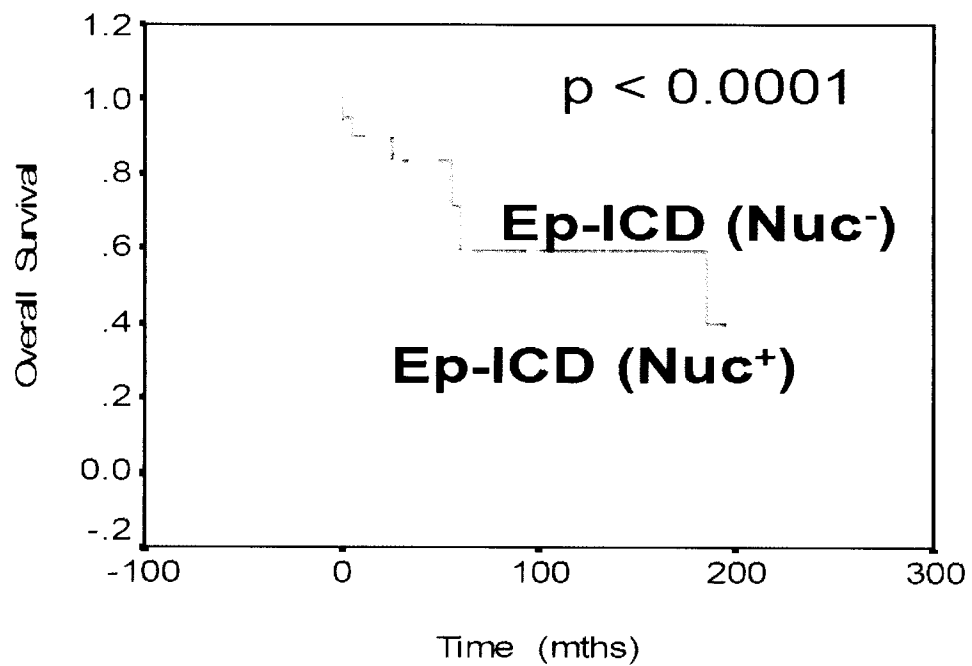
Median DFS, EpCAM Nuc+= 5 mths
Median DFS, EpCAM Nuc- = 185 mths

Figure 5

(C)
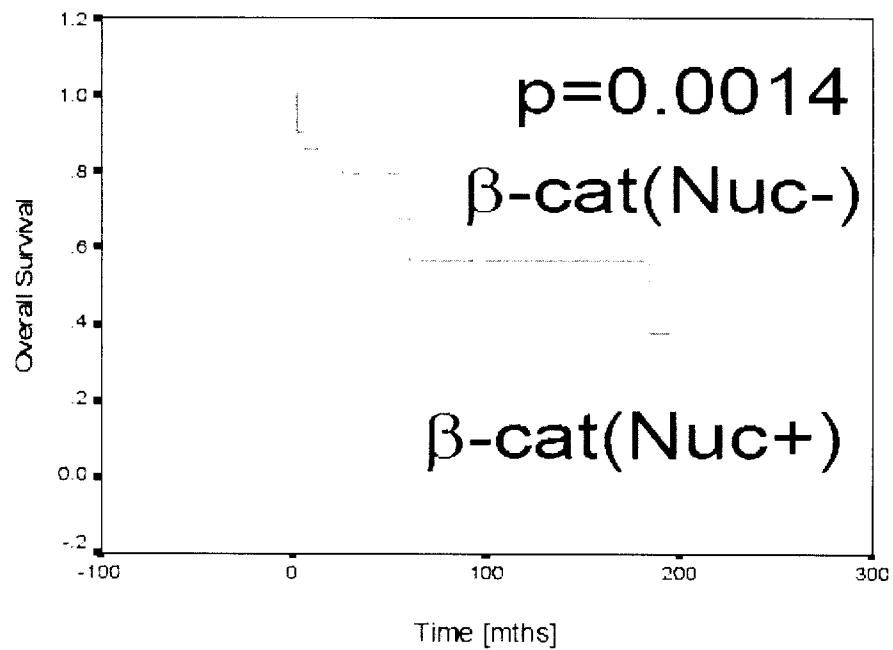
Median DFS, β-cat Nuc+ = 5 mths
Median DFS, β-cat Nuc- = 185 mths

Figure 5

(D)
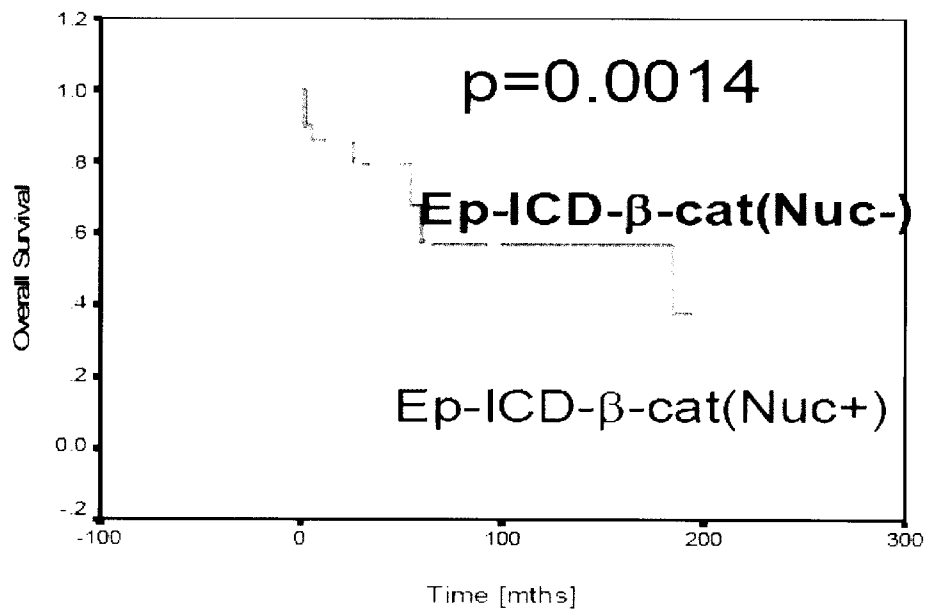
Median DFS, Ep-ICD-β-cat Nuc+= 5 mths
Median DFS, Ep-ICD-β-cat Nuc- =185 mths

Figure 6

(A)

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Figure 6

(B)

Cell line	ARO	WRO	TT	CAL-62	TPC-1
I. MOC-31					
II. EpICD					
III. DAPI					
IV. Merged					

Figure 6

(C)

cell line ARO CAL WRO TT TPC-1

EpICD

β-Actin

Figure 6

(D)

Relative Quantification of EpCAM
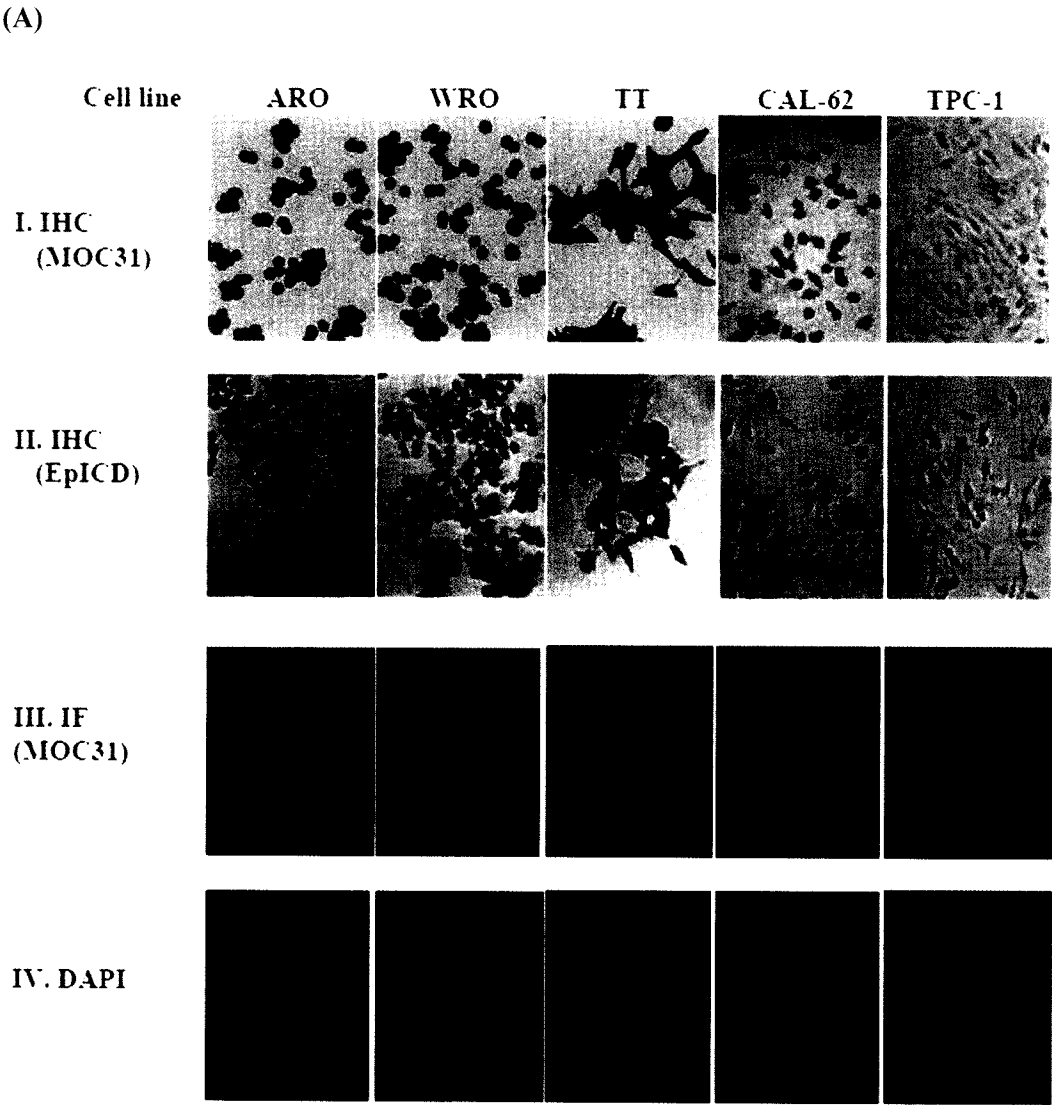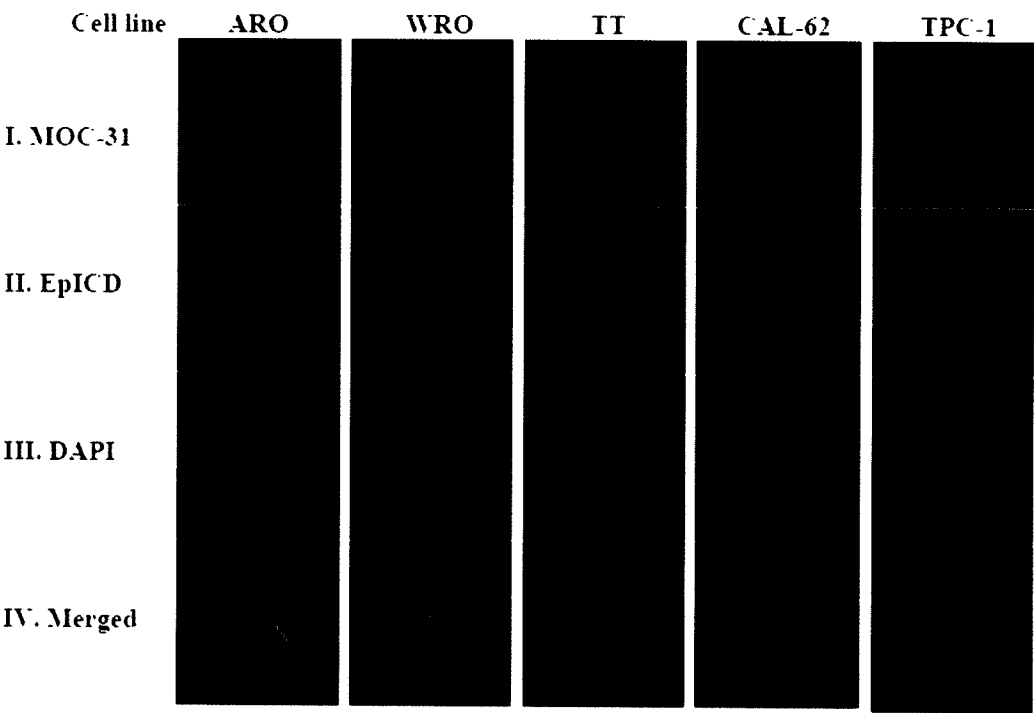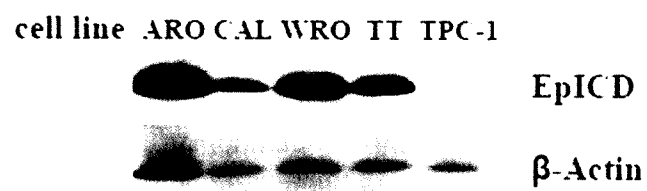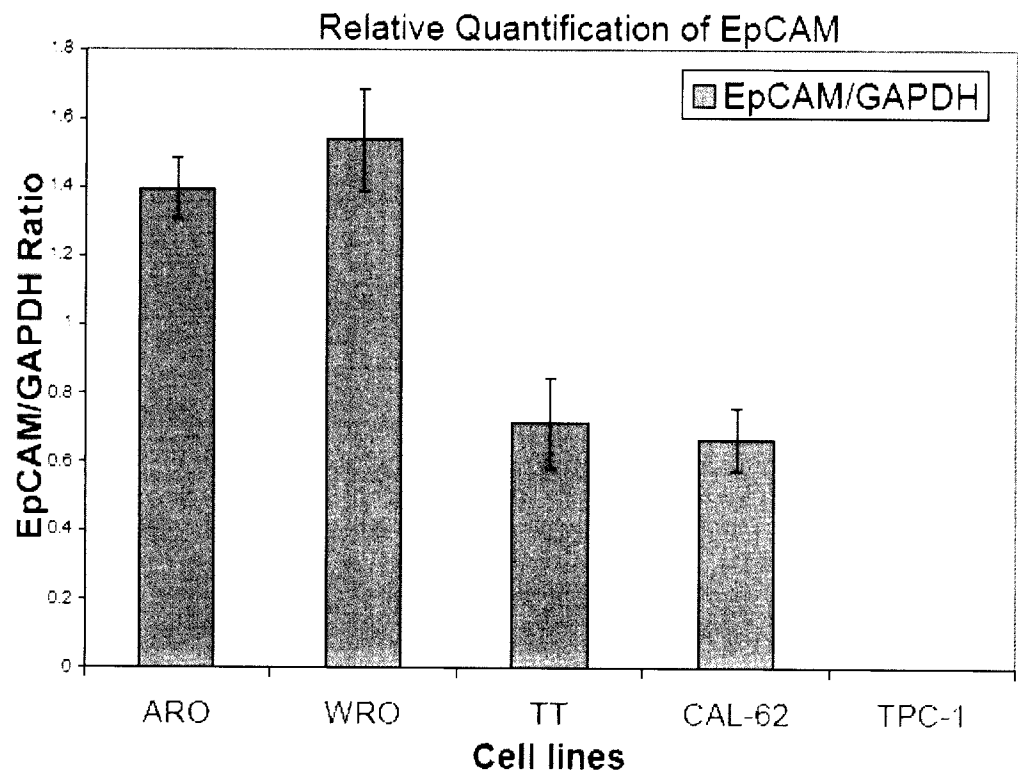
Figure 6

(E)
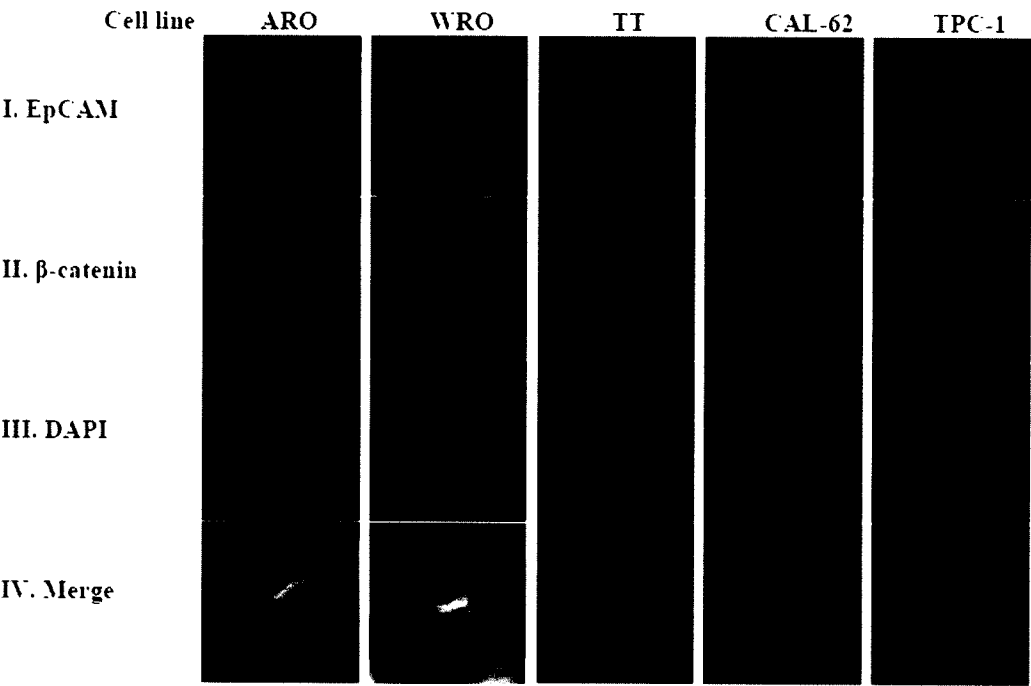
Figure 6

(F)

Figure 7
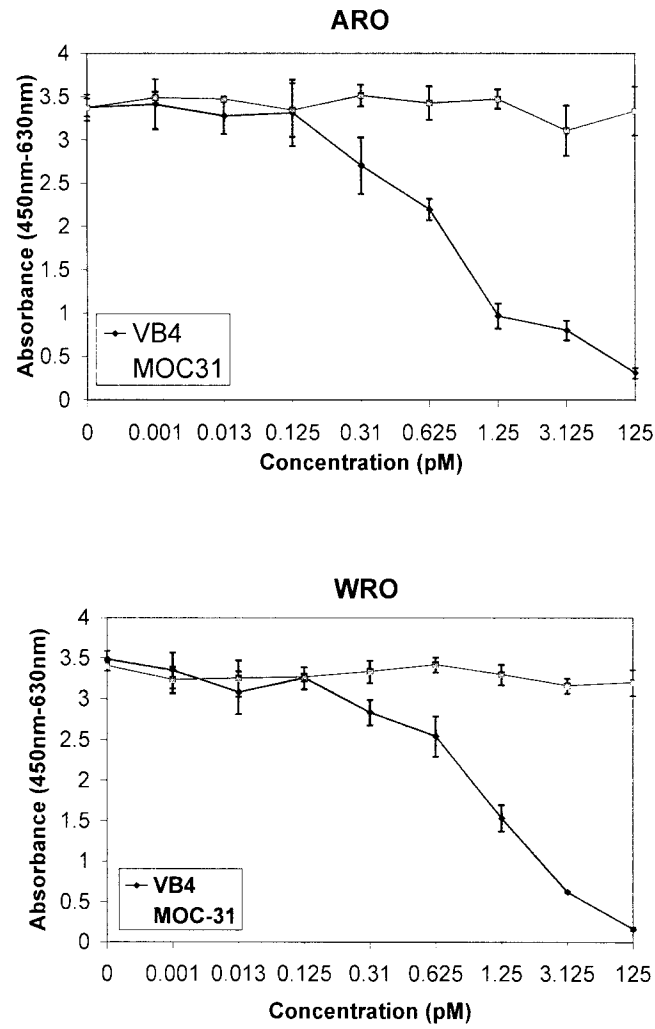
Figure 7
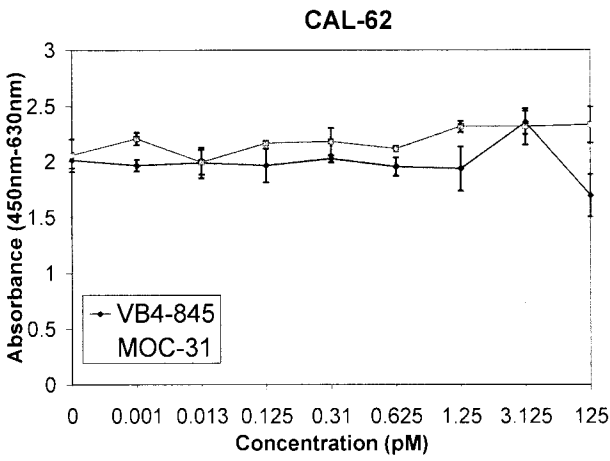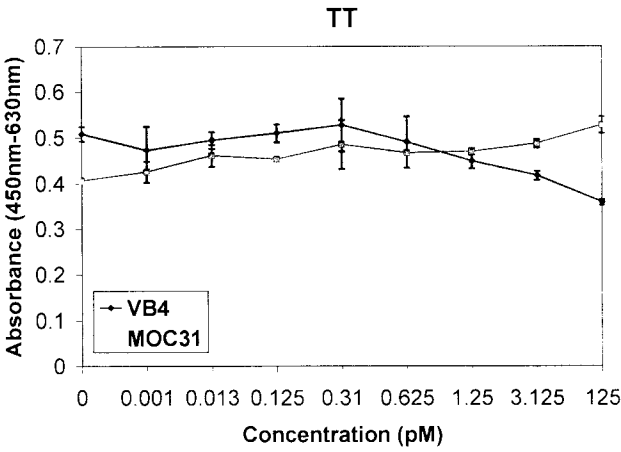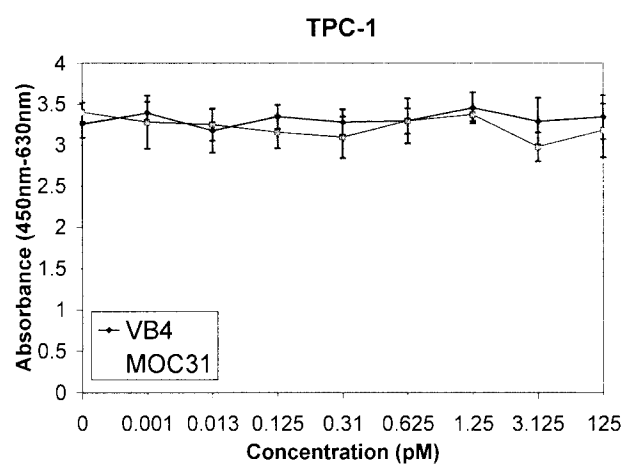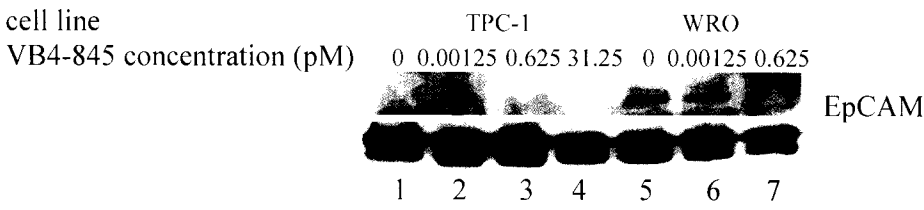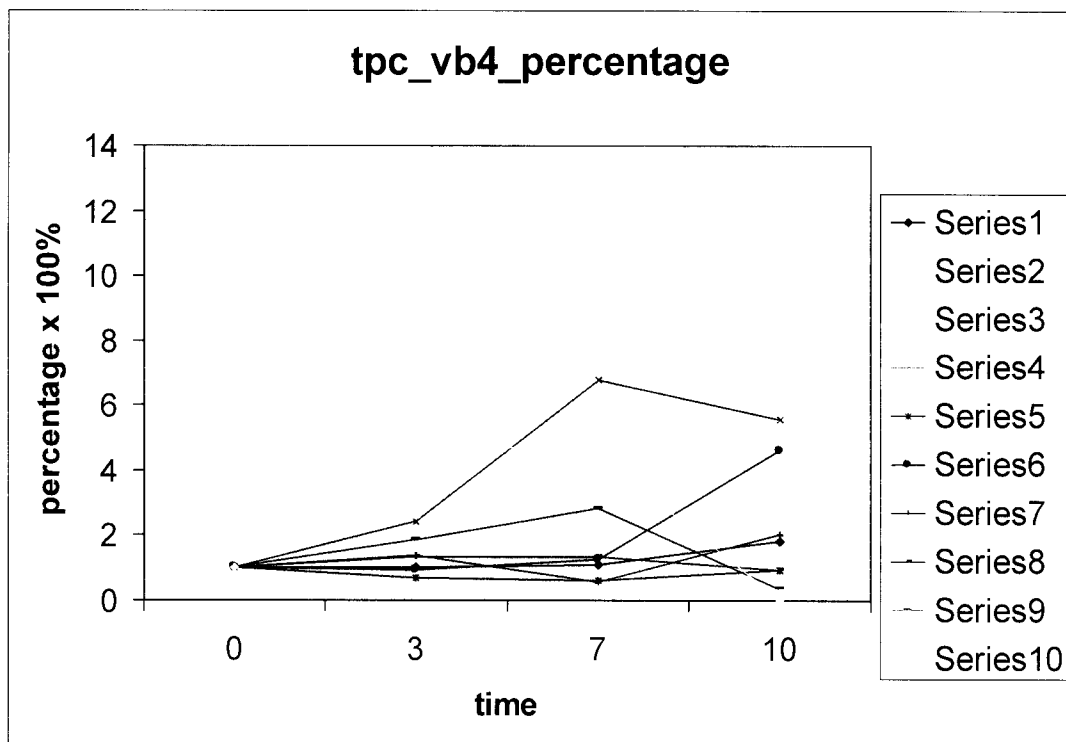
TT

CAL-62

Figure 7

Figure 8

cell line   TPC-1   WRO

VB4-845 concentration (pM)   0   0.00125   0.625   31.25   0   0.00125   0.625

EpCAM

1   2   3   4   5   6   7

Figure 9

(A)

Figure 9

(B)

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Figure 10

Thyroid Cancer Subtype

Figure 11

Figure 12

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Figure 13

Thyroid Cancer Subtype

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Figure 14

Figure 15

ROC Curve
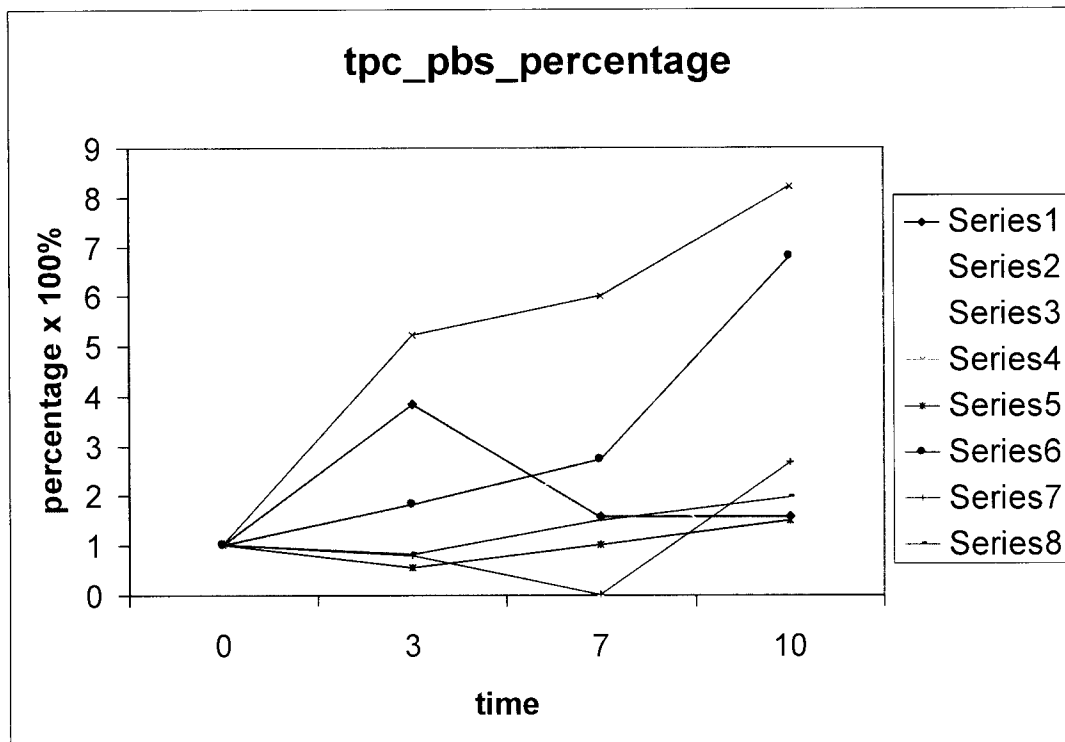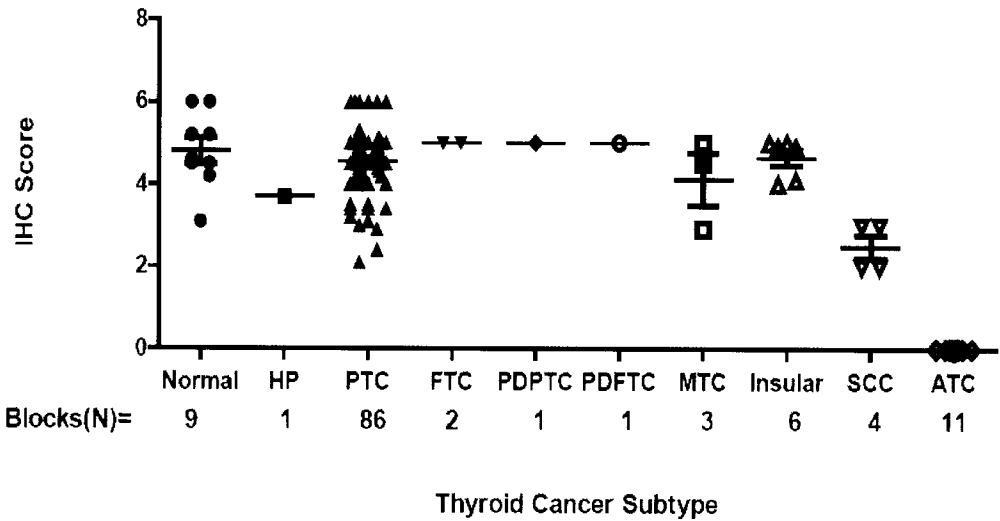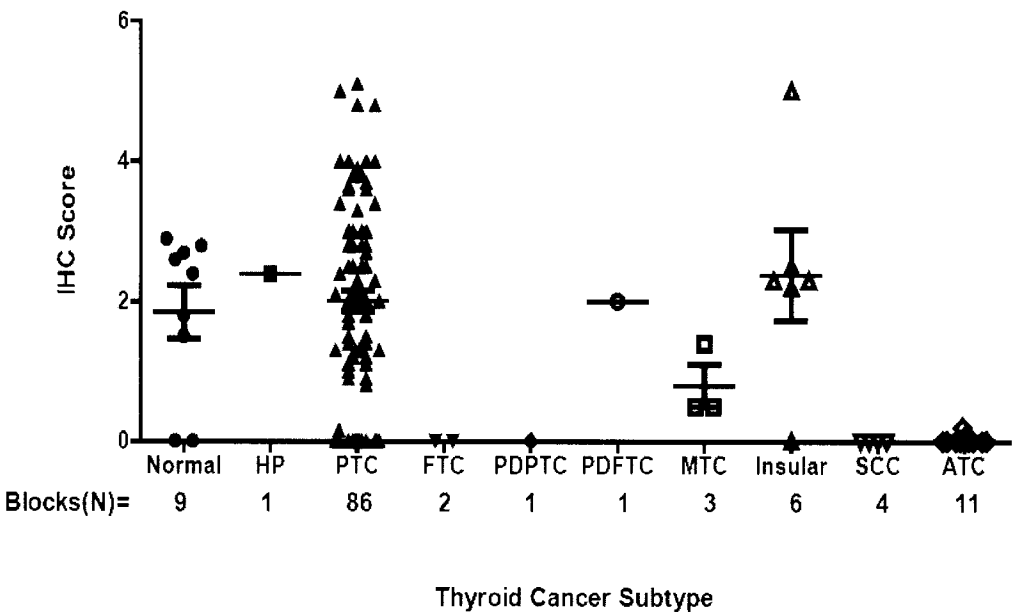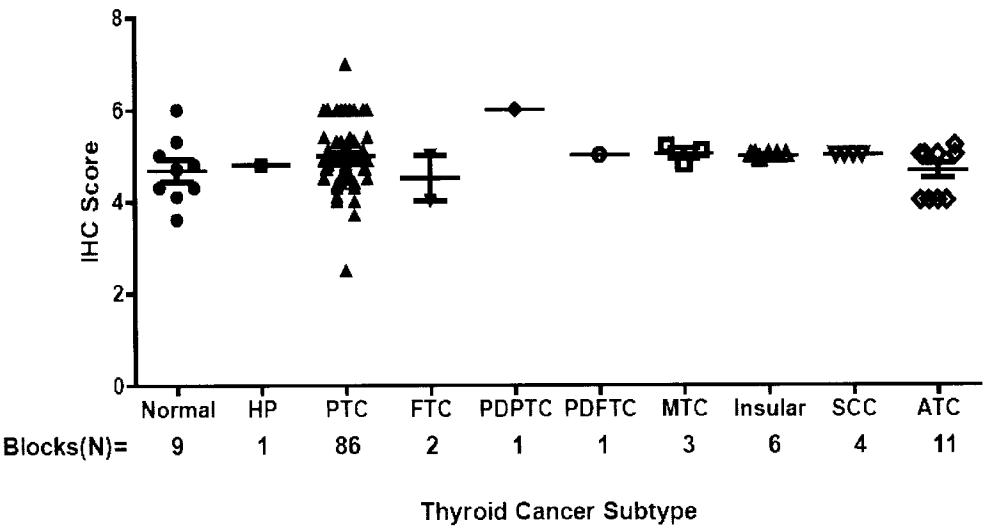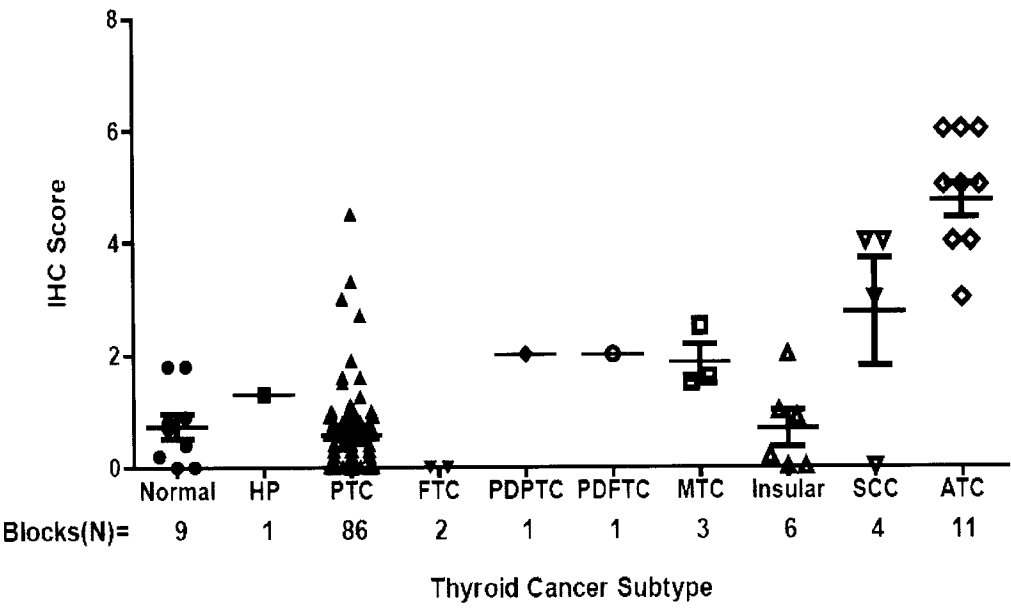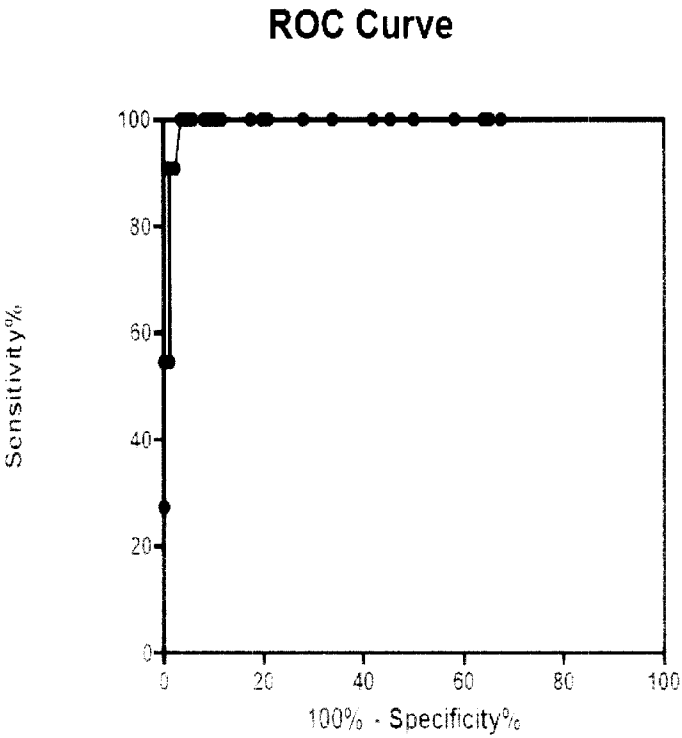
AUC	0.9931
*Using 4 as cut off Specificity	98.80%
PPV	90.90%

Figure 16

ROC Curve
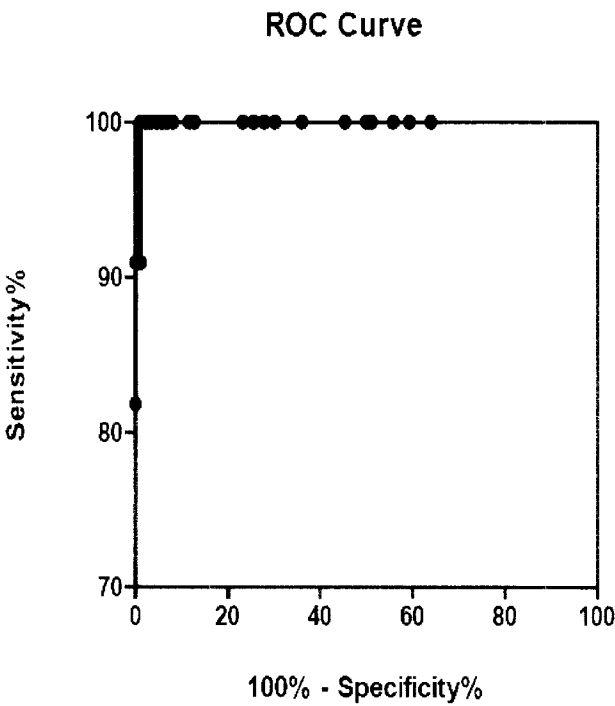
AUC	0.9989
*Using 4 as cut off Specificity	98.80%
PPV	91.67%

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Figure 17

Figure 18

37/40

Figure 19

Figure 20

A. Membrane EpEx
Malignant vs Benign Thyroid Tumors

B. Nuclear EpICD
Malignant vs Benign Thyroid Tumors

C. Membrane EpEx
Aggressive vs Nonaggressive Cancers

D. Nuclear EpICD
Aggressive vs Nonaggressive Cancers

Figure 21

(A)

Loss of Membrane EpEx (Moc31) Expression

N (cases) =   9   11   10

(B)

EpICD Nuclear Expression

N (cases) =   9   11   10

Figure 22

A.

EpEx (Moc31) Membrane Expression

	Benign	Nonaggressive TC	Aggressive TC
N (cases) =	9	11	10

Note: A cutoff score of ≤4 was used to determine positivity (Loss of Membrane expression).

B.

EpICD Nuclear Expression

	Benign Tumor	Nonaggressive TC	Aggressive TC
N (cases) =	9	11	10

Note: A cutoff score of ≥4 was used to determine positivity.

A.	CLASSIFICATION OF SUBJECT MATTER

IPC: *C12Q 1/68* (2006.01) , *A61K 39/395* (2006.01) , *A61P 35/00* (2006.01) , *G01N 33/48* (2006.01) , *G01N 33/574* (2006.01) , *G01N 33/68* (2006.01), *C40B 30/04* (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)
C12Q 1/68 (2006.01) , *A61K 39/395* (2006.01) , *A61P 35/00* (2006.01) , *G01N 33/48* (2006.01) , *G01N 33/574* (2006.01) , *G01N 33/68* (2006.01), *C40B 30/04* (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)
Canadian Patent Database, Epoque (EPODOC), Total Patent, PubMed, Scopus
Keywords: thyroid, cancer, tumour, catenin, EpCAM, epithelial cell adhesion molecule, intracellular domain, ectodomain, marker

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WISEMAN, S.M. et al. "Anaplastic thyroid carcinoma: expression profile of targets for therapy offers new insights for disease treatment". ANNALS OF SURGICAL ONCOLOGY. 10 November 2006 (10-11-2006), V. 14(2), p. 719-729. ISSN: 1068-9265. whole document	1-8 and 10-31
X	WISEMAN, S.M. et al. "Identification of molecular markers altered during transformation of differentiated into anaplastic thyroid carcinoma". ARCHIVES OF SURGERY. August 2007 (08-2007), V. 142(8), p. 717-729. ISSN: 0004-0010. whole document.	1-9 and 11-31
X	GARCIA-ROSTAN, G. et al. "β-catenin dysregulation in thryoid neoplasms: down regulation, abberant nuclear expression, and CTNNB1 exon mutations are markers for aggressive tumor phenotypes and poor prognosis". AMERICAN JOURNAL OF PATHOLOGY. March 2001 (03-2001), V. 158(3), p. 987-996. ISSN: 0002-9440. whole document.	1-9 and 11-31

[X] Further documents are listed in the continuation of Box C.	[X] See patent family annex.

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

Date of the actual completion of the international search	Date of mailing of the international search report
22 November 2010 (22-11-2010)	7 January 2011 (07-01-2011)

Name and mailing address of the ISA/CA	Authorized officer
Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Tera Mosher (819) 994-4248

Box No. I	Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

    a.   (means)

         [X]   on paper

         [X]   in electronic form

    b.   (time)

         [X]   in the international application as filed

         [X]   together with the international application in electronic form

         [   ]   subsequently to this Authority for the purposes of search

2. [X] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3.   Additional comments :

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. [X] Claim Nos. :   1, 3, 8, 9, 12, 14-21, 23, 24, 26, 27 and 31

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

    Claims 1, 3, 8, 9, 12, 14-21, 23, 24, 26, 2 and 31 are directed to a method for treatment of the human or animal body by surgery or therapy which the International Search Authority is not required to search. However, this Authority has carried out a search based on the alleged purposes or uses of the product defined in claims 1, 3, 8, 9, 12, 14-21, 23, 24, 26, 27 and 31.

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 1-31 (partially) are directed to methods for detecting or diagnosing thyroid cancer using β-catenin. Methods to select test agents using β-catenin as well as the use of and kits comprising antibodies to β-catenin are also claimed.
Group B - Claims 1-31 (partially) are directed to methods for detecting or diagnosing thyroid cancer using Ep-ICD. Methods to select test agents using Ep-ICD as well as the use of and kits comprising antibodies to Ep-ICD are also claimed.
Group C - Claims 2-4, 6, 7, 10, 12, 14-21 and 31(partially) are directed to methods for detecting or diagnosing thyroid cancer using EpEx.

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all

    searchable claims.

2. [X] 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.

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ENSINGER, C. et al. "EpCAM overexpression in thyroid carcinomas: a histopathological study of 121 cases". JOURNAL OF IMMUNOTHERAPY. September/October 2006 (09/10-2006), V. 29(5), p. 569-573. ISSN: 1524-9557. whole document.	31
P,X	RALHAN, R. et al. "EpCAM nuclear localization identifies aggressive thyroid cancer and is a marker for poor prognosis". BMC CANCER. 25June 2101 (25-06-2010), V. 10, p.331-341. ISSN: 1471-2407. whole document.	1-31
P, X	CHO, N.L. et al. "Sulindac reverses aberrant expression and localization of β-catenin in papillary thyroid cancer cells with the BRAF^{V600E} mutation". THYROID. 01 June 2010 (01-06-2010), V.20(6), p. 615-622. ISSN: 1557-9077.	1-8 and 11-31
A	WO 2007/141029 A1 (MAETZEL, D. et al.) 13 December 2007 (13-12-2007).	
A	US 2008/0075741 A1 (LEE, M. et al.) 27 March 2008 (27-03-2008).	

atent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
US2008075714A1	27 March 2008 (27-03-2008)	AU2003273542A1	19 December 2003 (19-12-2003)
		AU2003273542A8	19 December 2003 (19-12-2003)
		AU2003293408A1	30 June 2004 (30-06-2004)
		CA2507581A1	24 June 2004 (24-06-2004)
		EP1530418A2	18 May 2005 (18-05-2005)
		EP1530418A4	12 October 2005 (12-10-2005)
		EP1567008A2	31 August 2005 (31-08-2005)
		EP1567008A4	08 October 2008 (08-10-2008)
		JP2005528115T	22 September 2005 (22-09-2005)
		JP2006508682T	16 March 2006 (16-03-2006)
		US2004018531A1	29 January 2004 (29-01-2004)
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		US7803783B2	28 September 2010 (28-09-2010)
		US2004171559A1	02 September 2004 (02-09-2004)
		WO03102215A2	11 December 2003 (11-12-2003)
		WO03102215A3	21 May 2004 (21-05-2004)
		WO2004053069A2	24 June 2004 (24-06-2004)
		WO2004053069A3	26 August 2004 (26-08-2004)
WO2007141029A1	13 December 2007 (13-12-2007)	EP1865001A1	12 December 2007 (12-12-2007)
		EP2041181A1	01 April 2009 (01-04-2009)
		US2010226922A1	09 September 2010 (09-09-2010)