Secretome Proteins as Candidate Biomarkers for Aggressive Thyroid Carcinomas

by

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Abstract

Using secretome proteomics and bioinformatics, analyses of non-aggressive and aggressive thyroid carcinoma (TC) cell lines revealed nine proteins as potential biomarkers for detection of aggressive TCs. Of these, APP, APLP2, AXL, PKM2, 14-3-3 zeta and SET were detected in patients' sera by western blotting and showed significant differences in subcellular expression in benign and malignant tissues using immunohistochemistry (IHC). Membrane and cytoplasmic ALCAM expression was reduced in patients with poorly/undifferentiated (aggressive) TC compared to well-differentiated (non-aggressive) TC ($p < 0.001$, AUC=0.865, sensitivity=82%, specificity=74%) as analyzed by IHC in 158 TCs and 45 benign nodules. Kaplan-Meier survival analysis revealed reduced disease-free survival for TC patients with lower ALCAM expression (median survival 7.3 years compared to 13.5 years for patients with higher expression, $p = 0.002$). ALCAM sera levels were elevated in aggressive versus non-aggressive TC patients ($p = 0.04$), as measured by ELISA. ALCAM may potentially serve as a useful diagnostic and prognostic marker for aggressive TC.

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List of Abbreviations

ADAM - a disintegrin and metalloproteinase
ALCAM - activated leukocyte cell adhesion molecule
APP - amyloid beta A4 protein
APLP2 - amyloid-like protein 2
ATCC - American Type Culture Collection
AJCC - American Joint Committee on Cancer
ATC - anaplastic thyroid carcinoma
ATP - adenosine triphosphate
AUC - area-under-curve
AXL - tyrosine protein kinase receptor UFO
BRAF - B-raf proto-oncogene
CI - confidence interval
CM - conditioned media
CCS - Canadian Cancer Statistics
DSMZ - German Collection of Microorganisms and Cell Culture
DTC - differentiated thyroid carcinoma
ELISA - enzyme-linked immunosorbent assay
EGFR - epidermal growth factor receptor
EMT - epithelial-mesenchymal transition
FFPE - formalin-fixed paraffin-embedded
FNAB - fine needle aspiration biopsy
FTC - follicular thyroid carcinoma
FVPTC - follicular variant papillary thyroid carcinoma
hnRNP K - heterogeneous nuclear ribonucleoprotein K
HR - hazard ratio
IPA - Ingenuity Pathway Analysis
LC-MS/MS - liquid chromatography-tandem mass spectrometry
L1CAM - L1 cell adhesion molecule
MAPK - mitogen-activated protein kinase
MLNR - metastatic lymph node ratio
MNG - multinodular goiter
MSH - Mount Sinai Hospital
MTC - medullary thyroid carcinoma
NCI - National Cancer Institute
NFGR - nerve growth factor receptor
NIS - sodium-iodide symporter
NPV - negative predictive value
OR - odds ratio
PDPTC - poorly-differentiated papillary thyroid carcinoma
PGK1 - phosphoglycerate kinase 1
PI3K - phosphatidylinositol 3'-kinase
PKM2 - pyruvate kinase muscle isozyme M2
PTC - papillary thyroid carcinoma

RAI (I^{131}) - radioactive iodine
RAS - rat sarcoma viral oncogene
REB - Research Ethics Board
RECIST - Response Evaluation Criteria in Solid Tumors
rhTSH - recombinant human thyroid stimulating hormone
RRA - radioactive remnant ablation
ROC - receiver operating characteristic
SEER - Surveillance Epidemiology and End Results
SET - phosphatase 2A inhibitor
SD - standard deviation
 T_3 - triiodothyronine
TACE - tumour necrosis factor α converting enzyme
TC - thyroid carcinoma
Tg - thyroglobulin
TGF- β - transforming growth factor β
TNM - tumour, lymph nodes, metastasis
TSH - thyroid stimulating hormone
VEGF - vascular endothelial growth factor
VEGFR - vascular endothelial growth factor receptor

Chapter 1

Literature Review

1 Introduction

1.1 Thyroid Carcinoma – Overview

Thyroid carcinoma (TC) is the most prevalent malignancy of the endocrine system (1, 2). The estimated new TC cases and deaths in the United States in 2012 are 56,460 and 1,780, respectively (National Cancer Institute statistics 2012; <http://www.cancer.gov/statistics>). 80% - 85% of all TCs are well-differentiated papillary thyroid carcinoma (PTC), with overall 10-year survival rates more than 95%, even with the presence of lymph node metastasis (1, 3). 10% - 15% of patients with differentiated thyroid carcinoma (DTC) present with or will develop distant metastases (4). In this set of patients, the 10-year survival rate drops to approximately 40% (3, 5). However, anaplastic thyroid carcinoma (ATC) is among the most aggressive and deadly cancers known, with a median survival of less than 6 months, although it accounts for only 2% of all thyroid tumours (6, 7). Thus, even though TCs generally have an impressive survival rate, there exists a subset of aggressive and poorly or undifferentiated thyroid tumours that account for the majority of the deaths attributed to this malignancy (3). Follicular thyroid carcinoma (FTC), ATC, PTC, and Hürthle cell carcinomas (an FTC variant) consist of tumours originating from thyroid follicular cells, whereas medullary thyroid carcinoma (MTC), a calcitonin-secreting tumour, originates from thyroid C cells. Although there are some common forms of treatment between TC subgroups, there lie critical differences in therapeutic intervention according to the tumour type. The differentiation between aggressive and non-

aggressive tumours, therefore, becomes vitally important for long-term management of TC patients and presents an unanswered question for translational cancer research.

1.2 Subtypes of Thyroid Carcinoma

Different subtypes of TC include papillary, follicular, medullary, and anaplastic TC. The percentage distribution of TC tumour types, 10-year survival, and deaths according to a study of 53, 856 patients are listed in Table I (2). There are 15 histological variants of PTC, some of which will be described in more detail.

TABLE I
Subtypes of thyroid carcinomas

Tumour Type	% Of all TC	10-year relative survival (%)	Deaths by Tumour Type (%)
Papillary¹	79	93	53
Follicular	13	85	18
Hürthle (FTC variant)	2	76	7
Medullary	4	75	9
Anaplastic	2	14	14

Table adapted from Sipos *et al.* (2)

1 - Papillary TC variants include: follicular, macrofollicular, tall cell, columnar cell, clear cell, oncocytic (Hürthle) cell, diffuse sclerosing, cribriform, solid, PTC with insular pattern, PTC with mucoepidermoid carcinoma, PTC with focal anaplastic spindle and giant cell carcinoma, PTC with medullary carcinoma, and PTC with fasciitis or fibromatosis-like stroma (8) .

Papillary Thyroid Carcinoma

PTC often presents as a thyroid nodule ranging in size from 1 centimeter or less (microcarcinoma) to several centimeters in diameter, and often may be in the background of a

multinodular goiter (MNG) (9). The primary tumour can be a combination of solid and cystic, or just solid, but rarely completely cystic. The diagnosis of PTC is based on the nuclear characteristics of the tumour, such as having an irregular “raisin-like” nuclear membrane that is usually oval, not round (9). As well, PTCs appear to have powdery to clear chromatin (“Orphan Annie eye nuclei”), nuclear overlapping, small nucleoli, nuclear grooves and sometimes nuclear pseudoinclusions (9). The papillary structures of the classic PTC are complex and typically appear as “finger-like” projections. There are several histological variants of PTC, some of which have been described as being biologically more aggressive than classic PTC, such as the tall cell variant, diffuse sclerosing variant, columnar cell variant, and the follicular variant.

Follicular Variant PTC

The follicular variant PTC (FVPTC) is the most common variant of PTC, following classic PTC. FVPTC has the same characteristic nuclear features of classic PTC but has a follicular rather than papillary architecture (9, 10). FVPTCs are often difficult to diagnose because of the cytological similarity to follicular adenomas (11). FVPTCs were believed to behave in a similar clinical manner as classic PTC, however, recent studies have suggested otherwise (10-12). Gupta *et al.* reported that it is important to understand the differences in the subtypes of FVPTC because they have clinical and genotypic differences that could account for the more aggressive behaviour of the tumour (11). They report that diffuse FVPTC has a higher rate of central lymph node metastasis and B-raf proto-oncogene (BRAF) mutation than the encapsulated and non-encapsulated FVPTC (11). Oncogenes are discussed in section 1.6.

Tall Cell Variant PTC

This variant is associated with higher recurrence rates and poorer prognosis, and usually presents in older males (9). The histology of these tumours is characterized by at least 50% cells with granular eosinophilic cytoplasm and a height twice that of the width (9, 13, 14). This tumour is often accompanied by vascular and extrathyroidal extension (9). A study showed that regardless of tumour size, age, sex, and confinement of the tumour to the thyroid, this variant shows a higher rate of lymph node and distant metastasis compared to classic PTC (14). The presence of BRAF mutation in tall cell variants could account for this increase in tumour aggressiveness (13).

Oncocytic (Hürthle cell) Variant PTC

The oncocytic (Hürthle cell, or oxyphilic) variant of PTC is comprised of large polygonal cells, nuclear features characteristic of PTC with prominent central nucleoli, and granular eosinophilic cytoplasm (9). The abundant mitochondria within the cytoplasm give an eosinophilic granularity (9). The clinical behaviour of this variant is believed to be similar to that of classic PTC. This variant should not be confused with the Hürthle cell carcinoma, which is a type of FTC that shows focal papillary architecture and presents with a more aggressive clinical course (15).

Diffuse Sclerosing Variant PTC

The diffuse sclerosing variant of PTC is described as having diffuse involvement of both thyroid lobes without the formation of a localized mass, though, in about 50% of the cases, a dominant nodule is present in one of the thyroid lobes (15). The tumour often arises in

the background of lymphocytic thyroiditis and is characterized as having abundant psammoma bodies, diffuse stromal fibrosis, dense lymphoid infiltration, extensive squamous metaplasia, and nuclei similar to classic PTC (9, 15). Most patients are young adult females, though there are also cases reported in young children and adolescents (15). These tumours show a more aggressive behaviour compared to classic PTC; they have higher lymph node and pulmonary metastases, however, there is no increase in mortality for this group (9, 15).

Columnar Cell Variant PTC

The columnar cell variant is an infrequent variant of PTC that demonstrates more aggressive behaviour involving extrathyroidal extension (9). The majority of this type of tumour consists of pseudostratified columnar cells with elongated hyperchromatic nuclei, as well as supra- and subnuclear cytoplasmic vacuoles, while the nuclear features of classic PTC are less apparent in this variant (9). The encapsulated form does not metastasize, or shows low metastatic potential; however, the unencapsulated form of this variant displays the more aggressive behaviour (15).

Papillary Carcinoma with Insular Pattern

The histological appearance of PTC with insular pattern consists of the presence of well-defined nests of tumour cells with scant cytoplasm and dark, round, monomorphic nuclei, cytologic uniformity, hypercellularity, and scant colloid (16). These tumours are typically greater than four centimeters in size and have a high nuclear to cytoplasmic ratio with the absence of nuclear grooves and intranuclear inclusions that are generally seen in classic PTC (16). This variant is a more aggressive variant of PTC, often debated whether they should be

labelled as a poorly-differentiated form of PTC because they can still uptake radioactive iodine (RAI, I^{131}), a process that does not occur in poorly or undifferentiated TCs. A study by Rufini *et al.* studied two groups of this insular variant, one group being predominantly insular (>50% of tumour), while the other a focal insular variant (<50% of tumour), and they showed that only 4 of 9 (44%) of the former group had the ability for I^{131} uptake, while 1 of 2 in the second group could uptake I^{131} (17). As well, they showed that the predominantly insular group was associated with aggressive features such as extrathyroidal extension, lymph node metastasis, distant metastasis, and a poor prognosis (17). Therefore, their histological and biological behaviour deem them to be in the middle of PTC and ATC in aggressiveness and require more aggressive treatment.

Anaplastic Thyroid Carcinoma (ATC)

In ATC, there are no features of differentiated tissue, but rather the tumour is extensively infiltrative and is characterized by atypical cells, either spindle, epithelioid, squamoid, giant, or osteoclast-like giant cells with abundant atypical mitotic figures, and multinucleated cells (1, 18). The spindle cell variants are sometimes arranged in fascicles to resemble sarcoma, while at other times are arranged to resemble fibrous histiocytoma (6). The giant cell variant is more pleomorphic and has numerous tumour giant cells, whereas the squamoid type is characterized as having irregular configuration and abundant cytoplasm (6). These variants are described as having tan white fleshy large tumours with large areas of hemorrhage, high mitotic activity, necrosis, marked invasiveness, and a low rate of apoptosis (19). It is suggested that well-differentiated TCs may progress towards ATC through dedifferentiation of insular variants of PTC or FTC and altered expression of p53 (6). This is

further supported by a finding that differentiated TC regions co-exist in up to 90% of ATCs (6).

Although ATC is rare with an age-adjusted annual incidence of 2 per million/year (20), its lethality is highlighted by the finding that of approximately 1,200 thyroid carcinoma deaths in the USA in 2006, over 50% were due to ATC despite accounting for only 2% of all thyroid carcinomas (18). Histologic appearance of ATC predicts poor prognosis; the mean survival of ATC patients after diagnosis is only 6 months, making this cancer one of the most aggressive types (1). Furthermore, distant metastatic sites include 80% lung, 6-15% bone, and 5-13% brain (21-24). Favourable prognostic factors for ATC patients include: younger age (< 45 years), female sex, small foci of ATC, no evidence of metastatic disease, and surgery for locoregional disease (25). The overall 5-year survival rates for stage IVA, IVB, and IVC ATC are 22.9%, 10.1%, and 0% respectively, according to the AJCC. Due to the lack of evidences of various therapeutic approaches, there is currently no consensus on the management of ATC patients. A combination of surgery, radiation therapy, and chemotherapy may improve ATC patient survival (25).

1.3 Incidence & Mortality

The estimated number of new TC cases in Canada for 2011 was 5,700 (1,200 males and 4,500 females) (Canadian Cancer Statistics 2011, CCS). According to the United States National Cancer Institute's Surveillance Epidemiology and End Results (SEER) database, as of January 1, 2009, there were approximately 496,901 people alive in the United States who had a history of thyroid malignancy (National Cancer Institute statistics 2012). Also, it is estimated

that 56,460 people (13,250 men and 43,210 women) will be diagnosed with and 1,780 men and women will die of TC in 2012 (SEER). Based on rates from 2007-2009, 1.03% of men and women born today will be diagnosed with TC sometime during their lifetime (SEER).

TABLE II
Thyroid cancer incidence and mortality by age in the United States

Age (y)	Incidence (%)	Mortality (%)
<20	1.8	0.1
20-34	15.5	0.8
35-44	20.4	2.2
45-54	24.3	8.3
55-64	19.0	16.9
65-74	11.7	24.7
74-85	5.9	29.8
85+	1.4	17.3

Data summarized from NCI SEER Database for data from years 2005-2009.

The median age for TC diagnosis is 50 years and the median age for death is 73 years (SEER). The trend in SEER cancer incidence for cancer of the thyroid between 1997-2009 for all races and genders showed a significant increase of 6.6, which clearly demonstrates that the incidence of thyroid malignancy is at an all-time high. A similar increase in incidence is found among the Canadian population with a 6.8% increase in incidence for males and 8.8% for females (CCS). The widespread increase in TC incidence over several decades and the cause of this increase is still controversial. Some studies suspected that the increase was due to increased patient exposure to diagnostic X-rays (26), especially among children (13). Another study suggested that the increased incidence is due to improved diagnostics that has been increasing the detection of small subclinical tumours, such as with the use of ultrasound-guided fine needle aspiration biopsies (FNAB) (27). Other factors that have been suggested to have an

influence on the increased incidence of thyroid carcinomas are radiation exposure (28) and increased body mass index (29). A 2006 study by Davies and Welch investigated TC cases in the US from 1973 to 2002 and found that the increase in incidence was attributed to detection of small PTC and subclinical disease with no significant increase in FTC and MTC, nor in true recurrence of cancer (30). Contrary to this belief, a study from the SEER database by Enewold *et al.* for TC cases diagnosed from 1980 to 2005 of 48, 403 patients, they found that incidence varied by gender, ethnicity, and histological type, and that increased incidence was only found in PTC and consistently among all racial/ethnic groups (31). Of 39, 706 PTC cases further analyzed, it was found that rates increased most rapidly for females. Also, between 1992-1995 and 2003-2005, rates increased almost 100% among White non-Hispanics and Black females, whereas only 20-50% among White Hispanics, Black males, and Asian/Pacific Islanders. They concluded that this data reveals that increased sensitivity in diagnostic procedures and medical surveillance cannot alone account for these increasing thyroid cancer trends, especially considering that tumours of all sizes are increasing at an equal rate.

1.4 Detection, Diagnosis, and Treatment

In the early stages of thyroid cancer, symptoms are usually absent. As the cancer progresses, some symptoms patients may present with are hoarseness of the voice, a lump in the front of the neck, swollen lymph nodes in the neck, trouble swallowing or breathing, and/or consistent pain in the throat or neck. However, most often these symptoms are not due to cancer, but rather either benign goiters/nodules, or infection. Benign or malignant nodules are often incidentally detected when a patient is seen for other conditions, whereby ultrasound, CT

scan, MRI, or other forms of medical surveillance are used. Blood tests are performed to measure the levels of TSH (thyroid stimulating hormone), which acts on the thyroid gland to stimulate the production of thyroid hormone thyroxine (T_4), as well as thyroid hormones (T_3 - triiodothyronine, and T_4) to determine how well the thyroid is functioning and to rule out any non-cancerous diseases such as hyper- or hypothyroidism.

Fine-Needle Aspiration Biopsy

Thyroid nodules are detected by ultrasound scans of the neck. FNAB is then performed to determine if the nodule is benign or malignant, and if possible, the type of malignancy. The accuracy of this procedure has increased through the use of ultrasound guided FNA. In this technique, a fine needle (22 to 27 gauge) is inserted into the thyroid and an aspirate is obtained, upon which cytological smears are made to assess if malignancy is present (32, 33).

The Bethesda system is currently used to classify and report thyroid cytopathology assessments obtained through the use of FNA (34). The six diagnostic categories of this system are (a) inadequate or non-diagnostic, (b) benign or hyperplastic, (c) atypia of undetermined significance/follicular lesion of undetermined significance, (d) follicular or Hürthle cell neoplasm, (e) suspicious for malignancy, and (f) malignant (32, 34). Approximately 60-70% of all thyroid FNAs have benign cytology (32). Seven to 10% of FNAB are non-diagnostic, and contributing factors to this rate are nodule composition, FNA technique, and on-site assessment of specimen adequacy (32). Three to 6% of FNAB diagnoses are atypia of undetermined significance, and this rate can be attributed by poor specimen preparation or by the presence of minimal atypical features in a predominantly benign appearing sample (34, 35). FNAs are often repeated for these reports. Follicular or Hürthle cell neoplasm on FNA represent 6-12%

of diagnoses (36-39), and carry a 15-30% risk for malignancy (35). Therefore, surgical assessment for vascular and/or capsular invasion, often by hemithyroidectomy, is required to distinguish a follicular cancer from a benign follicular adenoma (40-42). Although most turn out to be benign follicular adenomas, those that do have a carcinoma require a second surgery to completely remove the thyroid (32). Mutational analyses for a panel of genes including BRAF, rat sarcoma viral oncogene (RAS), RET/PTC, and PAX8/PPAR γ is reported to have a sensitivity ranging from 38-85%, with a > 95% specificity for detection of malignancy (36-38, 43, 44). Specimens that are suspicious for malignancy on cytology account for 3-5% of FNA cases, and this is due to the presence of some but not all the nuclear features of the cancer, and these features are not accounted for in the majority of the sample (32, 34, 45). The risk of malignancy is 60-70% for this group, therefore surgery is often recommended (32). Lastly, 3-7% of FNAs have a diagnosis of malignancy with conclusive features of TC, and a total thyroidectomy is often performed, unless there is the presence of metastatic disease, lymphoma, and PDPTC, whereby surgery may not necessarily be performed (32).

Tumour Stage Classification

Criteria for TC tumour staging according to the American Joint Committee on Cancer (AJCC) guidelines include patient age, tumour type, tumour size, presence and location of lymph node metastases, and presence of distant metastases (AJCC Cancer Staging Manual, 6th Ed. 2002). The following table lists the TNM (tumour, lymph nodes, metastasis) classification system for TC (Table III). All ATCs (undifferentiated TC) are stage IV, while no PTCs or FTCs are stage III or IV for patients under the age of 45 years.

TABLE III
AJCC thyroid cancer TNM staging system

Stage	Papillary or Follicular		Medullary	Anaplastic
	Patient age <45y	Patient age ≥45y	Any age	Any age
I	any T, any N, M0	T1, N0, M0	T1, N0, M0	
II	any T, any N, M1	T2, N0, M0	T2, N0, M0	
III		T3, N0, M0	T3, N0, M0	
		T1, N1 _a , M0	T1, N1 _a , M0	
		T2, N1 _a , M0	T2, N1 _a , M0	
		T3, N1 _a , M0	T3, N1 _a , M0	
IVA		T4 _a , N0, M0	T4 _a , N0, M0	T4 _a , any N, M0
		T4 _a , N1 _a , M0	T4 _a , N1 _a , M0	
		T1, N1 _b , M0	T1, N1 _b , M0	
		T2, N1 _b , M0	T2, N1 _b , M0	
		T3, N1 _b , M0	T3, N1 _b , M0	
		T4 _a , N1 _b , M0	T4 _a , N1 _b , M0	
IVB		T4 _b , any N, M0	T4 _b , any N, M0	T4 _b , any N, M0
IVC		any T, any N, M1	any T, any N, M1	any T, any N, M1

Table adapted from AJCC Cancer Staging Manual, Sixth Edition and Cooper *et al.* 2009 (46)

T1 - Primary tumour diameter ≤ 2 cm and is limited to the thyroid

T2 - Primary tumour diameter between 2 and 4 cm and is limited to the thyroid

T3 - Primary tumour diameter ≥ 4 cm and is limited to the thyroid or with minimal extrathyroidal extension

T4_a - Any size tumour extending beyond the thyroid capsule to invade subcutaneous soft tissues, larynx, trachea, esophagus, or recurrent laryngeal nerve
(For ATC, T4_a defines an intrathyroidal ATC - surgically resectable)

T4_b - Any size tumour invading prevertebral fascia or encasing the carotid artery or mediastinal vessels
(For ATC, T4_b defines an extrathyroidal ATC - surgically unresectable)

N1_a - Metastasis to level VI nodes (pretracheal, paratracheal, and prelaryngeal/Delphian)

N1_b - Metastasis to unilateral, bilateral, contralateral cervical, or superior mediastinal nodes

M0 - There is no distant metastasis

M1 - There is distant metastasis

Radioactive Iodine Therapy and Thyroglobulin

After surgical removal of the thyroid for patients with DTC, I^{131} is administered to destroy any remaining thyroid tissue, and also allows for subsequent RAI scans to detect locoregional and/or metastatic disease (47). Efficacy of this treatment is assessed by serum thyroglobulin (Tg) levels. Tg is used as a marker for DTC management and detection of residual/recurrent disease. Tg is a prohormone of T_4 and T_3 and is synthesized by follicular cells of the thyroid and released into the serum. Therefore, residual Tg after I^{131} therapy is indicative of malignancy (47). Determinants of serum Tg concentrations are (a) mass of thyroid tissue present (b) presence of thyroid injury due to either post-FNA, thyroidectomy or RAI therapy, or during thyroiditis (c) and amount of TSH receptor stimulation (i.e. endogenous TSH, recombinant human TSH (rhTSH), serum human chorionic gonadotropin, TSH receptor antibodies associated with autoimmune thyroid disease) (48). Anti-Tg antibodies may also alter the detected level of serum Tg and may result in false low levels, thereby presenting a limitation in this assay especially for patients with autoimmune diseases where levels of anti-Tg antibodies may be higher than normal (49). Approximately 10% of the general population has anti-Tg antibodies compared to 20% for DTC patients (50). Serum Tg levels prior to surgery are not a reliable indicator of TC because both normal and malignant thyroid cells release Tg. Since serum Tg is cleared with a half-life of 30 hours, it is expected that it be undetectable if the patient is cured after total thyroidectomy and RAI ablation (51). However, it may take up to one year or longer for the levels to fall below detection (52). The sensitivity of most serum Tg assays is 0.9 ng/mL, however, the sensitivity has increased to 0.1 ng/mL or lower with recent available assays (53, 54). The sensitivity of this assay is increased when Tg

levels are measured after TSH stimulation, which includes thyroid hormone withdrawal or administration of rhTSH.

Serum Tg is emerging as an important indicator for the requirement of radioactive remnant ablation (RRA) therapy post total thyroidectomy for patients with well-differentiated PTC. The need for RRA therapy post total thyroidectomy is highly debated. The American Thyroid Association, European Thyroid Association, British Thyroid Association, and the Mayo Clinic set different guidelines as to which PTC patients should receive RRA therapy based on posed risk for recurrent disease. Some factors considered include TNM stage, presence of lymph node metastasis, extrathyroidal extension, and vascular invasion (55). A study led by Dr. Walfish at Mount Sinai Hospital introduced the application of post-surgical stimulated Tg for RRA selection in low risk PTC (55). Patients with Tg levels $<1\text{ng/mL}$ were not administered RRA whereas patients with Tg levels $>5\text{ ng/mL}$ received RRA (55). Patients with Tg levels $1\text{-}5\text{ ng/mL}$ were selected for RRA based on certain histology, nodes, gender, neck ultrasound, and patient's choice (55). Of 104 patients, 59 had undetectable stimulated-Tg after thyroidectomy, 35 patients had Tg values of $1\text{-}5\text{ ng/mL}$, and 10 patients had stimulated-Tg levels $>5\text{ ng/mL}$. RRA was administered to only 1 patient with Tg $<1\text{ng/mL}$, 6 patients with Tg between 1 and 5 ng/mL , and 9 patients with Tg $>5\text{ ng/mL}$. Three year follow-up of patients who had not received RRA based on these Tg levels showed no disease recurrence, as assessed by ultrasound scans and serum Tg levels. In comparison to current guidelines set by the thyroid associations that would have suggested 67-85% of patients receive RRA, this study suggested RRA for only 15.4% of patients, an 82% reduction in the use of RRA. Therefore, they conclude that stimulated-Tg measurement performed after total thyroidectomy is an objective parameter that can assist in RRA decision-making for patients with low risk DTC. Patients included in the study did not have detectable interfering serum anti-Tg antibodies, a necessary

selection criterion to avoid misleading serum Tg levels. Patients not receiving RRA therapy are spared from potential side effects such as sialadenitis, lacrimal gland/duct injury, and infertility (56-58).

Detection and Treatment of Anaplastic Thyroid Carcinoma (ATC)

ATCs are usually identified as rapidly growing masses, often > 5cm as either a single nodule or multiple nodules upon initial presentation (6). This mass is associated with pressure symptoms and hoarseness (6). The diagnosis of ATC is confirmed with the use of FNAB, which is shown to be 90% accurate (22). Failure of FNAB to diagnose ATC may be due to sampling an area that consists of well-differentiated TC foci or sampling areas with abundant presence of necrosis, fibrosis, or hemorrhage (6). ATC can also be incidentally found in patients who have undergone surgery with a known diagnosis of well-differentiated TC (6). The majority of patients with ATC do not undergo surgery due to the tumour being beyond the bounds of any meaningful resection (6). In a subset of patients where the disease is localized, complete resection of the disease without affecting surrounding vital structures may lead to prolonged patient survival (6). Most ATC patients die from uncontrolled local symptoms. Sometimes surgery followed by chemoradiation is performed on ATC patients with non-localized disease as a palliative measure to prevent death by asphyxiation and possibly increase survival by a few months (6). Radiotherapy alone is also sometimes used as a palliative measure to increase local control and short-term patient survival (59). A study reported that for 51 ATC patients treated with radiotherapy for local control, the median survival was found to be 7.5 months, versus 1.6 months for patients without local control (59). However, some

issues pertaining to radiotherapy include timing of the therapy, dose administered, and pattern of delivery (6).

Because many ATC patients present with metastases, chemotherapy becomes important in the multimodal management of ATC. However, whether using monotherapy (doxorubicin) or combination therapy (i.e. cisplatin, bleomycin, melphalan, paclitaxel), disappointing response rates have been reported (6). Most studies report only a few patients with improvements, but no patients with a complete response, often due to the chemoresistance of ATC cells (6).

For multimodal treatment of ATC, controversy still remains regarding the timing of the different modes of treatment. One study suggests chemoradiation to be given before surgery (24), while another study suggests chemoradiation after surgery (60) .

1.5 Prognostic Factors

Histology

Patient outcome is strongly influenced by tumour histology. As stated in detail above, there are aggressive TCs as well as aggressive variants of PTC. ATC is known to have the worst prognosis, followed by insular carcinoma/PDPTC, and medullary thyroid carcinoma (MTC). The aggressive variants of PTC include the tall cell, follicular, columnar cell, and diffuse sclerosing variant. Despite there being 15 variants of PTC with different characteristic histological features, many have similar clinical behaviour (2).

Tumour Size

The size of the tumour correlates with prognosis for patients with PTC, and the larger the tumour size, the more likely the presence of locoregional and distant metastases (61) . Also, the risk of recurrent disease and cancer-specific mortality is increased linearly with tumour size (61) . A study by Mazzaferri *et al.* reported that tumours <1.5cm in size had a 0.4% 30-year cancer-specific mortality rate as compared to 22% for tumours >4.5cm (61) .

Age

Age at the onset of this disease is a critical predictor of patient outcome. An age cut-off of 45 years is currently used by TC staging guidelines of the AJCC. Patients under this cut-off are considered stage I if there is no distant metastasis, and are considered stage II with distant metastasis, therefore, no patient in this group can be stage III or IV. Conversely, patients above this age cut-off have stage III disease if their tumour is larger than 4 cm, has minimal extrathyroidal extension, or has regional lymph node metastasis. Stage IV category includes patients with greater extrathyroidal extension, lateral lymph node metastasis, distant metastasis, or having an ATC. Studies have concluded that patients younger than 45 years have a survival advantage compared to their older counterparts (62, 63) . Other studies have suggested different age cut-offs, ranging from 50 to 60 years (64, 65). However, a recent study by Oyer *et al.* aimed to elucidate the relationship between age and prognosis for DTCs, such as PTC and FTC, by investigating a total of 42, 209 DTC cases (66) . Patients 45 years and older had significantly worse survival than younger patients, and that a significant decrease in disease-specific survival was first seen in patients aged ≥ 35 years and this survival steadily decreased with each additional decade of age (66) . They concluded that patients ≥ 35 years were 14

times more likely to die from DTC than patients who were younger, and that increasing age represented by a continuum starting at 35 years is associated with poorer survival (66) .

Gender

Mortality rates and recurrence rates are higher among men than women (2, 61) . Furthermore, men have higher rates of extrathyroidal invasion than women (51% vs. 39%), higher rate of locoregional lymph node involvement (40% vs. 32%), and over twice the rate of distant metastases (9% vs. 4%) (2). A recent study evaluating the disease-specific survival of women and men of different age groups concluded that women had improved survival compared to men when diagnosed under the age of 55, however this survival became similar to men for women diagnosed at 55-69 years of age and above 70 years (67) . They hypothesized that older age influences the effect of gender on survival outcomes due to hormonal alterations caused by menopause in women (67) .

Lymph node Metastasis

Prior to the use of ultrasonography, approximately 15-30% of patients had locoregional lymph node involvement at presentation, however, with the widespread use of ultrasonography and the ability to identify small nodes, these rates have increased (68, 69) . There has been much controversy regarding the clinical importance of lymph node metastasis, and while some studies have found no difference in survival for patients with or without lymph node metastasis (70, 71) , others report an increased risk of recurrence (61, 72) and reduced survival (73) . However, a study of the SEER database concluded that patient age was an important factor for survival for patients with lymph node metastasis, whereby patients aged <45 years had no

effect on survival as compared to 46% increased risk of death for patients ≥ 45 years ($p < 0.001$) (74) . The AJCC includes nodal status as a parameter for TNM staging for PTC; however, it only takes into account the presence/absence and location of lymph node metastasis but not the extent of involvement (46). A recent study by some in our group has shown that the metastatic lymph node ratio (MLNR; ratio between number of metastatic nodes and total number of lymph nodes resected) is an independent predictor of PTC recurrence and enhances the predictive value of TNM nodal classification (75) . For 35 patients with recurrent disease of a total of 253 patients analyzed, TNM nodal classification with total MLNR had greater accuracy in predicting recurrence than the TNM nodal classification alone (75). Other studies have shown that elevated MLNR reduces survival in well-differentiated TC (76) and MTC (77) . Therefore, the MLNR may be a more reliable means for identifying risk of recurrence than the current staging system alone.

Extrathyroidal Extension

Approximately 30% of patients with PTC have extrathyroidal extension (61) . Gross extension of the cancer into surrounding musculature, esophagus, or trachea is associated with high risk of recurrence (78) . These patients require aggressive surgical debridement (79, 80) and may also benefit from external beam radiotherapy (81) . Microscopic thyroid extension is also associated with an increased risk for recurrent disease (82) , as well as higher risk of lymph node metastasis (83) and a higher mortality rate than patients without this spread (61).

Distant Metastases

Although only 5-10% of patients initially present with distant metastases, it is the main cause of death for PTC patients (5, 84) . An additional 2.5-5% of patients will develop them after initial radioiodine ablation (85) . Mortality is high for these patients; one study stated a 50% survival at 3.5 years, however, survival is improved in younger patients (86) , patients with microscopic disease(5), and patients with iodine-avid tumours (5, 86) . Fifty percent of patients with distant metastasis develop it in the lungs, 25% have only metastasis to the bone, 20% have both lung and bone metastases, and about 5% develop distant metastases to other sites (5, 85) .

Risk of Recurrence

The AJCC staging criteria was developed to aid in prognostication of TC patients, predict risk of death, and to aid in making decisions on postoperative therapy. For risk of recurrence, patients are stratified into three categories depending on certain features that account for increased risk. Low-risk patients have (i) no local or distant metastases; (ii) fully resected macroscopic tumours; (iii) no tumour invasion of locoregional tissues or structures; (iv) no aggressive histology (i.e. tall cell or insular variant) or vascular invasion or; no I¹³¹ uptake outside the thyroid bed (46, 87). Intermediate-risk patients have (i) microscopic invasion of tumour into the perithyroidal soft tissues at initial surgery; (ii) cervical lymph node metastases or I¹³¹ uptake outside the thyroid bed (88) ; (iii) tumour with aggressive histology or vascular invasion (46). High-risk patients have macroscopic tumour invasion, incomplete tumour resection, and distant metastases (46). Depending on patient risk, the primary aim of the first dose of RAI after total thyroidectomy may be 1) remnant ablation - destroys residual

thyroid tissue and facilitates detection of recurrent disease and initial staging, 2) adjuvant therapy - decreases risk of recurrence and mortality by destroying suspected metastatic disease, or 3) RAI therapy - treats known persistent disease (46).

1.6 Oncogenes and Tumourigenesis

It is estimated that 3.2% to 6.2% of TCs can be explained by familial factors. Most of the familial TCs are PTC, and a 4 to 10 fold increase in the incidence of PTC is reported in relatives of PTC patients (89). Familial PTC cases are known to have an early onset of disease and a more aggressive phenotype (90). There have been several oncogenes studied and associated with particular subtypes of TC, which has led researchers to a better understanding of this disease. The mitogen-activated protein kinase (MAPK) signalling pathway is a key pathway that is constitutively activated in the presence of some mutations, and this pathway includes important cell surface receptors such as RET, guanosine triphosphate-binding G-proteins such as RAS and intracellular kinases such as RAF (91). MAPK pathway plays a critical role in cell proliferation, differentiation, and apoptosis (1, 92).

B-raf Proto-Oncogene (BRAF) Mutation

The most extensively studied mutation in TC is the BRAF mutation, and it is associated with 29% to 83% of PTC tumours (1, 93, 94), 10% of poorly-differentiated, and 15% of undifferentiated tumours (1). This mutation is the most potent isoform in stimulating the MAPK pathway (95). The BRAF mutation results in a 500 fold increase in activation of the MAPK pathway and is associated with the silencing of many thyroid-specific iodine-

metabolizing genes (96). Although the MAPK pathway is affected by many genetic alterations in PTC, the histological phenotype and expression profile is not necessarily the same among all, therefore, suggesting that other pathways may be involved (91). The exact clinical implications of this mutation are debated. Some studies have found this mutation to be associated with persistent disease and death, extrathyroidal extension, multifocality, presence of nodal metastases, advanced-stage disease, poor iodine uptake, and older age at initial presentation (91, 97-99). Other studies have shown an absence of significant relationships between clinical outcome and BRAF mutation (93, 97, 100). Since the BRAF mutation is exclusive to PTC, or ATC originating from PTC, it can be used to aid in the diagnoses of PTC patients (101). One study showed that the mutation was present in 72% of tumour samples and that examination of this mutation in 16% of the indeterminate FNA biopsies resulted in a diagnosis (102). In another study, a diagnosis was established for 5 of 15 indeterminate FNA samples using this mutation analysis (2). Since poorly-differentiated and anaplastic TC harbour the BRAF mutation, but not RET/PTC, it is believed that the BRAF mutation may predispose PTCs to de-differentiation (103). A study by Knauf *et al.* showed that targeted expression of BRAF in transgenic mice results in de-differentiation of PTC (104).

RET/PTC, RAS, and PAX8/PPAR γ Mutations

Another mutation associated with TC is the RET/PTC mutation. This mutation is present in 20% of classic PTCs (91). The BRAF and RET/PTC mutations are mutually exclusive; therefore they do not occur in the same tumour (91). The RAS mutation is present in 20-40% of benign nodules (follicular adenomas), 40-50% of FTC, and 10-20% of follicular

variant PTC, and is associated with less lymph node and more distant metastases (91), while the PAX8/PPAR γ mutation is present in 2-10% of follicular adenomas and 35% of FTC (91) .

p53

The p53 gene is a tumour suppressor gene that plays an important role in its nuclear transcription factor production and cell cycle regulation, DNA repair, and apoptosis. p53 is mutated or absent in approximately 50% of all human cancers and 52% of ATC (105). Mutations in this gene result in angiogenesis, growth, and dedifferentiation (18). Interestingly, reintroduction of the wild type p53 in ATC cell lines resulted in the redifferentiation of ATC (106) .

PI3K/Akt Pathway

Aberrant activation of the phosphatidylinositol 3'-kinase (PI3K) /Akt pathway has also been shown to play a role in thyroid tumourigenesis and promotes the progression of a thyroid adenoma to FTC and to ATC (18). This pathway has many key roles, including cell survival and proliferation. Hou *et al.* report genetic alterations in 31% of benign thyroid adenomas, 55% of FTC, 24% of PTC, and 58% of ATC (107) .

EGFR and VEGF

Epidermal growth factor receptor (EGFR) is a cell membrane receptor that plays a major role in tumourigenesis and cancer progression (18). Induction of EGFR by stimulation with its ligands or activating mutations of its tyrosine kinase domains leads to a cascade of responses including cell growth, proliferation, invasion, cell survival, and angiogenesis (108).

Overexpression of EGFR has been associated with advanced tumour stage, metastasis, and poor clinical outcome in several human cancer such as breast, cervical, lung, bladder, and head and neck cancers (18). At least one-third of all ATCs demonstrate EGFR overexpression (109). Many kinds of EGFR-targeted therapies are being investigated including anti-receptor monoclonal antibodies, anti-ligand monoclonal antibodies, ligand-toxin conjugates, and tyrosine kinase inhibitors (18).

Vascular endothelial growth factor (VEGF) functions in the tumourigenic process by binding to the tyrosine kinase receptor VEGFR, thereby activating downstream signalling pathways involved in angiogenesis (18). Therefore, VEGF is implicated in accelerated growth, local invasion, and metastasis (18). Current therapeutic approaches involve targeting both the EGFR and VEGF signalling pathways in combination.

1.7 New Therapies for Thyroid Cancer

Recently, new therapies have entered clinical trials using multi-targeted kinase inhibitors for patients with advanced or progressing metastatic thyroid cancers. Agents tested commonly target growth factor receptor pathways and have similar drug toxicities. Since a common feature of TC is increased vascularization with elevated expression of VEGF compared to normal tissue, many drugs are chosen for their targeting of its receptor (VEGFR) (110). Several clinical trials using different tyrosine kinase inhibitors that have been conducted and/or are ongoing are listed in Table IV (111-117). Patient response to treatment is determined as per the Response Evaluation Criteria in Solid Tumors (RECIST) (118). A lack in drug response through targeted inhibition could be attributed by compensatory signalling pathways that can rescue tumour growth (110).

TABLE IV
Clinical trial agents for thyroid cancer patients

Drug	Pathway Targeted	TC Type	Patient Response	Author & Year of Study
Sorafenib	RAF, RET, VEGFR	30 DTC	23% - partial response 53% - stable disease	Gupta-Abramson <i>et al.</i> 2008
Sorfenib	RAF, RET, VEGFR	41 PTC 11 FTC 4 ATC	15% - partial response 56% (PTC) - stable disease No response for FTC and ATC	Kloos <i>et al.</i> 2009
Sorfenib	RAF, RET, VEGFR	32 DTC	25% - partial response 34% - stable disease	Hoftijzer <i>et al.</i> 2009
Sunitinib	VEGFR	37 DTC 6 MTC	11% (DTC) - partial response 57% (DTC) - stable disease 33% (MTC) - stable disease	Cohen <i>et al.</i> 2008
Sunitinib	VEGFR	28 DTC 7 MTC	3% - complete response 29% - partial response 46% - stable disease	Carr <i>et al.</i> 2010
Axitinib	VEGFR, c-Kit, PDGFR	60 of many types	30% - partial response 28% - stable disease	Cohen <i>et al.</i> 2008
Pazopanib	VEGFR, PDGFR	39 de-differentiated DTC	46% - partial response	Bible <i>et al.</i> 2010
Gefitinib	EGFR	27 of many types	No response	Pennel <i>et al.</i> 2008

Table adapted from Antonelli *et al.* 2011 (110)
c-Kit : a protein receptor that binds to stem cell factors
PDGFR : platelet-derived growth factor receptor

In vitro tests are shown to be 60% effective in predicting chemosensitivity *in vivo* (119), while negative chemosensitivity tests *in vitro* are associated with 90% treatment ineffectiveness *in vivo* (120), therefore *in vitro* studies are an important preliminary measure for the selection of clinical trial drugs. These *in vivo* studies allow researchers to avoid administration of ineffective chemotherapeutics (110) .

Several other drug groups and targets are currently being tested *in vitro* or in clinical trials, many of which are tailored to the treatment of ATC. Combretastatin A4 phosphate is a

vascular disrupting agent that inhibits tumour blood flow through already existing vessels (105). AZD2171 is another tyrosine-kinase inhibitor of VEGFR, and it has been shown to block tumour growth and extend the survival of ATC-bearing mice (121). Histone deacetylase inhibitors show promise for TC treatment as well. Through hyperacetylation, these agents are able to induce cell differentiation, cell cycle arrest, apoptosis, and they also have the potential to enhance drug cytotoxicity (105) .

The sodium-iodide symporter (NIS) is an intrinsic plasma membrane protein that mediates follicular thyroid cells to actively transport iodide into the thyroid gland (18) . Subsequent iodide accumulation through this mediation allows for effective therapeutic application of RAI and diagnostic thyroid scintigraphy (18) . This function is lost in ATCs due to decreased expression of NIS resulting in a loss of iodide accumulation (18) . Transfection of human NIS into ATC-bearing mice tumours effectively inhibited tumour growth when a therapeutic dose of I^{131} was administered to the mice (122). Furthermore, gene therapy targeting of transcription factors TTF-1 and PAX8, which cooperate in the transcriptional activation of thyroid-specific genes such as Tg and NIS, can lead to the re-activation of these genes and improve the ability of ATC cells to uptake radioiodine (18) .

1.8 Summary

There are many subtypes of TC and each subtype presents not only different histological characteristics, but also different clinical outcomes. There are limitations in pre-operative diagnoses of TC, and there are currently no biochemical markers that could aid in identifying aggressive subtypes of TC, nor to aid in prognostication. Although serum Tg is an

important marker for management of TC patients, it is limited to differentiated carcinomas and absence of anti-Tg antibody interference. Also, there is much controversy regarding the extent of treatment to be given to TC patients, such as whether total thyroidectomy or lobectomy is required, or which patients require RAI ablation. Therefore, new therapeutic strategies and targets are required for time and cost-effective management of TC patients.

Chapter 2

Rationale and Objectives

2 Rationale and Objectives

Currently there are no molecular markers that can identify and predict aggressive TC cases, particularly those that may result in distant metastases and reduced survival (123). Although ultrasound-guided FNAB is the most accurate technique for pre-operative diagnosis of TC patients, about 20-40% of all cytological reports are limited by indeterminate, suspicious, or atypical findings (124). This often results in many patients undergoing unnecessary total thyroidectomies, as many of the suspected nodules turn out to be benign (91, 125). Despite the plethora of criteria to differentiate aggressive TCs from non-aggressive cases, lack of universal consensus and controversy generating ongoing debates resulted in only a select few being considered in the currently recommended TNM staging system (2). Accurate staging and risk stratification are critical determinants guiding the postoperative management of TC patients. This includes the need for I¹³¹ therapy, or alternatives such as conservative management without ablation, surgical re-intervention, or external-beam radiation therapy, while deciding the long-term follow-up strategy (126). Therefore, there is an urgent need to identify biomarkers that could aid in the detection, diagnosis, and management of TC, especially those with an aggressive potential. This could help set up clear criteria for patients who require adjuvant treatment, especially RAI ablation where discrepancy currently exists (55).

Advances in proteomic technologies in combination with bioinformatics have provided opportunities to reveal important molecular pathways implicated in cancer progression and for

the discovery of biomarkers. The challenges of blood proteomics, stemming from the complexity of the fluid, have led researchers to seek alternate sources for the discovery of circulating cancer biomarkers, such as the cancer “secretome” (127) . The term secretome refers to proteins released by cells by different mechanisms of secretion (128) . These proteins may be detected by analyzing the conditioned media (CM) of cancer cell lines. Secretome proteins are of particular interest because they are more likely to be detectable in blood, and therefore have the potential for use as minimally-invasive blood-based biomarkers to aid in disease management (129-135). Furthermore, secretome proteins have been shown to be implicated in the interactions between cancer cells and stromal cells that are actively involved in tumour progression and are often differentially expressed in cancer tissues (136-138) .

2.1 Goals

The goal of this study was to analyze the secretomes of aggressive and non-aggressive thyroid carcinoma cell lines using proteomics and bioinformatics to identify potential diagnostic and/or prognostic biomarkers for aggressive TC. The proteins of interest would then be further analyzed in TC patients’ sera and tissues.

2.2 Hypotheses

HYPOTHESIS 1: Analysis of proteins secreted from non-aggressive and aggressive TC cell lines may lead to the identification of candidate biomarkers based on important potential biological interactions.

HYPOTHESIS 2: Of a panel of secretome proteins chosen for further investigation, some are likely to display differential expression in patients' tissues and sera between benign and malignant thyroid tumours. Furthermore, some proteins may serve as diagnostic biomarkers for the detection of more aggressive forms of TC when assessing different clinico-pathological parameters.

HYPOTHESIS 3: Proteins expressions associated with malignancy may also serve as prognostic markers and correlate with disease-free survival of TC patients.

2.3 Specific Aims

AIM 1: Proteomic analysis of secretomes of aggressive (anaplastic-derived) and non-aggressive (papillary-derived) TC cell lines in tandem with bioinformatics for identification of candidate biological markers for aggressive TCs.

AIM 2: Confirm the detectability of a panel of secretome proteins in TC patients' sera using western blotting.

AIM 3: Verification of proteins in TC patients' tissues using immunohistochemistry to determine differences in subcellular expression and to correlate protein staining with clinico-pathological parameters and patient survival.

AIM 4: Quantify protein levels in TC patients' sera using enzyme-linked immunosorbent assay and correlate levels with clinico-pathological parameters to assess their potential for use as diagnostic blood-based biomarkers.

Chapter 3 Methods

3 Methods

3.1 General Schematic

A general schematic of the workflow and experimental design used in this study is provided in Figure. 1. Using a secretome approach, candidate biomarkers were identified for potential use in blood-based assays. Our experimental approach included: (i) identifying high confidence secretome proteins from conditioned media of cultured PTC and ATC cells using liquid chromatography - tandem mass spectrometry (LC-MS/MS) proteomic analysis; (ii) using Ingenuity Pathway Analysis (IPA) to identify key protein-protein interactions in signaling pathways pivotal in cancer; (iii) selecting a panel of proteins from this analysis for further investigation in patients' sera using western blots and/or ELISA; (iv) investigating potential alterations in expression of these proteins in patients' tumours using IHC.

Figure 1. Schematic of experimental workflow

Figure 1. **Schematic of experimental workflow.** PTC (BCPAP) and ATC (C643, SW1736) cell lines were cultured. Conditioned media was collected, proteins were precipitated, and high confidence secretome proteins were identified using liquid chromatography - tandem mass spectrometry (LC-MS/MS). Ingenuity Pathway Analysis was used to identify key protein-protein interactions in cell signalling networks that are pivotal in cancer, as well as literature review of protein expression and their roles in cancer. Nine proteins were chosen to study their expression in human sera and tissues using western blots, ELISA, and IHC to assess their applicability as potential biomarkers for aggressive TC.

3.2 Detailed Methods

Cell Lines

Three TC cell lines, non-aggressive BCPAP (PTC-derived), and aggressive SW1736 and C643 (ATC-derived) were used in this study. SW1736 was provided by Dr. E. Heldin (Rudbeck Laboratory, Uppsala University, Finland), and C643 by Dr. G. Salvatore (University of Naples, Italy). BCPAP was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschh, Germany). Short tandem repeat (STR) profiles of all the cell lines were determined and matched to those of the original TC-derived cell lines to confirm their identity (139). These cell lines have been demonstrated to express thyroid specific genes, thereby confirming their thyroid origin (139) .

Cell culture, conditioned media collection and protein precipitation

TC cell lines (BCPAP, SW1736, and C643) were propagated in RPMI-1640 containing 100 µg/mL streptomycin and 100 U/mL penicillin, 10% FBS and 1X non-essential amino acids in 150 mm dishes to about 65% confluence at 37°C in a humidified atmosphere of 5% CO₂ - 95% air. For secretome analysis, cells were grown in serum-free medium for 48 h as previously described by us (140). Trypan blue staining was performed following collection of the conditioned media at 48 h to estimate the number of dead cells. Since $\geq 98\%$ cells were viable at 48 h, this time period was chosen for further study. This was done to maintain secretome integrity by limiting secretome contamination from lysis of dead cells. Thereafter, the CM were collected, centrifuged at 5000 g for 5 minutes at 4°C, filtered using a 0.2 µm nylon filter, snap frozen, and stored at -80°C until further use. Proteins were precipitated from

the pooled CM using sodium deoxycholate-trichloroacetic acid method and prepared for LC-MS/MS analysis as described earlier (140). Briefly, following 2 h incubation on ice, the samples were centrifuged at 11,000 g for 30 minutes and washed twice with ice-cold acetone. The precipitated proteins were then dissolved in 50 mM NH_4HCO_3 buffer, pH 7.5. The protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA). Protein samples were then heated for 1 h at 65°C in the presence of 5 mM dithiothreitol, cooled to room temperature, and incubated in dark for 1 h with 10 mM iodoacetamide for alkylation. Sequencing grade trypsin (Promega, WI) at 1:20 (w/v) in 50 mM ammonium bicarbonate buffer was subsequently added and the samples were incubated at 37°C overnight. The trypsin-digested samples were then dried under vacuum and dissolved in 10 μL of 0.1% formic acid. Experiments were repeated twice and each set was analyzed separately using LC-MS/MS as described below.

Liquid chromatography – tandem mass spectrometry

Samples were analyzed by online nanoflow LC-MS/MS using the Eksigent Tempo LC (Dublin, CA) fitted with a 10- μL sample loop. A part of the trypsin-digested samples were loaded onto a 5-mm RP C18 precolumn (LC Packings, Amsterdam, The Netherlands) at 20 $\mu\text{L}\cdot\text{min}^{-1}$ and washed for 5 min before switching the precolumn inline with the separation column. The separation column was a 75- μm -internal diameter x 150-mm-length capillary column (Integrafit capillary from New Objective, Woburn, MA) packed in-house with 3.5- μm C18 beads with 100-Å pores from Kromasil (Akzo Nobel/EKA Chemicals inc, NY). Gradient elution was performed at a flow rate of 200 nL/min with solvent A being 5% acetonitrile and 0.1% formic acid in water, and solvent B 95% acetonitrile in 0.1% formic acid. The gradient

was as follow: start at 95 % A, 5 % B, hold until $t = 5$ min.; B increased to 15% by $t = 10$ min, then to 35% by $t = 70$ min, and finally to 80% by $t = 85$ min, hold until $t = 95$ min; then B is lowered to 5% by $t = 98$ min, and the column equilibrated until $t = 135$ min. This HPLC was connected on-line to a Q-Star Elite mass spectrometer (AB SCIEX, Foster City, CA) via a nanoflow II electrospray ionization interface. Samples were analyzed in information-dependent acquisition mode with the scan cycles set to perform a 1-s MS survey scan with a mass range of 400-1500 Da followed by MS/MS scans of the four most abundant peaks with a mass range of 80-2000 Da for 2 s each, The collision energy (CE) was automatically controlled by the IDA CE Parameters script. Switching criteria were set to ions with $m/z \geq 400$ and ≤ 1500 , charge states of 2-4, and abundances of ≥ 10 counts. Former target ions were excluded for 30 s, and ions within a 6-Da window were ignored. The controlling software was Analyst QS 2.0 (AB SCIEX). Performance of the analytical system, including sensitivity, resolution, and mass accuracy, was evaluated and re-optimized once every day, using 95 fmol of a bovine serum albumin tryptic digest as control.

Bioinformatics

The MS/MS spectra were processed by ProteinPilot version 2 (Applied Biosystems), using the Paragon algorithm in Thorough Search mode, with iodoacetamide selected as the cysteine modification, and the search option 'emphasis on biological modifications' checked (129). The confidence threshold was set at 95% (ProteinPilot protein Unused Score of 1.3), and the search was performed against a Swiss-Prot database concatenated in June 2010, containing 66 082 distinct human protein entries. SignalP (<http://www.cbs.dtu.dk/services/SignalP> 3.0) was used to analyze identified proteins for

classical protein secretion features (141). SignalP predicts the presence and the location of signal peptide cleavage sites in the amino-acid sequences by a combination of artificial neural networks and hidden Markov model algorithms to detect signal peptides from input protein sequences. The presence of secretory signal peptide sequences was determined with a probability ≥ 0.9 . SecretomeP (<http://www.cbs.dtu.dk/services/SecretomeP> 2.0) was used for non-classical and leaderless protein secretion (142). A given protein was considered non-classically secreted if it contains a non-classical (non-signal) peptide-triggered protein secretion with score ≥ 0.5 ; only proteins that did not contain a signal peptide as determined by SignalP were legitimate candidates for this analysis. IPA (Ingenuity Systems, www.ingenuity.com) was used to determine the subcellular localization and biological functions of the identified proteins (for detailed information on IPA, visit www.ingenuity.com). We classified a protein as “secreted” if it satisfied at least one of the following four criteria: (i) its subcellular location is extracellular or membrane-bound, according to IPA; (ii) it is classically secreted, according to SignalP; (iii) it is non-classically secreted, according to SecretomeP; or (iv) it is non-classically secreted by the exosome pathway, according to the literature. IPA was used to generate potential cellular networks for all proteins to aid in the selection of a panel of proteins for immunohistochemical and serological analysis. Proteins were considered for further analysis if they were identified with at least 2 unique peptides ($\geq 95\%$ confidence) and were secreted from ATC-derived cell lines. 14-3-3 zeta and activated leukocyte cell adhesion molecule (ALCAM/CD166) were identified with only one high confidence peptide, yet these proteins were also considered for further verification because 14-3-3 zeta was previously identified as a candidate biomarker in head and neck squamous cell carcinomas by some in our group (143), and ALCAM has recently been reported to play a potential tumourigenic role in head and neck and in TC cells (143, 144), and

also identified as a predictor of disease progression and reduced survival in oral cancer patients (145) .

Patient specimens

The study was approved by Mount Sinai Hospital (MSH) Research Ethics Board (REB), Toronto, Canada. Informed consent for the scientific use of anonymized patients' data and tumour tissues had been obtained from all patients as per REB guidelines. All data were analysed anonymously. Five mL of blood was collected from TC patients and patients with benign nodules attending the thyroid clinic at MSH. Blood was allowed to coagulate for 30 min at room temperature, centrifuged at 1000 g for 10 min, and sera stored in 1.5 mL freezer vials at -80°C. Archived formalin-fixed paraffin-embedded (FFPE) tissue blocks from the MSH Tumour Bank were retrieved and reviewed by the pathologist (CM) to select tissues from patients with benign nodules, early stage PTC and variants of PTC, and advanced stage TCs of different subtypes. Clinico-pathological parameters were obtained from histopathological analyses and the clinical database (IK). Sera and tissue samples from patients with benign thyroid nodules, non-aggressive TCs, and aggressive TCs were analyzed. Benign nodules were obtained from patients with multinodular goiters, Grave's disease, Hashimoto's thyroiditis, or hyperplastic nodules. Non-aggressive TCs were those of well-differentiated tumour histology and determined to be Stage I or II according to the AJCC tumour staging criteria and did not have lymph node metastasis, distal metastasis, and/or extrathyroidal extension. Aggressive carcinomas were those that were determined to be Stage III or IV according to AJCC tumour staging criteria, and/or have poorly differentiated histology, lymph node metastasis or distal metastasis, or extrathyroidal extension. Patients under 45 years of age with stage II TC were

classified under aggressive carcinomas, as these patients have distant metastases. The age of patients ranged from 16 to 85 years, median 47 years, including 47 males and 156 females. Forty-five benign thyroid nodules and 158 malignant tissues were analyzed for ALCAM expression.

Detection of proteins in thyroid carcinoma and benign patients' sera by western blot

Six benign (non-cancerous) sera and 12 sera from TC patients were analyzed for detection of 9 proteins namely, ALCAM, tyrosine protein kinase receptor UFO (AXL), amyloid beta A4 protein (APP), amyloid-like protein 2 (APLP2), heterogeneous nuclear ribonucleoprotein K (hnRNP K), phosphoglycerate kinase 1 (PGK1), phosphatase 2A inhibitor (SET), 14-3-3 zeta, and pyruvate kinase muscle isozyme M2 (PKM2) using western blots. Of the carcinoma patients, 6 were diagnosed with non-aggressive TC and 6 with aggressive TC. Sera (24 μ L) from TC patients were treated and concentrated using the ProteoPrep 20 Immunodepletion Kit (Sigma) as described (140). Proteins were separated by denaturing electrophoresis on polyacrylamide gels and blotted onto a polyvinylidenedifluoride (PVDF) membrane (Millipore, Billerica, MA). Blots were pre-incubated at room temperature for 1 h in a blocking solution consisting of 5% (w/v) skimmed milk powder in 20 mM Tris-HCl buffer pH 7.6, 140 mM sodium chloride (TBS) containing 0.1% Tween 20 (TBST). Primary antibodies were diluted with 5% (w/v) skimmed milk powder in TBS prior to incubation (2 h) with the following antibodies from Abcam (Cambridge, MA): ALCAM monoclonal mouse antibody (1:500 dilution); APLP2 rabbit polyclonal antibody (1:100); SET rabbit polyclonal antibody (1:1000); PKM2 rabbit polyclonal antibody (1:250); APP rabbit monoclonal antibody

(1:750); AXL rabbit polyclonal antibody (1:100); and 14-3-3 zeta mouse monoclonal antibody obtained from Imgenex (1:200). Following incubation, blots were washed (3 times, 5 min) with TBST and incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:2000 in TBS containing 5% skimmed milk powder. Blots were washed again with TBST and developed using Western Lightning *Plus*-ECL Enhanced Chemiluminescence Substrate (PerkinElmer Inc, Waltham, MA, USA). Ponceau S staining was used to confirm equal protein loading in different lanes.

Immunohistochemical analysis in human thyroid tissues

Immunohistochemistry was performed on archived FFPE human benign and TC tissue sections to determine the expression patterns of ALCAM, APP, APLP2, AXL, SET, PGK1, 14-3-3 zeta, PKM2 and hnRNP K. Serial TC and benign tissue sections (4µm thickness) were deparaffinised in xylene and hydrated with graded alcohol series as described earlier by us (143). All incubations were conducted at room temperature. The VECTASTAIN rapid protocol was used. The slides were incubated with 10% blocking serum (normal horse serum for anti-mouse secondary antibodies or normal goat serum for anti-rabbit secondary antibodies) for 20 min. After blocking the non-specific binding sites, sections were incubated with the following anti-human antibodies from Abcam (Cambridge, MA): ALCAM mouse monoclonal antibody (1:200), APP rabbit monoclonal antibody (1:200), APLP2 rabbit polyclonal antibody (1:100), hnRNP K mouse monoclonal (1:500), PKM2 rabbit polyclonal (1:50), SET rabbit polyclonal (1:200), AXL rabbit polyclonal (1:100), PGK1 mouse monoclonal (1:100) from Santa Cruz Biotechnology (Santa Cruz, CA), and 14-3-3 zeta mouse

monoclonal antibody (1:100) from Imgenex (San Diego, CA) for 1 h. Tissues were then treated with 3% H₂O₂ in TBS for 10 min to block the endogenous peroxidase activity, and were subsequently incubated with a biotinylated secondary antibody (horse anti-mouse or goat anti-rabbit) for 10 min. The sections were finally incubated with VECTASTAIN Elite ABC Reagent (Vector labs, Burlingame, CA) for 10 min and diaminobenzidine was used as the chromogen. Negative control tissues were incubated with biotinylated secondary antibody (horse anti-mouse or goat anti-rabbit) with the omission of primary antibody, following the same protocol. Slides were counterstained with haematoxylin.

Evaluation of immunohistochemistry

The immunostaining scores were based on percentage positivity and staining intensity. Sections were scored as positive if epithelial cells showed immunoreactivity in the membrane, cytoplasm and/or nucleus when observed by two evaluators (SC, IK). Percentage positive scores were assigned according to the following scale: 0, <10% cells; 1, 10-30% cells; 2, 31-50% cells; 3, 51-70% cells; and 4, >70%. Staining intensity was then also scored semi-quantitatively as follows: 0, none; 1, mild; 2, moderate; and 3, intense. A total score was then obtained (ranging from 0 to 7) by adding the percentage positivity scores and intensity scores for each section. Average scores of three representative areas for the histotype on diagnosis of the tissues were calculated to obtain a final score for the immunostained slide. The average scores of the two evaluators were used for subsequent analyses; inter-examiner variability was minimal. Scatter plots were used to determine the distribution of total score of membrane, cytoplasmic, and/or nuclear expression for proteins analyzed. Also, to assess the total expression of ALCAM in the tissues, the membrane and cytoplasm scores were added to obtain

a total score out of 14. Box plots were used to determine the distribution of membranous, cytoplasmic, and total expression IHC scores for ALCAM.

Enzyme Linked Immunosorbent Assay

For ALCAM, sera from 44 patients with benign thyroid nodules, 29 with non-aggressive TC, and 17 with aggressive TC were analyzed using ELISA. For AXL, sera from 48 patients with benign thyroid nodules, 32 with non-aggressive TC, and 18 with aggressive TC were analyzed. Serum levels of ALCAM and AXL proteins were analyzed in duplicates using commercially available human AXL ELISA kit and CD166 (ALCAM) ELISA kit (R & D Systems, Minneapolis, MN). For both ALCAM and AXL, primary antibodies were diluted to a concentration of 2.0 µg/mL in PBS and 100 µL of the diluted antibody was used to coat 96-well microtiter plates overnight at room temperature. The plate was then washed three times with 0.05% Tween 20 in PBS after each step and aspirated prior to blocking with 1% BSA in PBS (reagent diluent) for 1 h. Then 100 µL of patient sera samples (diluted 1:500 with reagent diluent), were added to each well and incubated for 2 h. Thereafter, 100 µL of secondary antibody (diluted to a concentration of 50 ng/mL with reagent diluent) was added for 2 h. After washing, 100 µL of streptavidin-HRP was added to each well of the plate for 20 min. The color was developed with 100 µL of tetramethylbenzidine for 20 min and the reaction was stopped with 50 µL of 2 N H₂SO₄. The absorbance was measured with a microtiter plate reader at 450 nm.

Statistical analyses

The immunohistochemical data were subjected to statistical analyses using SPSS 20.0 software (SPSS Inc., Chicago, IL). Frequencies were reported for categorical variables and mean $\pm$ standard deviation was reported for continuous variables. Area-under-curve (AUC), sensitivity, and specificity were determined using receiver operating characteristic (ROC) analysis. The relationships between protein expression and clinico-pathological parameters were tested using Chi-Square and/or Fischer's exact test. Two-sided p values were calculated and $p < 0.05$ was considered to be significant. For ALCAM IHC data, cut-off values of ≥ 3 , ≥ 4.5 , and ≥ 7 were defined as positive criteria of immunoreactivity for membrane, cytoplasmic, and total ALCAM expression, respectively, based on sensitivity and specificity values, for statistical analysis. The relationships between ALCAM expression (positive/ negative) and categorical variables- clinico-pathological parameters were tested using Chi-square and/or Fischer's exact test. Positive predictive value (PPV), negative predictive value (NPV), odds ratio (OR), and 95% confidence interval (CI) were also calculated. Kaplan-Meier survival plots were used to determine correlation of ALCAM expression and disease-free survival in TC patients, to determine the suitability of ALCAM as a prognostic marker. Disease-free survival analysis was thyroid cancer specific, whereby an event (no survival) was defined as thyroid cancer recurrence, metastasis, or death during patient follow-up. Hazard ratios (HR) were also calculated. Cox-regression (multivariate) analysis was performed to determine the prognostic potential of ALCAM expression in comparison with known clinical and pathological parameters. Student's t test was used to analyze serum ALCAM and AXL data, separately.

Chi-Square and Fischer's exact test was used to analyze the two proteins in combination, and a cut-off sera value of 42 ng/mL was used as the positive criteria for each ALCAM and AXL in the analysis. All scatterplots were produced using GraphPad Prism 5.0 Software (La Jolla, CA, USA).

Chapter 4 Results

4 Results

4.1 Protein Identification and Bioinformatics

To identify potential serum biomarkers for aggressive TC, the secretomes of two aggressive TC cell lines (anaplastic-derived; SW1736, C643) and a non-aggressive (papillary-derived; BCPAP) cell line were systematically analyzed. All 83 high confidence ($\geq 95\%$) secretome proteins identified by proteomics were analyzed by IPA to investigate their potential interactions in cellular networks. A refined network of important interactions of our secretome proteins is shown in Figure 2. The network generated suggests that several of the identified secretome proteins (shown in red and highlighted in yellow, Figure 2) interact with cytoplasmic proteins via membrane-bound receptors such as EGFR, L1 cell adhesion molecule (L1CAM), and nerve growth factor receptor (NGFR). These membrane receptors play key roles in regulating cancer progression. Furthermore, AXL was found to interact with GRB2 and SRC, both intermediates of EGFR signaling pathways. As mentioned earlier, EGFR is associated with tumour aggressiveness and is often chosen as a target for different therapeutic agents used in clinical trials of cancer patients. 14-3-3 zeta (YWHAZ) is a major cytoplasmic hub protein having direct interactions (shown with bold lines in Figure 2) with a large number of identified secretome proteins and EGFR-signalling pathways. ALCAM was found to have direct interactions with L1CAM and NGFR, and indirect interactions with EGFR and 14-3-3 zeta signalling. From this network, 14-3-3 zeta, APLP2, APP, ALCAM, AXL, hnRNP K, PGK1, PKM2, and SET were selected for further analyses in sera and tissues of TC patients.

This panel of nine proteins was chosen based on their reported roles in tumour progression in the literature (144, 146-153), and importantly, these proteins were all identified in the CM of anaplastic-derived cell lines. Furthermore, the potential direct or indirect interactions these secretome proteins have in cancer relevant pathways, namely EGFR-activated pathways, make them of great interest to further investigate. Some of these proteins have been previously studied in several different types of cancer, though some of them have not been investigated in thyroid carcinomas (144, 146-153). The peptide sequences identified by mass spectrometric analysis for these nine proteins are listed in Appendix I and their mass spectra and information are given in Appendix II.

Figure 2. Ingenuity Pathway Analysis

Figure 2. Ingenuity Pathway Analysis reveals several important protein-protein interactions of identified secretome proteins. The network generated suggests several extracellular secretome proteins (labelled in red and highlighted in yellow) interact with cytoplasmic proteins via membrane-bound receptors such as EGFR, L1CAM, and NGFR. Furthermore, AXL interacts with GRB2 and SRC, both intermediates of EGFR signalling pathways. 14-3-3 zeta (YWHAZ) was also observed to be a potential central cytoplasmic hub mediating interactions between many of the secretome proteins and EGFR-signalling pathways. ALCAM was found to have interactions with L1CAM and NGFR, and indirect associations to the EGFR-activated pathways. From this network, 14-3-3 zeta, APLP2, APP, ALCAM, AXL, hnRNP K, PGK1, PKM2, and SET were selected for further analysis in sera and/or tissues of thyroid cancer patients. Bold lines represent direct protein-protein interactions and dashed lines represent indirect interactions.

4.2 Detection of Secretome Proteins in Human Sera by Western Blots

To confirm that these proteins are secreted and can be detected in TC patients' sera, the presence of these 9 proteins was examined in sera from patients with benign nodules (n = 6), non-aggressive TC (n = 6), and aggressive TC (n = 6). As shown in Figure 3, AXL (A1,A2), APP (B1, B2), APLP2 (C1, C2), ALCAM (D), 14-3-3 zeta (E), PKM2 (F), and SET (G) were all detected in sera of patients with benign thyroid nodules, non-aggressive and aggressive TCs. hnRNP K and PGK1 could not be detected in sera of either benign or TC patients analyzed in this study. Differences were observed between the sera of patients with benign nodules and TCs for ALCAM, APP, AXL, 14-3-3 zeta, and PKM2. Two immunoblot panels are shown for AXL, APP, and APLP2 in Figure 3 (A-C) to depict the variations observed in sera from patients with benign nodules, non-aggressive TC and aggressive TC. The variations observed within these groups for AXL were also observed using ELISA (section 4.4). Ponceau S staining was performed to confirm equal protein loading (Figure 3H, full blot Appendix III). Although the immunodepletion of abundant proteins in sera may cause off-target size bands, the observed size of protein bands were as would be expected according to what is reported in the literature and the antibody datasheets.

Figure 3. Identified secretome proteins are detected in benign and thyroid cancer patients' sera using western blot analysis. Protein samples (50 μ g) were prepared from the sera of cancer-free individuals and TC patients. AXL (**A1**, **A2**), APP (**B1**, **B2**), APLP2 (**C1**, **C2**), ALCAM (**D**), 14-3-3 zeta (**E**), PKM2 (**F**), and SET (**G**) were all detected in sera of patients with benign thyroid nodules, non-aggressive and aggressive TCs using western blot analysis, and show potential differences between TC subgroups. Aggressive cancer group consisted of individuals with PTC with stage IV TC and/or having distant metastasis, and non-aggressive cancer as stage I PTC with no metastasis. Single blots are shown, except for AXL, APP, and APLP2, which are represented by two blots to show variations in sera levels within different patient groups. Ponceau S stained blot is shown to demonstrate equal loading (**H**).

4.3 Immunohistochemical Analysis of Identified Proteins in Human Thyroid Tissues

Immunohistochemical analysis of proteins APLP2, APP, AXL, PGK1, hnRNP K, SET, PKM2, and 14-3-3 zeta was carried out to investigate alterations in their expression in benign and malignant thyroid tissues (Fig.4). Scatter plots showing the distribution of immunohistochemical scores for these proteins are shown in Figure 5. The clinico-pathological information of patients is listed in Appendix IV. All eight proteins were detected in benign and malignant thyroid tissues and showed differential expression (Fig. 4 and Table V). An increase in cytoplasmic expression of APLP2 was observed in cancer as compared to benign tissues ($p = 0.044$; Fig. 4IA). Nuclear APP expression was significantly downregulated in malignant as compared to benign tissues ($p < 0.001$, AUC = 0.937; Fig. 4IB). ROC curves for all protein expressions are shown in Appendix V, and AUC, sensitivity, specificity, PPV, and NPV values for statistical tests of protein IHC scores are listed in Appendix VI. No significant difference was observed in the cytoplasmic APP expression in benign and malignant thyroid tissues. Nuclear AXL expression could distinguish malignant tumours from benign nodules with the greatest accuracy in comparison to the rest of the proteins. AXL nuclear expression was decreased in TCs compared to benign nodules ($p < 0.001$, AUC = 0.995, sensitivity = 100%, specificity = 90%, PPV = 90.9%, NPV = 100%). Cytoplasmic AXL expression was also decreased in malignant tissues ($p = 0.001$, AUC = 0.788; Fig. 4IC). A decrease in nuclear expression of SET was observed in TCs as compared to the benign tissues ($p < 0.001$, AUC = 0.963; Fig. 4ID). Both nuclear and cytoplasmic staining was observed for PKM2 with a significant decrease in nuclear expression in TCs ($p < 0.001$, AUC = 0.977; Fig. 4IIE).

Figure 4. Immunostaining of secretome proteins

II

Figure 4. Continued

Figure 4. Immunostaining of proteins in human benign and thyroid cancer tissues shows differences in subcellular localization. Tissues were classified as benign, non-aggressive (Stage 1 PTC) or aggressive (stage 4 PTC, insular, PDPTC, or ATC). Differential nuclear (N) or cytoplasmic (C) expression of all proteins was observed in benign and malignant tissues using immunohistochemistry. **(IA)** Cytoplasmic and nuclear expression of APLP2 is shown in benign and malignant tissues; **(IB)** A decrease in nuclear APP expression in both aggressive and non-aggressive TC tissues is shown compared to benign tissue; **(IC)** A decrease in nuclear and cytoplasmic AXL expression is observed for cancer compared to benign tissues; **(ID)** Decreased SET nuclear expression observed in cancer tissues compared to benign tissues; **(IE)** Cytoplasmic staining for PKM2 is shown while nuclear expression is only observed in benign tissues; **(IF)** Nuclear and cytoplasmic staining for PGK1 was observed in benign tissues, and decrease in nuclear and cytoplasmic staining was observed in cancer tissues; **(IG)** Decreased nuclear and cytoplasmic staining of hnRNP K is shown for cancers compared to benign tissue; **(IHH)** An increase in cytoplasmic staining of 14-3-3 zeta is shown for non-aggressive cancer compared to benign, while a decrease is observed in aggressive tissue compared to non-aggressive. Magnification is 400X.

Figure 5. Scatterplot analysis for immunohistochemical thyroid tissue scores

Figure 5. Scatterplot analysis for immunohistochemical (IHC) scores of proteins in thyroid tissues. Each symbol corresponds to an individual patient. Thick horizontal bars denote mean IHC score values for each group $\pm$ standard error. Significantly reduced nuclear expression of APP, AXL, PKM2, PGK1, hnRNP K and SET was observed in malignant compared to benign tissues. Cytoplasmic expression of AXL, PGK1 and hnRNP K was significantly decreased in malignant tissues compared to benign, while increased APLP2 expression was observed in malignant tissues. N=nuclear and C=cytoplasmic.

TABLE V

Immunohistochemical analysis of secretome proteins in benign versus malignant thyroid tissues

Proteins	Total Cases	Nuclear Positivity		<i>p</i>	Cytoplasmic Positivity		<i>p</i>
	N	N	%		N	%	
APLP2							
Benign	20	13	65	1.000	13	65	0.044
Cancer	20	13	65		19	95	
APP							
Benign	19	12	63	<0.001	11	58	0.745
Cancer	21	0	0		14	67	
AXL							
Benign	20	20	100	<0.001	16	80	0.001
Cancer	23	2	9		6	26	
hnRNP K							
Benign	20	20	100	<0.001	15	75	0.002
Cancer	39	22	56		12	31	
PGK1							
Benign	20	20	100	<0.001	20	100	<0.001
Cancer	21	8	38		9	43	
PKM2							
Benign	21	18	86	<0.001	7	33	0.350
Cancer	20	0	0		10	50	
SET							
Benign	20	20	100	<0.001	4	20	0.480
Cancer	19	5	26		6	32	
14-3-3 zeta							
Benign	20	7	35	0.067	5	25	0.058
Cancer	21	2	9.5		12	57	

Reduced PGK1 nuclear and cytoplasmic expression was observed in malignant tissues as compared to benign nodules ($p < 0.001$, AUC = 0.926 and $p < 0.001$, AUC = 0.950, respectively; Fig. 4IIF). A decrease in nuclear ($p < 0.001$, AUC = 0.962) and cytoplasmic ($p = 0.002$, AUC = 0.816) expression of hnRNP K was also observed in TCs as compared to the benign tissues (Fig. 4IIG). There was an increase in 14-3-3 zeta cytoplasmic expression in malignant compared to benign tissues, though not significant (Fig. 4IIH).

Immunohistochemical analysis of ALCAM in human thyroid tissues

Immunohistochemical analysis of ALCAM was carried out to determine differences in its subcellular localization and levels of expression, including total expression, in benign thyroid tissues and different subtypes of TC in a large patient cohort. ALCAM expression in relation to patients' clinico-pathological characteristics is listed in Table VI. Representative tissues sections showing the immunostaining pattern of ALCAM in benign thyroid nodules and several variants of TCs are shown in Figure 6. Intense membrane and cytoplasmic staining was observed in Stage I classic PTC compared to benign thyroid tissues (Fig. 6a, 6b), whereas decreased membrane expression was observed in the stage IV PTC (Fig. 6c). No membrane staining and very mild cytoplasmic staining was seen in ATCs (Fig. 6d). The PDPTC and insular carcinomas (poorly-differentiated TCs) showed reduced membrane and cytoplasmic staining (Fig. 6e, 6f). Differences in patterns of staining were observed among the PTC variants. The tall cell variant PTCs displayed strong membrane and cytoplasmic staining (Fig. 6g); follicular variant showed decreased positivity and mild membrane staining with mild to moderate cytoplasmic staining (Fig. 6h); while the oncocytic variant showed moderate cytoplasmic staining and mild membrane staining (Fig. 6i).

TABLE VI

Analysis of ALCAM expression with clinical and pathological characteristics

Clinico-pathological Feature	Total Cases	Membrane Positivity N (%)	<i>p</i> value	OR (95% CI)	Cytoplasmic Positivity N (%)	<i>p</i> value	OR (95% CI)	Total Positivity N (%)	<i>p</i> value	OR (95% CI)
Age										
<45	91	70 (76.9)	0.010	2.31 (1.24-4.28)	77 (84.6)	0.576	1.30 (0.62-2.73)	80 (87.9)	0.020	2.48 (1.16-5.32)
≥45	110	65 (48.1)			89 (53.6)			82 (50.6)		
Gender										
Male	47	26 (55.3)	0.051	1.99 (1.02-3.90)	35 (74.5)	0.121	1.98 (0.90-4.37)	33 (70.2)	0.055	2.22 (1.04-4.74)
Female	156	111 (71.2)			133 (85.3)			131 (84)		
Pathology										
Benign	45	41 (91.1)	<0.001	6.62 (2.26-19.40)	44 (97.8)	0.001	12.07 (1.60-90.77)	44 (97.8)	<0.001	13.93 (1.86-104.55)
Cancer	158	96 (60.8)			124 (78.5)			120 (75.9)		
Histological Grade										
Well-Differentiated	135	90 (66.7)	<0.001	5.67 (2.09-15.36)	116 (85.9)	<0.001	11.45 (4.27-30.68)	114 (84.4)	<0.001	15.38 (5.43-43.54)
Poorly/Un-Differentiated	23	6 (26)			8 (34.8)			6 (26)		
Histotype										
Classic PTC	77	54 (70.1)	0.009	_____	67 (87)	<0.001	_____	66 (85.7)	<0.001	_____
Follicular PTC	44	28 (63.6)			37 (84)			37 (84)		
Tall cell PTC	5	4 (80)			4 (80)			4 (80)		
Oncocytic PTC	9	4 (44.4)			8 (88.9)			7 (77.8)		
PDPTC	2	0 (0)			2 (100)			2 (100)		
Insular TC	8	2 (25)			3 (37.5)			2 (25)		
ATC	13	4 (30.8)			3 (23.1)			2 (15.4)		
AJCC Stage										
I	93	64 (68.8)	0.290	_____	77 (82.8)	0.380	_____	78 (83.9)	0.472	_____
II	11	5 (45.5)			9 (81.8)			8 (72.7)		
III	10	5 (50)			10 (100)			8 (80)		
IV	24	14 (58.3)			18 (75)			17 (70.8)		
LN Metastasis	50	34 (68)	0.581	0.76 (0.37-1.58)	41 (82)	0.813	1.18 (0.47-2.95)	40 (80)	0.822	1.14 (0.47-2.75)
Distant Metastasis	7	2 (28.6)	0.096	5.00 (0.93-26.86)	3 (42.9)	0.017	7.51 (1.56-36.25)	4 (57.1)	0.148	3.19 (0.67-15.19)
Extrathyroidal Extension	38	22 (57.9)	0.327	1.48 (0.69-3.18)	25 (65.8)	0.002	4.21 (1.68-10.53)	26 (68.4)	0.052	2.62 (1.09-6.29)
Multifocality	85	50 (58.8)	0.153	1.75 (0.85-3.61)	68 (80)	0.659	1.31 (0.54-3.18)	67 (78.8)	1.000	1.10 (0.48-2.55)
Perineural Invasion	6	2 (33.3)	0.187	3.83 (0.68-21.68)	5 (83.3)	1.000	0.92 (0.10-8.21)	3 (50)	0.094	4.36 (0.83-22.89)

Figure 6. Immunostaining of ALCAM in different thyroid cancer subtypes

Figure 6. Immunostaining of ALCAM shows different expression between benign, non-aggressive, and aggressive human thyroid cancer tissues. Paraffin-embedded sections of benign thyroid nodules and different subtypes of thyroid carcinomas were immunostained using anti-CD166 monoclonal antibody as described in the Methods section. The photomicrographs of representative immunostained tissues depict: **(a)** benign nodule showing ALCAM membrane and cytoplasmic expression; **(b)** classic PTC showing strong membrane and cytoplasmic expression; **(c)** stage IV PTC showing mild membrane and cytoplasmic staining; **(d)** ATC showing no membrane and scant cytoplasmic expression; **(e)** PDPTC and **(f)** insular carcinoma showing some mild membrane and cytoplasmic expression; **(g)** tall cell variant PTC showing strong membrane and moderate cytoplasmic expression; **(h)** follicular variant PTC depicting some membrane expression and moderate cytoplasmic expression; **(i)** oncocytic cell variant PTC showing predominantly cytoplasmic expression; and **(j)** negative control showing no immunostaining. Arrows show membranous (M) and cytoplasmic (C) ALCAM expression. Magnification is 400x.

Comparison of the staining patterns of histological variants of TC revealed significant differences in levels of ALCAM staining intensity and percentage positivity among the different groups for both total and subcellular ALCAM expression; membrane $p = 0.009$, cytoplasm $p < 0.001$, and total $p < 0.001$ (Table VI). Membrane, cytoplasmic, and total ALCAM expression were significantly decreased in TCs as compared to benign tissues ($p < 0.001$). Comparing ALCAM staining across the four stages of TC did not reveal significance, though an overall decrease in expression was observed in advanced stage TC. Membrane, cytoplasmic, and total ALCAM expression was also decreased in TC cases with distant metastasis. Cytoplasmic ALCAM was positive in 3 of 7 distant metastasis cases, while only 2 of 7 cases were positive for membrane ALCAM expression. Decreased cytoplasmic ALCAM expression was significantly associated with extrathyroidal extension ($p = 0.002$, Table VI).

To evaluate the ability of ALCAM to serve as a marker for aggressive TC, the malignant cases were subdivided into two groups according to histological grade; well-differentiated (non-aggressive) and poorly/undifferentiated (aggressive) TC (Figure 7A). Significantly decreased membrane ($p < 0.001$), cytoplasmic ($p < 0.001$), and total ALCAM ($p < 0.001$) expression was observed in the aggressive group compared to non-aggressive. Using ROC analysis, AUC for total ALCAM expression was 0.865 (Figure 7B) and the sensitivity and specificity were 82% and 74% respectively (OR = 15.38, 95% CI = 5.43 – 43.54; PPV= 95%, NPV = 45%, Tables VI and VII). The AUC values were 0.790 and 0.802 for membrane and cytoplasmic ALCAM expression, respectively. Membrane expression sensitivity and specificity were 62.2% and 78.3% respectively; PPV was 93.8% and NPV was 27.4%, and cytoplasmic expression sensitivity and specificity were 80% and 69.6% respectively (PPV = 93.5%, and NPV = 44.1%; Tables VI and VII).

Figure 7. ALCAM expression is decreased in poorly/undifferentiated thyroid cancer tissues. Categories include benign nodules, well-differentiated PTC (WD-PTC), and poorly-differentiated PTC (PDPTC) or undifferentiated TC. **(A)** Box plots for immunohistochemical scores are shown, thick horizontal bars denote mean values for each group $\pm$ standard deviation. Membrane, cytoplasmic, and total ALCAM expression significantly varied among the groups ($p < 0.001$) and showed decreased staining in poorly/undifferentiated tumours. **(B)** Receiver operating characteristics curve of ALCAM immunohistochemical scores for well-differentiated versus poorly/undifferentiated TC. Membrane, cytoplasmic, and total ALCAM area-under-curve (AUC) values were 0.790, 0.802, and 0.865 respectively.

TABLE VII

Receiver operating curve analysis of ALCAM expression in association with clinico-pathological parameters*

ALCAM	Sensitivity	Specificity	PPV	NPV	AUC
Membrane Staining					
Age <45 vs. ≥45	59.1	23.1	48.1	31.8	0.567
Benign vs. Cancer	91.1	39.2	93.9	29.9	0.814
Well vs. poorly/undifferentiated	62.2	78.3	93.8	27.4	0.790
Distant metastasis vs. none	66.7	71.4	97.7	10.6	0.763
Extrathyroidal extension vs. none	67.0	42.1	75.3	32.7	0.604
Cytoplasmic Staining					
Age <45 vs. ≥45	80.9	15.4	53.6	40.0	0.551
Benign vs. Cancer	97.8	21.5	26.2	97.1	0.808
Well vs. poorly/undifferentiated	80.0	69.6	93.5	44.1	0.802
Distant metastasis vs. none	84.9	57.1	97.3	17.4	0.731
Extrathyroidal extension vs. none	89.0	34.2	78.1	54.2	0.629
Total Staining					
Age <45 vs. ≥45	75	12	51	28	0.539
Benign vs. Cancer	97.8	24.1	26.8	97.4	0.868
Well vs. poorly/undifferentiated	82.0	74.0	95.0	45.0	0.865
Distant metastasis vs. none	81.0	42.9	96.2	11.1	0.803
Extrathyroidal extension vs. none	85.0	31.6	76.6	34.4	0.626

* Chi-square analysis was used to generate sensitivity, specificity, PPV, and NPV

Kaplan-Meier survival analysis of follow-up data of 134 TC patients showed significantly reduced disease-free survival for TC patients with decreased ALCAM membrane expression ($p = 0.016$, median survival 8.3 years, range 0.2 to 11.8 years), as compared to median survival of 13.7 years for those with higher ALCAM membrane expression (Figure 8A). Similarly, TC patients with decreased ALCAM cytoplasmic expression had reduced disease-free survival ($p < 0.001$, median survival 6 years) as compared to a median survival of 13.7 years for those with higher ALCAM cytoplasmic expression (Figure 8B). Total ALCAM expression also emerged as a prognostic marker; overall decreased expression correlated with poor prognosis ($p = 0.002$), with a median survival of 7.3 years for patients with decreased expression as compared to 13.5 years for patients with higher ALCAM expression (Figure 8C).

Cox-regression (multivariate) analysis was performed to determine the prognostic potential of ALCAM expression in comparison to clinical and pathological parameters that were independently significant in Kaplan-Meier analysis. The parameters tested were ALCAM staining (membrane, cytoplasmic, total), age, histological grade, extrathyroidal extension, as well as combined ALCAM staining with each of the clinico-pathological parameters. The most significant parameters for prognosis were combined cytoplasmic ALCAM expression and histological grade ($p < 0.001$, HR = 21.71, 95% CI = 6.71 – 106.25), and extrathyroidal extension ($p = 0.002$, HR = 9.78, 95% CI = 1.80 – 12.91).

Figure 8. Kaplan-Meier analysis of ALCAM tissue expression

Figure 8. Decreased ALCAM expression is associated with reduced patient disease-free survival. Kaplan-Meier estimation of cumulative proportion of disease-free survival was assessed. **(A)** Significantly reduced disease-free survival for thyroid cancer patients with decreased ALCAM membrane expression ($p = 0.016$, median survival 8.3 years), as compared to a median survival of 13.7 years for those with higher ALCAM membrane expression. **(B)** TC patients with decreased cytoplasmic ALCAM expression had reduced disease-free survival ($p < 0.001$, median survival 6 years) as compared to a median survival of 13.7 years for those with higher cytoplasmic ALCAM expression. **(C)** TC patients with decreased total ALCAM expression had reduced disease-free survival ($p = 0.002$, median survival 7.3 years) as compared to a median survival of 13.5 years for those with higher total ALCAM expression. Absence of ALCAM expression is defined as IHC scores below the respective cut-offs, whereas presence of ALCAM is defined as IHC scores above the respective cut-offs (see Appendix VI).

4.4 Comparison of Serum ALCAM and AXL levels Between Benign, Non-Aggressive, and Aggressive TC Patients

To build upon the efforts established with western blot and IHC analysis to evaluate the diagnostic potential of the proteins, ELISA was used to quantify the levels of the proteins in TC patients' sera. To evaluate their potential as blood-based biomarkers, serum ALCAM and AXL levels were measured in benign, non-aggressive and aggressive TC patients' pre-surgical sera and correlated with clinico-pathological parameters (Table VIII and Fig. 9). Increased ALCAM levels were associated with advanced AJCC tumour stage ($p = 0.007$) and presence of lymph node metastasis ($p = 0.018$). An increase in ALCAM levels was observed in patients with extrathyroidal extension; however, it did not reach statistical significance ($p = 0.078$). Serum AXL levels were increased in patients with extrathyroidal extension ($p = 0.027$). The circulating levels of ALCAM and AXL varied among the PTC variants, with tall cell, diffuse sclerosing, PDPTC, and Hürthle cell (follicular) carcinoma patients showing higher ALCAM and/or AXL sera levels compared to those with non-aggressive variants of PTC (Table VIII). Circulating ALCAM levels were significantly higher in aggressive TC patients (mean $\pm$ standard deviation (SD), $67.2 \text{ ng/mL} \pm 36.4$) as compared to non-aggressive ($50.0 \text{ ng/mL} \pm 13.7$, $p = 0.04$, Fig. 9A). Serum ALCAM levels distinguished aggressive from non-aggressive TCs with a sensitivity of 65%, specificity 38%, and area-under-curve (AUC) of 0.606 based on ROC curve analysis with a cut-off value of 42 ng/mL. Serum AXL levels were not significantly different between the non-aggressive ($49.2 \pm 20.2 \text{ ng/mL}$) and aggressive TC patient groups ($55.0 \pm 24.8 \text{ ng/mL}$, Fig. 9B). Furthermore, combined ALCAM and AXL sera positivity (level above 42 ng/mL) could not more accurately detect aggressive TCs than the independent use of ALCAM (data shown in Appendix VII).

TABLE VIII

Correlation of ALCAM and AXL sera levels with clinico-pathological characteristics

Clinico-pathological Parameter	Total Cases	s-AXL Mean $\pm$ SD	<i>p</i>	95% CI	Total Cases	s-ALCAM Mean $\pm$ SD	<i>p</i>	95% CI
Age								
<45	34	55.3 $\pm$ 23.6	0.171	-2.893 – 16.027	27	48.2 $\pm$ 24.3	0.470	-14.427 – 6.241
$\geq$ 45	64	48.8 $\pm$ 21.9			63	51.8 $\pm$ 20.2		
Gender								
Male	18	52.2 $\pm$ 23.7	0.820	-13.091 – 10.389	17	57.5 $\pm$ 31.5	0.308	-24.929 – 8.813
Female	80	50.8 $\pm$ 22.4			73	49.2 $\pm$ 18.3		
Pathology								
Benign	48	50.8 $\pm$ 23.5	0.920	-9.560 – 8.633	44	46.9 $\pm$ 14.3	0.091	-16.411 – 1.233
Cancer	50	51.3 $\pm$ 21.9			46	54.4 $\pm$ 26.2		
TC Aggressiveness								
Non-aggressive	32	49.2 $\pm$ 20.2	0.379	-7.255 – 18.742	29	50.0 $\pm$ 13.7	0.040	0.985 - 39.503
Aggressive	18	55.0 $\pm$ 24.8			17	67.2 $\pm$ 36.4		
Histotype								
Classic PTC	22	44.3 $\pm$ 16.9	0.158	-	21	50.7 $\pm$ 23.3	0.080	-
Follicular PTC	19	54.7 $\pm$ 22.5			17	51.9 $\pm$ 21.8		
Tall cell PTC	2	82.8 $\pm$ 54.1			2	79.0 $\pm$ 58.0		
Oncocytic PTC	3	46.6 $\pm$ 16			3	43.8 $\pm$ 10.3		
Diffuse sclerosing PTC	3	58.3 $\pm$ 23.7			2	78.3 $\pm$ 48.4		
Poorly-differentiated PTC	1	68.5			-	-	-	-
Hurthle Cell Carcinoma	-	-	-	-	1	113		
AJCC Stage								
I	38	47.7 $\pm$ 18.3	0.168	-	35	49.2 $\pm$ 20.9	0.007	-
II	5	56.3 $\pm$ 24.3			5	55.8 $\pm$ 21.8		
III	3	66.8 $\pm$ 30.4			2	62.0 $\pm$ 34.6		
IV	4	67.7 $\pm$ 38.4			4	95.0 $\pm$ 42.1		
LN metastasis								
Absent	36	47.7 $\pm$ 19.6	0.064	-0.790 – 26.239	33	46.2 $\pm$ 13	0.018	14.125 – 44.256
Present	14	60.4 $\pm$ 25.5			13	75.4 $\pm$ 38.2		
Distal Metastasis								
Absent	49	50.9 $\pm$ 22.0	-	-	45	53.1 $\pm$ 24.9	-	-
Present	1	68.5			1	113		
Extrathyroidal Extension								
Absent	42	48.3 $\pm$ 18.8	0.027	2.234 – 34.822	39	49.1 $\pm$ 17.6	0.078	-5.378 – 76.112
Present	8	66.8 $\pm$ 30.9			7	84.4 $\pm$ 44		
Angioinvasion								
Absent	44	51.4 $\pm$ 22.7	0.904	-20.518 – 18.190	41	52.6 $\pm$ 24	0.418	-33.560 – 66.897
Present	6	50.3 $\pm$ 16.6			5	69.3 $\pm$ 40.9		
Multifocality								
Absent	25	50.0 $\pm$ 20.3	0.684	-10.002 – 15.114	23	49.6 $\pm$ 15.6	0.348	-8.217 – 22.602
Present	25	52.6 $\pm$ 23.7			22	56.8 $\pm$ 31.9

Figure 9. **ALCAM and AXL expression in thyroid cancer patient sera using enzyme-linked immunosorbent assay.** Pre-surgery blood samples were obtained from patients with benign nodules, aggressive TC (stage IV TC, poorly-differentiated tumours, and/or having distant metastasis), and non-aggressive TC (stage I or II PTC, no metastasis). **(A)** ALCAM sera levels were significantly higher in the aggressive TC patients ($p=0.04$, mean = $67.2 \text{ ng/mL} \pm 36.4$ standard deviation) compared to non-aggressive ($50.0 \text{ ng/mL} \pm 13.7$). **(B)** AXL sera levels were not significantly different between the non-aggressive ($49.2 \text{ ng/mL} \pm 20.2$) and aggressive TC ($55.0 \text{ ng/mL} \pm 24.8$).

Chapter 5

Discussion, Conclusions, Future Directions

5.1 Discussion

The first aim of this study was the identification of candidate biomarkers for tumour aggressiveness using a secretome-based LC-MS/MS analysis of proteins secreted by aggressive and non-aggressive thyroid cancer cell lines in tandem with bioinformatics. In light of bioinformatics advances, the use of tools such as IPA software has directed identification of several important potential interactions that may otherwise not have been considered by researchers. However, experimental verification of protein-protein interactions is warranted to establish their functional significance in thyroid malignancy and aggressiveness of TC. Furthermore, proteins secreted by aggressive TC cell lines are important to consider because of the significance of tumour cell interactions with the extracellular environment that play vital roles in tumour growth, invasion, and metastasis (137, 138). Investigation of the thyroid cancer secretome is of particular importance because of the urgent need for blood-based clinical biomarkers to aid in disease management. Secreted proteins, in addition to the role they have within cells, also have the potential to be detectable in blood, which will allow for future development of minimally-invasive blood-based assays to aid in the management of TC (129, 130). This is of paramount importance in preventing unnecessary surgery and its inherent complications and morbidity. The development of these assays will also allow healthcare resources and efforts to be directed in a focused manner to high risk patients.

We initially identified 188 proteins using LC-MS/MS, of which 83 had high confidence peptide identifications and were chosen for further analyses. Quantitative proteomics was not used in this study and this was a limitation. Although the number of proteins identified herein is substantially smaller than that of other studies using more recent advanced methods, we did identify enough secreted proteins having key roles in cancer development for subsequent analyses.

The IPA networks that were generated herein suggested nine secretome proteins potentially interacted, either directly or indirectly with components of EGFR-activated pathways. EGFR is a cell membrane receptor that plays an important role in cancer development and progression (108), and it is suggested that overexpression of the EGFR gene is correlated with aggressive tumour behaviour and poor clinical outcome in several cancers including lung, colorectal, breast, bladder, and head and neck carcinomas (154, 155). It is noteworthy that activation of EGFR signalling has recently been reported to play an important role in TC tumour aggressiveness, favouring progression towards an angiogenic, poorly-differentiated, and TSH-independent phenotype (154). Importantly, epithelial-mesenchymal transition (EMT) has been demonstrated by other studies to be an important step in the progression of well-differentiated TC to aggressive TC such as PDPTC and ATC (156-158). EMT is involved in the disassembly of junctional structures of epithelial cells and in the remodelling of the extracellular matrix, thereby making the cells more migratory (159). These events are key to the metastatic potential of cancer cells. Furthermore, a growing body of evidence has found that EGFR pathway overexpression may be a critical part of this progression, and that EGFR signaling promotes EMT (154, 160-162). Also in support of this were findings by Grande *et al* that overexpression of EGF and transforming growth factor β (TGF- β) in pig thyrocytes led to their progression towards EMT phenotypes (163).

The IPA network analysis also revealed a potential direct interaction of AXL, an identified secretome protein, with SRC and GRB2. SRC and GRB2 are both components of the EGFR signalling pathway and have important roles in several cell processes including apoptosis, cell migration, and gene transcription (146). In TC cell lines, AXL has been shown to have a high level of tyrosine phosphorylation due to the constitutive expression of its ligand GAS6 (146). Furthermore, knockdown of AXL decreased TC cell viability and invasiveness, while also reducing growth of thyroid tumour xenografts in nude mice (146). AXL overexpression and its association with invasiveness and metastasis has been reported in several cancer types including breast, prostate, and lung cancer (164-166). In our study, IHC analysis on patient tissues revealed decreased nuclear and cytoplasmic AXL expression in malignant compared to benign tissues. AXL could be a potential therapeutic target because of its function as a tyrosine kinase receptor. AXL could be targeted by available drugs being used for the treatment of other cancers that are regulated by tyrosine kinases (167). As discussed previously under new therapies for thyroid cancer, several agents tested in clinical trials for TC patients target tyrosine kinase receptors that subsequently affect the RAF, EGFR, and/or VEGFR pathways. Therefore, the type and function of this protein deem it of interest to investigate on several different levels.

14-3-3 zeta emerged as a major cytoplasmic hub in IPA analysis, mediating many of the aforementioned potential interactions. This is experimentally supported by other recent studies which have shown that depletion of 14-3-3 zeta leads to reduced EGFR signaling in breast cancer (168). Another important interaction of 14-3-3 zeta includes that with filamin A, a protein that is involved in EMT of cells, and is also proposed to play a role in response to DNA damage via interactions with BRCA1 and BRCA2 (169-172). 14-3-3 zeta is an adaptor protein that functions as an intracellular signalling molecule that can affect cell migration and

metastatic properties of tumour cells (173). Some types of molecules that 14-3-3 zeta can bind to include transcription factors, biosynthetic enzymes, cytoskeletal proteins, signalling molecules, apoptosis factors, and tumour suppressors. There are seven isoforms in the family of 14-3-3 proteins.

Overexpression of 14-3-3 zeta has been reported in esophageal cancer tissues (174), and decreased serum levels have been reported in ovarian cancer (175). The sera and tissue expression of 14-3-3 zeta observed herein is in support of these findings. Our western blots showed decreasing 14-3-3 zeta levels with increasing aggressiveness, though this must be taken as only a preliminary trend that requires further verification using the ELISA. We also observed an overall increase cytoplasmic expression of 14-3-3 zeta in malignant compared to benign tissues, though this was not significant as there was a large variation in the expression. A recent thyroid cancer study demonstrated 14-3-3 zeta expression by proteomic profiling of a total of 9 FTC and 10 PTC well-differentiated tissues (176). They reported 14-3-3 zeta cytoplasmic expression in 6 FTCs and 8 PTCs. Tissue proteomic profiling could have alternatively been used for our study if the aim was to search for tissue-based biomarkers. Since our ultimate goal is to find a blood-based biomarker for use in minimally-invasive assays, we deemed secretome proteomics of thyroid cancer cell lines more suitable to identify secreted proteins. Using secretome proteomics does not limit one to the discovery of blood-based biomarkers. As we have observed in this study, differences in the subcellular localization of several of these proteins were confirmed in thyroid cancer patients' tissues, and these proteins can be taken further as tissue biomarkers, if not as blood biomarkers.

Other secretome proteins investigated in our selected panel of candidate markers have also been shown to have important biological functions relating to cancer. Although APP is known for its role in the development of Alzheimer's disease, recent studies have shown that

the proteolytic cleavage of APP is associated with growth factors and NF- κ B pathway activation, and alteration of calcium signalling and cytoskeletal dynamics (177). These interactions, among others, suggest a role for APP in cancer development. Overexpression of APP has been reported in pancreatic, prostate, colon, and oral squamous cell carcinoma tissues (150, 177-181). We report a loss in nuclear APP expression in thyroid carcinomas as compared to benign tissues with no notable changes in cytoplasmic expression. A study by Krause et al. demonstrated an increase in cytoplasmic expression of the endo-domain of APP and an increase in cell surface expression of the ecto-domain in thyroid cancer compared to benign tissues (182). APLP2 is a homologue of APP which is similarly cleaved, and interestingly, shown to have functional redundancy to APP (183). Herein, we report increased cytoplasmic APLP2 expression in malignant compared to benign tissues. Unlike APP, no apparent changes were observed in nuclear APLP2 expression.

Some of the secretome proteins investigated in this study are involved in cell metabolism. PGK1 is well known as a glycolytic enzyme that catalyzes the conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate for the generation of adenosine triphosphate (ATP). However, studies have shown PGK1 to have other functions important in disease progression. In the nucleus, PGK1 affects DNA replication and repair; therefore, it is assumed that its effect on glycolysis-related genes plays a critical role in tumorigenesis (184). In prostate cancer, overexpression of PGK1 reduces VEGF and increases angiostatin, a vascular inhibitor (184). Therefore, they demonstrate that overexpression of PGK1 may restrict tumour growth by limiting angiogenesis (184). Though interestingly, they report that at metastatic sites, the chemokine signalling responsible for metastatic growth reduces PGK1 expression, thereby releasing the angiogenic response (184). Another study reports that PGK1-overexpressing lung cancer cells reduce cyclooxygenase 2 expression and promote anti-tumour immunity in mice

(185). These findings suggest a coordinated control in PGK1 expression between its effects on energy consumption and metastatic potential as the tumour progresses. We report both decreased nuclear and cytoplasmic PGK1 expression in thyroid cancer tissues compared to benign tissues. Therefore, the regulatory functions suggested in these PGK1 studies could explain the events behind thyroid tumourigenesis and account for the decreased expression we have observed in cancer tissues. PGK1 is expressed in several other cancers such as breast, ovarian, pancreatic, and gastric cancer (186-189) .

PKM2 is another important protein involved in cell metabolism. PKM2 has dual roles that are critical for tumourigenesis: cancer cell metabolism regulation and gene transcription required for cell proliferation. Two important mechanisms underlying tumour development by regulation of metabolic and non-metabolic functions of PKM2 have been delineated: (i) PKM2 enhances aerobic glycolysis (190, 191) and (ii) upon EGFR activation, PKM2 promotes tumour cell proliferation by binding to and transactivating Y333-phosphorylated β -catenin (192). Thus, the coordinated control of metabolism and proliferation by PKM2 is essential for tumourigenesis (192). In our immunohistochemical analysis, we reported nuclear loss of PKM2 in malignant tissues. This could be accounted for by other studies which suggest that nuclear translocation of PKM2 induces cell death; therefore, PKM2 nuclear loss in thyroid cancer could suggest a decrease in apoptosis due to this change in expression that would allow for tumour growth.

SET protein is an oncoprotein known for its role in regulating DNA replication, gene transcription, cellular proliferation, apoptosis, and possibly cell cycle progression by inhibiting cyclin B-cdk1 and protein phosphatase 2A (193). As expected, we observed nuclear and cytoplasmic expression of protein SET. Nuclear expression of SET was decreased in malignant compared to benign tissues.

hnRNP K plays a role in cancer relevant pathways as well, and is involved in tumour development and progression (194-196). Decreased nuclear expression of hnRNP K in patients with Dukes C stage colorectal cancer has been correlated with poor survival (197), while Hope *et al.* showed significant aberrant cytoplasmic immunoreactivity in tumour cells related to colorectal cancer with lymph node metastasis (198). We similarly observed decreased nuclear and cytoplasmic expression of hnRNP K in TC tissues.

As a prelude towards evaluating the potential of these secretome proteins to serve as serum-based biomarkers, we sought to first determine the detectability of these proteins in TC patients' sera. Indeed, western blot analysis found the secretome proteins were detectable and suggested a potential for differential expression in some cases. PGK1 and hnRNP K were undetectable using this analysis. This may be due to lower circulating levels and/or the inability of this method and the chosen antibodies to detect the proteins. Importantly, due to the limited sample size and the semi-quantitative nature of western blots, findings obtained using this method must be confirmed with a more accurate quantitative method such as the ELISA, as will be later discussed.

Another aim of our study was to verify the expression of the secretome proteins in thyroid tissues and to observe the pattern of subcellular expression of the proteins using IHC. We also aimed to determine the potential of the secretome proteins for differential subcellular expression when comparing benign nodules and malignant tissues. To our knowledge, this is the first report to detect the expression of APLP2, 14-3-3 zeta, PKM2, PGK1, SET, and hnRNP K in benign and malignant thyroid tissues and/or sera. IHC analysis of the selected panel of secretome proteins showed differential expression between benign and malignant thyroid tissues. Interestingly, expression of all proteins was found to be significantly decreased in the nucleus and/or cytoplasm of TCs except APLP2 and 14-3-3 zeta, which both showed

increased cytoplasmic expression in TC compared to benign thyroid nodules. Although increased cytoplasmic expression of 14-3-3 zeta was not found to be statistically significant, this may possibly be due to the small sample size. Increased expression of 14-3-3 zeta is even more interesting when considering the proposed cellular network generated by IPA, which suggested that 14-3-3 zeta acts as a cytoplasmic hub, mediating interactions between many secretome proteins and important pathways. The sample size for the proteins, excluding ALCAM, was small, nevertheless, our findings provide the proof of principle of biomarker potential of these candidates.

When initially immunostaining a small tissue sample size for ALCAM, we observed a large variation in ALCAM expression among the malignant thyroid tissues. We chose to expand the study of this protein to determine if it is associated with different clinico-pathological parameters that are correlated with TC aggressiveness. IHC analysis of tissue specimens from a large cohort of patients, including those with benign nodules, several variants of PTC and aggressive features of TC, revealed ALCAM expression to be negatively correlated with increased tumour aggressiveness. Namely, decreased membrane, cytoplasmic, and total ALCAM expression was associated with poor differentiation, presence of metastasis, and extrathyroidal extension, all of which are characteristic features of aggressive TC. Most importantly, the data demonstrated decreased membrane, cytoplasmic, and total ALCAM expression to be associated with decreased disease-free survival, underscoring its potential applicability as a prognostic marker for TC.

In support of our findings, ALCAM down-regulation has been reported in breast cancer and correlated with poor prognosis, recurrence, and death (199), as well as in high-grade prostate cancer (200). Decreased or lost ALCAM membrane expression was also associated with poorer outcome in epithelial ovarian cancer, whereas cytoplasmic expression

of ALCAM was found in these patients (201). In contrast, further stratification based on immunohistochemical staining and subcellular localization demonstrated that elevated cytoplasmic ALCAM was significantly associated with shortened disease free survival in breast cancer (202, 203). Also, elevated ALCAM expression has been correlated with the vertical phase proliferation in melanomas (204) and poor prognosis (lymph node metastasis, tumour aggressiveness and T3/T4 stage cancers) in esophageal squamous cell carcinoma (205). High ALCAM expression in the primary tumour was reported to be associated with reduced survival or unfavourable prognosis in other tumour types including oral cancer (145, 206), pancreatic cancer (207), colorectal cancer (208, 209), and neuroblastoma (210). ALCAM has been shown to be a predictive marker in cervical cancer; and interestingly, high expression levels correlated with poor disease free survival and cancer specific survival in patients who did not receive any further therapy after primary tumour excision, whereas high ALCAM was associated with favourable prognosis in patients receiving adjuvant chemoradiation (211). Similar association of high ALCAM expression with favourable prognosis was reported in chemotherapy treated breast cancer patients, which might be explained by its influence on apoptosis (212, 213). Collectively these reports underscore the prognostic potential of ALCAM in human cancers, pointing to divergent roles played by ALCAM in progression of different tumour types. Thus, in depth understanding of biological roles of ALCAM is needed to explain the divergent variations in ALCAM expression reported in human cancers.

The molecular basis to explaining the diverse roles that ALCAM plays in TC progression and aggressiveness might lie in the critical triggering event that initiates the change of focus from homotypic adhesion to heterotypic tumour cell interaction with surrounding cell types. The clinical relationship of membrane ALCAM loss with aggressiveness of TC may relate to the process of proteolytic cleavage of ALCAM by a disintegrin and metalloproteinase

(ADAM)/ tumour necrosis factor α converting enzyme (TACE) and its shedding. The experimental evidence for this rationale has been provided in a recent study that addressed the role of ALCAM in thyroid tumour biology (144). ALCAM was detected in proteomic analysis of cell lysates from TC cell lines (TPC-1 and SKOV-3) and in their conditioned media, as well as in 4 PTCs and 2 medullary thyroid carcinomas by immunostaining (144). These authors demonstrated extracellular surface domain of ALCAM is cleaved by metalloproteinases ADAM17/TACE, which subsequently translocates to the cytoplasm where it facilitates aggressive transformation of tumour cells. The decreased membrane expression of ALCAM in stage IV TCs we observed, while maintaining cytoplasmic expression, could be explained by this tumourigenic event. In the initial stages of tumour formation the cells must be able to attach to each other, therefore adhesion molecules are required. This was observed in our study as increased expression and intense ALCAM membrane staining was present in the non-aggressive PTC. As the cancer progresses and the initiation of metastases begins as tumour cells originally attached to a particular organ start to spread to distant sites, cells must detach from the primary tumour, and therefore the expression of adhesion molecules are altered to facilitate this process. Herein, ALCAM membrane expression was absent, with low to moderate cytoplasmic expression in the most aggressive TC cases. The increased TACE mediated proteolysis of ALCAM in aggressive TC could account for the loss of membrane ALCAM in these tumours, and consequent relocalization from the cell membrane to the cytoplasm might ultimately enhance the migratory potential of malignant cells facilitating metastasis. Additionally, EGFR has been found to cooperate with the protease ADAM17/TACE to promote ALCAM shedding and soluble ALCAM release into the extracellular fluid (214). The involvement of TACE in increasing the motility of papillary thyroid cancer cells (TPC-1) has been reported (144). The loss of membrane ALCAM by

proteolysis from the membrane might likely be accompanied by increased levels of cytoplasmic ALCAM. However, in our study we also observed reduced cytoplasmic ALCAM levels in the most aggressive TC, such as ATC. This might be explained by recently reported promoter methylation that mediates silencing of ALCAM, thereby consequently reducing protein expression in these tumours, as was shown in breast cancer (202).

The final aim of our study was to quantify protein levels using the ELISA to assess secretome protein potential as diagnostic blood-based biomarkers. The ability of ALCAM to distinguish aggressive from non-aggressive tumours using IHC made this protein of particular interest to study its potential to serve as a serum-based biomarker. AXL was also of interest because of the variable expression seen in the sera blots and in tissues, as well as its appealing nature as a tyrosine kinase receptor that may be therapeutically targeted. Both of these proteins were also chosen for their key functional significance in cancer progression. The pre-surgical levels of ALCAM were found to correlate with important clinicopathologic characteristics including overall aggressiveness, AJCC tumour stage, lymph node and metastasis. AXL was found to be elevated in the sera of TC patients with extrathyroidal invasion. A limitation of this analysis was a small sample size of patients with aggressive features and/or histotypes of TC for sera analysis. Blood collection from TC patients was performed in a relatively small time frame, therefore presenting difficulty in acquiring large sample numbers from patients with the less prevalent, or aggressive, forms of TC. Also, we did not have sera samples from normal (tumour-free) individuals as a comparison for ALCAM levels to patients with benign nodules. Despite these limitations, these early results provide a look at the potential of these biomarkers. In particular, serum ALCAM has the potential to serve as a diagnostic, or even prognostic, marker given the observed differences between the pre-surgical sera levels with respect to important clinico-pathological parameters associated with TC aggressiveness.

5.2 Conclusions

Although thyroid carcinomas are the most prevalent endocrine malignancy, there still lies many questions about the progression of the disease from a non-aggressive subtype with overall excellent prognosis to an aggressive subtype with significantly reduced survival rates. Furthermore, there are no clinical biomarkers that can objectively identify an aggressive variant of TC before using surgical assessment. The aim of our study was to investigate potential protein biomarkers for the detection of aggressive thyroid carcinomas. These markers then may be presented as tissue or blood-based biomarkers for thyroid cancer patient diagnosis and management.

We hypothesized that analysis of proteins secreted from non-aggressive and aggressive thyroid cancer cell lines may lead to the identification of candidate biomarkers based on important protein-protein interactions. Of 83 high confidence secretome proteins identified using liquid chromatography tandem mass spectrometry, nine were selected for further analyses, which were 14-3-3 zeta, APP, APLP2, AXL, ALCAM, hnRNP K, PGK1, PKM2, and SET. These secretome proteins were chosen based on their function in cell cycle regulation, metabolism, and tumour progression. Also, several of these proteins are novel to thyroid cancer and have not been previously studied in TC patients' tissues and/or sera. Our protein selection was also based on Ingenuity Pathway Analysis, which revealed several important protein-protein interactions among our secretome proteins, including those with EGFR. These interactions may prove to be of importance in thyroid tumourigeneses upon verification in future experiments.

Also, we hypothesized that some of the proteins may distinguish malignant from benign tumours when analyzing thyroid cancer patients' tissues and sera. All proteins were

detectable in TC patients' sera using western blot analysis, except for hnRNP K and PGK1. Some proteins showed variability in protein expression among the non-aggressive and aggressive TC patients' sera. Significant differences in subcellular expression in 8 of 9 secretome proteins were found in TC patients' tissues between benign and malignant tumours, as assessed by immunohistochemistry. All proteins showed a decrease in nuclear, cytoplasmic, and/or membrane expression in cancer as compared to benign tissues except for APLP2 and 14-3-3 zeta, whereby a slight increase in cytoplasmic expression was observed.

More importantly, we sought to determine if any of these proteins were associated with thyroid cancer tumour aggressiveness. The study of ALCAM in TC patients' tissues was expanded to answer this question. ALCAM was associated with TC tumour aggressiveness and extrathyroidal extension. Decreased membrane and cytoplasmic expression was observed in poorly/undifferentiated TC tumours as compared to well-differentiated tumours. ALCAM is also a potential clinically useful prognostic biomarker for thyroid cancer. Reduced ALCAM expression correlated with reduced overall disease-free survival.

Increased serum ALCAM levels were associated with some aggressive features of thyroid cancer. ALCAM may be useful in the stratification of aggressive and non-aggressive TC patients.

In conclusion, the objectives of this study were met and the hypotheses are supported. The detection of these proteins in TC patients' sera and tissues underscores their potential for indepth investigation either singly or in combination for future development of minimally invasive markers for aggressive TCs.

5.3 Future Directions

The six proteins, AXL, APP, SET, PGK1, PKM2, and hnRNP K, that were able to distinguish benign from malignant tumours is certainly worthy of further investigation. The sample size of tissues was relatively small in comparison to the number of proteins analyzed as a panel. By expanding the sample size to include at least a total of 200 cases, we can determine if 1) the results are reproducible on large-scale; 2) statistical significance and accuracy in distinguishing benign from malignant nodules is maintained; 3) the variability in immunostaining of each protein observed is decreased; and 4) if some of these proteins can function as a panel for increased accuracy in distinguishing malignant from benign tumours.

This panel of candidate biomarkers has the potential to enhance the diagnostic capacity of FNAB in the early stages of TC detection. TC patients' FNABs should be collected to stain for these proteins using the aforementioned immunohistochemistry method. If these proteins are shown useful on FNAB, they can be potentially classified as tissue-based biomarkers. Also, we can take this even further and analyze their expression in sera of TC patients using the ELISA to demonstrate their potential as blood-based biomarkers. Although AXL did not show significant differences in patient sera levels between benign and malignant samples, others proteins may show otherwise. Details on sera analyses will be discussed with ALCAM.

Our results demonstrate ALCAM as a potential clinically useful diagnostic and prognostic tissue and/or serum-based biomarker for TC aggressiveness. Although a total of 203 cases were analyzed for ALCAM tissue expression, the number of aggressive subtypes of TC analyzed was small in comparison to the non-aggressive PTC. More tissues from patients with advanced stage TC, PDPTC, insular carcinoma, and ATC should be analyzed. This would

allow for the assessment of how well ALCAM can stratify aggressive variants of TC. MTC, which is not of follicular origin as the rest of the TC types, was not analyzed in our study since our focus was on PTC and ATC and the proteins secreted from these thyroid cancer cell lines. However, it would be interesting to collect MTC tissues for ALCAM staining to determine how this aggressive variant of TC stains for ALCAM, and if ALCAM expression can be used as a marker for the detection of MTCs.

Serum ALCAM levels may be useful in the stratification of aggressive and non-aggressive TC patients. Investigation of ALCAM in a large cohort of aggressive TC patients is required to determine its applicability as a blood-based marker for aggressive TC, as this was a limitation in our study. This can be achieved through multi-centre collaboration to recruit more patients. Stratification of aggressive TCs can potentially reduce the large sera level variation observed in this group. It is important to also obtain blood from normal (tumour-free) individuals to compare ALCAM sera levels with cancer-free patients who have benign nodules.

Furthermore, blood collection from TC patients pre-surgery and post-surgery would allow us to assess the ability of the protein to serve as a prognostic marker for TC management. Complete patient follow-up information including all forms of treatment given post-surgery, such as RAI or chemotherapy, should be well documented. Correlating the sera levels with different patient treatments may reveal interesting associations and the standardization of this protein for use in blood-based assays. Similar sera analyses can be performed on the rest of the secretome proteins identified in this study.

The IPA network suggested several important protein-protein interactions that warrant further investigation. This can be achieved by TC cell line experimentation using siRNAs to knockdown expression at the mRNA level. Alternatively, inhibitors may be used to

downregulate protein expression and function in cancer-relevant processes. Applying this method individually to some of the major protein hubs or regulators in our IPA network would allow for the elucidation of its effects on downstream signalling pathways and possible down/upregulations in other secretome proteins that we identified as having potential direct or indirect interactions with one another. This would be important in understanding the mechanistic aspect of TC tumour progression and could reveal a pathway worthy of investigating as a therapeutic target. This experiment can be implemented in already available thyroid cancer cell lines, such as those used herein, or in cells cultured from TC patient tumours obtained at the time of surgery, along with surrounding normal thyroid tissue as a control.

It would be important to assess the success of the targeted knockdown of expression in the suggested experiment. This can be achieved by a real-time reverse transcription polymerase chain reaction (qRT-PCR) to quantify mRNA levels and compare them to the control (untreated group). On the other hand, if protein inhibitors are used to downregulate protein expression, western blot analysis may be used to assess if protein downregulation is achieved compared to controls. Also, a cell viability assay could be performed to determine the effect of this knockdown/inhibition on cell survival. Once downregulation of the protein has been achieved, the effects on the levels of downstream molecules can be measured, either by western analysis or flow cytometry. Depending on the function of the protein analyzed, certain functional assays can be performed. This work can be expanded *in vivo* in TC tumour xenograft mice. Some of the clinical trial drugs listed in this report can also be used in these assays to determine the effect on tumour growth and downstream protein expression.

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Appendices

Appendix I

Supplementary Table S1 - Summary of high-confidence proteins in BCPAP, C643, and SW1736 thyroid cancer cell lines identified by mass spectrometry at York University

Accession #	Protein	Protein-pilot Unused Score	# Unique peptides	Sample	Peptide sequence	Precursor m/z	Charge	Peptide confidence
spt P63104	14-3-3	2.42	1	BCPAP-SFM 17/05	DSTLIMQLLR	595.3297	2	99
spt P63104	14-3-3	4		C643-SFM	DSTLIMQLLR	595.2571	2	99
spt P63104	14-3-3	3.15		SW-SFM 04/27	DSTLIMQLLR	595.3312	2	99
spt Q06481	APLP2	6.02	3	SW-SFM 04/27	C[Cam]LVGEFVSDVL LVPEK	902.4816	2	99
spt Q06481	APLP2			SW-SFM 04/27	M[Oxi]ALE[Dhy]NYL AALQSDPPRPHR	545.0292	4	99
spt Q06481	APLP2			SW-SFM 04/27	VPYVAQEIQEEIDEL LQEQR	810.4114	3	99
spt Q06481	APLP2	3.52		SW-SFM 05/13	VPYVAQEIQEEIDEL LQEQR	811.0769	3	99
spt P05067	APP	2.16	2	SW-SFM 04/27	LEVPTDGNAGLLAE PQIAMFC[Cam]GR	820.4079	3	99
spt P05067	APP	3.7		SW-SFM 05/13	C[Cam]LVGEFVSDAL LVPDK	881.4588	2	99
spt P30530	AXL	1.7	2	BCPAP-SFM 05/17	APLQGTLLGYR	594.8381	2	98
spt P30530	AXL	2.35		SW-SFM 05/13	APLQGTLLGYR	594.8384	2	99
spt P30530	AXL	2.01		SW-SFM 04/27	LAYQGQDTPEVLMD IGLR	1010.015	2	99
spt P61978	hnRNP K	2	2	C643-SFM	IILDLISESPIK	670.824	2	99
spt P61978	hnRNP K	2		SW-SFM 04/27	ILSISADIETIGEILK	857.9958	2	99
spt P00558	PGK1	1.7	1	C643-SFM	VLPGVDAISNI	549.2493	2	97
spt P14618	PKM2	2.64	2	C643-SFM	APIIAVTR	420.7204	2	97
spt P14618	PKM2	2.01		SW-SFM 04/27	FGVEQDVDMVFASF IR	620.6364	3	99
spt Q01105	SET	2	2	SW-SFM 04/27	IPNFWVTTFVNHPQV SALLGEEDEEALHYL TR	932.2211	4	99
spt Q01105	SET	2.77		SW-SFM 05/13	IDFYFDENPYFENK	920.9081	2	99
spt Q6PEY4	ALCAM	2	1	BCPAP 04/22	VLHPLEGAVVIFKK	555.0458	3	99

Proteinpilot Unused score: a score ≥ 1.3 corresponds to a confidence score $\geq 95\%$

Legend of amino-acid modifications: C[Cam] = Carbamidomethyl-Cystein; M[Oxi] = Oxidized Methionine; E[Dhy] = Dehydrated Glutamic acid.

Appendix II

Supplementary Tables and Figures S2 - MS/MS spectra of the identified peptides at York University

Protein: 14-3-3; Sample: BCPAP-SFM 17/05

Residue	b	b+2	y	y+2
D	116.034	58.5207	1189.66	595.334
S	203.066	102.037	1074.63	537.821
T	304.114	152.561	987.602	494.305
L	417.198	209.103	886.554	443.781
I	530.282	265.645	773.47	387.239
M	661.323	331.165	660.386	330.697
Q	789.381	395.194	529.346	265.177
L	902.465	451.736	401.287	201.147
L	1015.55	508.278	288.203	144.605
R	1171.65	586.329	175.119	88.0631

Protein: 14-3-3; Sample: C643-SFM

Residue	b	b+2	y	y+2
D	116.034	58.5207	1418.72	709.863
I	229.118	115.063	1303.69	652.35
C[CAM]	389.149	195.078	1190.61	595.808
N	503.192	252.1	1030.58	515.793
D	618.219	309.613	916.535	458.771
V	717.287	359.147	801.508	401.258
L	830.371	415.689	702.44	351.723
S	917.403	459.205	589.356	295.181
L	1030.49	515.747	502.324	251.665
L	1143.57	572.289	389.24	195.123
E	1272.61	636.811	276.155	138.581
K	1400.71	700.858	147.113	74.06

Protein: 14-3-3; Sample: SW-SFM 04/27

Residue	b	b+2	y	y+2
D	116.0342	58.5207	1189.6609	595.3341
S	203.0662	102.0368	1074.634	537.8206
T	304.1139	152.5606	987.6019	494.3046
L	417.198	209.1026	886.5543	443.7808
I	530.2821	265.6447	773.4702	387.2387
M	661.3225	331.1649	660.3861	330.6967
Q	789.3811	395.1942	529.3457	265.1765
L	902.4652	451.7362	401.2871	201.1472
L	1015.5492	508.2783	288.203	144.6051
R	1171.6504	586.3288	175.119	88.0631

Protein: APLP2; Sample: SW-SFM 04/27

Residue	b	b+2	y	y+2
C[CAM]	161.0379	81.0226	1803.956	902.4817
L	274.122	137.5646	1643.926	822.4664
V	373.1904	187.0988	1530.841	765.9243
G	430.2119	215.6096	1431.773	716.3901
E	559.2545	280.1309	1374.752	687.8794
F	706.3229	353.6651	1245.709	623.3581
V	805.3913	403.1993	1098.641	549.8239
S	892.4233	446.7153	999.5721	500.2897
D	1007.45	504.2288	912.5401	456.7737
V	1106.519	553.763	797.5131	399.2602
L	1219.603	610.305	698.4447	349.726
L	1332.687	666.847	585.3606	293.184
V	1431.755	716.3812	472.2766	236.6419
P	1528.808	764.9076	373.2082	187.1077
E	1657.851	829.4289	276.1554	138.5813
K	1785.946	893.4764	147.1128	74.06

Protein: APLP2; Sample: SW-SFM 04/27

Residue	b	b+2	y	y+2
M[Oxi]	148.0427	74.525	2177.092	1089.05
A	219.0798	110.0435	2030.057	1015.532
L	332.1639	166.5856	1959.02	980.0134
E[Dhy]	443.1959	222.1016	1845.936	923.4714
N	557.2388	279.123	1734.903	867.9554
Y	720.3021	360.6547	1620.861	810.9339
L	833.3862	417.1967	1457.797	729.4022
A	904.4233	452.7153	1344.713	672.8602
A	975.4604	488.2339	1273.676	637.3416
L	1088.545	544.7759	1202.639	601.8231
Q	1216.603	608.8052	1089.555	545.281
S	1303.635	652.3212	961.4962	481.2518
D	1418.662	709.8347	874.4642	437.7357
P	1515.715	758.361	759.4373	380.2223
P	1612.768	806.8874	662.3845	331.6959
R	1768.869	884.938	565.3317	283.1695
P	1865.921	933.4644	409.2306	205.119
H	2002.98	1001.994	312.1779	156.5926
R	2159.082	1080.044	175.119	88.0631

Protein: APLP2; Sample: SW-SFM 04/27

Residue	b	b+2	y	y+2
V	100.0757	50.5415	2429.22	1215.113
P	197.1285	99.0679	2330.151	1165.579
Y	360.1918	180.5995	2233.098	1117.053
V	459.2602	230.1337	2070.035	1035.521
A	530.2973	265.6523	1970.967	985.9869
Q	658.3559	329.6816	1899.929	950.4684
E	787.3985	394.2029	1771.871	886.4391
I	900.4825	450.7449	1642.828	821.9178
Q	1028.541	514.7742	1529.744	765.3757
E	1157.584	579.2955	1401.686	701.3464
E	1286.626	643.8168	1272.643	636.8251
I	1399.71	700.3588	1143.6	572.3039
D	1514.737	757.8723	1030.516	515.7618
E	1643.78	822.3936	915.4894	458.2483
L	1756.864	878.9356	786.4468	393.7271
L	1869.948	935.4777	673.3628	337.185
Q	1998.007	999.5069	560.2787	280.643
E	2127.049	1064.028	432.2201	216.6137
Q	2255.108	1128.058	303.1775	152.0924
R	2411.209	1206.108	175.119	88.0631

Protein: APLP2; Sample: SW-SFM 05/13

Residue	b	b+2	y	y+2
V	100.076	50.5415	2429.22	1215.11
P	197.129	99.0679	2330.15	1165.58
Y	360.192	180.6	2233.1	1117.05
V	459.26	230.134	2070.04	1035.52
A	530.297	265.652	1970.97	985.987
Q	658.356	329.682	1899.93	950.468
E	787.399	394.203	1771.87	886.439
I	900.483	450.745	1642.83	821.918
Q	1028.54	514.774	1529.74	765.376
E	1157.58	579.296	1401.69	701.346
E	1286.63	643.817	1272.64	636.825
I	1399.71	700.359	1143.6	572.304
D	1514.74	757.872	1030.52	515.762
E	1643.78	822.394	915.489	458.248
L	1756.86	878.936	786.447	393.727
L	1869.95	935.478	673.363	337.185
Q	1998.01	999.507	560.279	280.643
E	2127.05	1064.03	432.22	216.614
Q	2255.11	1128.06	303.178	152.092
R	2411.21	1206.11	175.119	88.0631

Protein: APP; Sample: SW-SFM 04/27

Residue	b	b+2	y	y+2
L	114.091	57.5493	2459.21	1230.11
E	243.134	122.071	2346.12	1173.56
V	342.202	171.605	2217.08	1109.04
P	439.255	220.131	2118.01	1059.51
T	540.303	270.655	2020.96	1010.98
D	655.33	328.169	1919.91	960.459
G	712.351	356.679	1804.88	902.945
N	826.394	413.701	1747.86	874.435
A	897.431	449.219	1633.82	817.413
G	954.453	477.73	1562.78	781.895
L	1067.54	534.272	1505.76	753.384
L	1180.62	590.814	1392.68	696.842
A	1251.66	626.333	1279.59	640.3
E	1380.7	690.854	1208.56	604.781
P	1477.75	739.38	1079.51	540.26
Q	1605.81	803.41	982.46	491.734
I	1718.9	859.952	854.401	427.704
A	1789.93	895.47	741.317	371.162
M	1920.97	960.99	670.28	335.644
F	2068.04	1034.52	539.24	270.123
C[CAM]	2228.07	1114.54	392.171	196.589
G	2285.09	1143.05	232.14	116.574
R	2441.2	1221.1	175.119	88.0631

Protein: APP; Sample: SW-SFM 05/13

Residue	b	b+2	y	y+2
C[CAM]	161.038	81.0226	1761.91	881.458
L	274.122	137.565	1601.88	801.443
V	373.19	187.099	1488.79	744.901
G	430.212	215.61	1389.73	695.367
E	559.255	280.131	1332.7	666.856
F	706.323	353.665	1203.66	602.335
V	805.391	403.199	1056.59	528.8
S	892.423	446.715	957.525	479.266
D	1007.45	504.229	870.493	435.75
A	1078.49	539.747	755.466	378.237
L	1191.57	596.289	684.429	342.718
L	1304.66	652.831	571.345	286.176
V	1403.72	702.366	458.261	229.634
P	1500.78	750.892	359.193	180.1
D	1615.8	808.405	262.14	131.574
K	1743.9	872.453	147.113	74.06

Protein: AXL; Sample: BCPAP-SFM 05/17

Residue	b	b+2	y	y+2
A	72.0444	36.5258	1188.67	594.84
P	169.097	85.0522	1117.64	559.322
L	282.181	141.594	1020.58	510.796
Q	410.24	205.624	907.5	454.253
G	467.261	234.134	779.441	390.224
T	568.309	284.658	722.42	361.713
L	681.393	341.2	621.372	311.19
L	794.477	397.742	508.288	254.648
G	851.499	426.253	395.204	198.106
Y	1014.56	507.785	338.182	169.595
R	1170.66	585.835	175.119	88.0631

Protein: AXL; Sample: SW-SFM 04/27

Residue	b	b+2	y	y+2
L	114.091	57.5493	2019.02	1010.01
A	185.129	93.0679	1905.94	953.472
Y	348.192	174.6	1834.9	917.954
Q	476.25	238.629	1671.84	836.422
G	533.272	267.14	1543.78	772.393
Q	661.33	331.169	1486.76	743.882
D	776.357	388.682	1358.7	679.853
T	877.405	439.206	1243.67	622.339
P	974.458	487.733	1142.62	571.816
E	1103.5	552.254	1045.57	523.289
V	1202.57	601.788	916.528	458.768
L	1315.65	658.33	817.46	409.234
M	1446.69	723.85	704.376	352.692
D	1561.72	781.364	573.336	287.171
I	1674.8	837.906	458.309	229.658
G	1731.83	866.417	345.225	173.116
L	1844.91	922.959	288.203	144.605
R	2001.01	1001.01	175.119	88.0631

Protein: AXL; Sample: SW-SFM 05/13

Residue	b	b+2	y	y+2
A	72.0444	36.5258	1188.67	594.84
P	169.097	85.0522	1117.64	559.322
L	282.181	141.594	1020.58	510.796
Q	410.24	205.624	907.5	454.253
G	467.261	234.134	779.441	390.224
T	568.309	284.658	722.42	361.713
L	681.393	341.2	621.372	311.19
L	794.477	397.742	508.288	254.648
G	851.499	426.253	395.204	198.106
Y	1014.56	507.785	338.182	169.595
R	1170.66	585.835	175.119	88.0631

Protein: hnRNPK; Sample: C643-SFM

Residue	b	b+2	y	y+2
I	114.091	57.5493	1340.8	670.905
I	227.175	114.091	1227.72	614.363
L	340.26	170.633	1114.64	557.821
D	455.286	228.147	1001.55	501.279
L	568.371	284.689	886.524	443.766
I	681.455	341.231	773.44	387.224
S	768.487	384.747	660.356	330.682
E	897.529	449.268	573.324	287.166
S	984.561	492.784	444.282	222.645
P	1081.61	541.311	357.25	179.129
I	1194.7	597.853	260.197	130.602
K	1322.79	661.9	147.113	74.06

Protein: hnRNPK; Sample: SW-SFM 04/27

Residue	b	b+2	y	y+2
I	114.091	57.5493	1714.98	857.996
L	227.175	114.091	1601.9	801.454
S	314.207	157.607	1488.82	744.911
I	427.292	214.149	1401.78	701.395
S	514.324	257.665	1288.7	644.853
A	585.361	293.184	1201.67	601.337
D	700.388	350.697	1130.63	565.819
I	813.472	407.24	1015.6	508.305
E	942.514	471.761	902.519	451.763
T	1043.56	522.285	773.477	387.242
I	1156.65	578.827	672.429	336.718
G	1213.67	607.337	559.345	280.176
E	1342.71	671.859	502.324	251.665
I	1455.79	728.401	373.281	187.144
L	1568.88	784.943	260.197	130.602
K	1696.97	848.99	147.113	74.06

Protein: PGK1; Sample: C643-SFM

Residue	b	b+2	y	y+2
V	100.076	50.5415	1097.62	549.314
L	213.16	107.084	998.552	499.78
P	310.213	155.61	885.468	443.237
G	367.234	184.121	788.415	394.711
V	466.302	233.655	731.393	366.2
D	581.329	291.168	632.325	316.666
A	652.367	326.687	517.298	259.153
L	765.451	383.229	446.261	223.634
S	852.483	426.745	333.177	167.092
N	966.526	483.766	246.145	123.576
I	1079.61	540.308	132.102	66.5546

Protein: PKM2; Sample: C643-SFM

Residue	b	b+2	y	y+2
A	72.0444	36.5258	840.53	420.769
P	169.097	85.0522	769.493	385.25
I	282.181	141.594	672.44	336.724
I	395.265	198.136	559.356	280.182
A	466.302	233.655	446.272	223.64
V	565.371	283.189	375.235	188.121
T	666.419	333.713	276.167	138.587
R	822.52	411.763	175.119	88.0631

Protein: PKM2; Sample: SW-SFM 04/27

Residue	b	b+2	y	y+2
F	148.076	74.5415	1859.9	930.454
G	205.097	103.052	1712.83	856.919
V	304.166	152.586	1655.81	828.409
E	433.208	217.108	1556.74	778.874
Q	561.267	281.137	1427.7	714.353
D	676.294	338.651	1299.64	650.324
V	775.362	388.185	1184.61	592.81
D	890.389	445.698	1085.54	543.276
M	1021.43	511.218	970.518	485.763
V	1120.5	560.753	839.477	420.242
F	1267.57	634.287	740.409	370.708
A	1338.6	669.805	593.341	297.174
S	1425.64	713.321	522.304	261.655
F	1572.7	786.856	435.271	218.139
I	1685.79	843.398	288.203	144.605
R	1841.89	921.448	175.119	88.0631

Protein: SET; Sample: SW-SFM 04/27

Residue	b	b+2	y	y+2
I	114.091	57.5493	3725.86	1863.43
P	211.144	106.076	3612.78	1806.89
N	325.187	163.097	3515.72	1758.37
F	472.255	236.631	3401.68	1701.34
W	658.335	329.671	3254.61	1627.81
V	757.403	379.205	3068.53	1534.77
T	858.451	429.729	2969.46	1485.24
T	959.499	480.253	2868.42	1434.71
F	1106.57	553.787	2767.37	1384.19
V	1205.64	603.321	2620.3	1310.65
N	1319.68	660.343	2521.23	1261.12
H	1456.74	728.872	2407.19	1204.1
P	1553.79	777.399	2270.13	1135.57
Q	1681.85	841.428	2173.08	1087.04
V	1780.92	890.962	2045.02	1023.01
S	1867.95	934.478	1945.95	973.479
A	1938.99	969.997	1858.92	929.963
L	2052.07	1026.54	1787.88	894.444
L	2165.15	1083.08	1674.8	837.902
G	2222.18	1111.59	1561.71	781.36
E	2351.22	1176.11	1504.69	752.849
E	2480.26	1240.63	1375.65	688.328
D	2595.29	1298.15	1246.61	623.807
E	2724.33	1362.67	1131.58	566.293
E	2853.37	1427.19	1002.54	501.772
A	2924.41	1462.71	873.494	437.251
L	3037.49	1519.25	802.457	401.732
H	3174.55	1587.78	689.373	345.19
Y	3337.62	1669.31	552.314	276.661
L	3450.7	1725.85	389.251	195.129
T	3551.75	1776.38	276.167	138.587
R	3707.85	1854.43	175.119	88.0631

Protein: SET; Sample: SW-SFM 05/13

Residue	b	b+2	y	y+2
I	114.091	57.5493	1840.81	920.907
D	229.118	115.063	1727.72	864.365
F	376.187	188.597	1612.7	806.851
Y	539.25	270.129	1465.63	733.317
F	686.318	343.663	1302.56	651.786
D	801.345	401.176	1155.5	578.251
E	930.388	465.698	1040.47	520.738
N	1044.43	522.719	911.426	456.217
P	1141.48	571.246	797.383	399.195
Y	1304.55	652.777	700.33	350.669
F	1451.62	726.311	537.267	269.137
E	1580.66	790.833	390.198	195.603
N	1694.7	847.854	261.156	131.082
K	1822.8	911.902	147.113	74.06

Protein: ALCAM; Sample: BCPAP 04/22

Residue	b	b+2	y	y+2
V	100.0757	50.5415	1663.031	832.0189
L	213.1598	107.0835	1563.962	782.4847
H	350.2187	175.613	1450.878	725.9427
P	447.2714	224.1394	1313.819	657.4132
L	560.3555	280.6814	1216.766	608.8868
E	689.3981	345.2027	1103.682	552.3448
G	746.4196	373.7134	974.6397	487.8235
A	817.4567	409.232	917.6183	459.3128
V	916.5251	458.7662	846.5811	423.7942
V	1015.594	508.3004	747.5127	374.26
I	1128.678	564.8424	648.4443	324.7258
I	1241.762	621.3844	535.3602	268.1838
F	1388.83	694.9187	422.2762	211.6417
K	1516.925	758.9661	275.2078	138.1075
K	1645.02	823.0136	147.1128	74.06

Appendix III

Supplementary Figure S3 - Representative control Ponceau S stained immunoblot

Appendix IV

Supplementary Table S4 - Outline of patient clinical information for tissues used for immunohistochemistry*

Patient #	Gender	Age at Surgery	Pathology	Histological Variant	Grade	TNM T	TNM N	TNM M	TNM Stage
1	M	67	ATC	Large cell undifferentiated carcinoma	PD	4	0	1	IVc
2	M	29	Benign	Multinodular goitre	WD				
3	F	55	Benign	Multinodular goitre	WD				
4	F	54	Benign	Hyperplastic nodule	WD				
5	F	27	Benign	Hashimoto's thyroiditis	WD				
6	F	16	Benign	Dominant nodule	WD				
7	M	56	Benign	Dominant nodule	WD				
8	F	34	Benign	Multinodular goitre	WD				
9	M	37	Benign	Follicular adenoma	WD				
10	F	76	Benign	Multinodular goitre	WD				
11	M	47	Benign	Multiple colloid/hyperplastic nodules	WD				
12	F	34	Benign	Multiple colloid/hyperplastic nodules	WD				
13	F	65	Benign	Hashimoto's thyroiditis, Multinodular hyperplasia	WD				
14	F	53	Benign	Multiple colloid/hyperplastic nodules	WD				
15	M	44	Benign	Colloid nodule	WD				
16	F	62	Benign	Grave's Disease	WD				
17	F	61	Benign	Multinodular goitre	WD				
18	M	61	Benign	Multiple colloid/hyperplastic nodules	WD				
19	F	38	Benign	Hashimoto's thyroiditis	WD				
20	F	63	Benign	Nodular hyperplasia	WD				
21	F	34	Benign	Multinodular goitre	WD				
22	F	47	Benign	Follicular lesion	WD				
23	F	53	Benign	Multinodular goitre	WD				
24	F	45	Benign	Follicular adenoma	WD				
25	M	76	Benign	Multinodular goitre	WD				
26	F	61	PTC	Classic variant	WD	4	1b	1	IVc

27	M	82	PTC	Classic variant	WD	4	1b	1	IVc
28	M	22	PTC	Classic variant	WD	4	1b	1	II
29	F	56	PTC	Classic variant	WD	1	0	0	I
30	F	43	PTC	Classic variant	WD	2	1a	0	I
31	M	71	PTC	Classic variant	WD				
32	M	53	PTC	Classic variant	WD	4	1b	1	IVc
33	F	33	PTC	Classic variant	WD	1	1a	0	I
34	F	75	PTC	Tall Cell	WD	1	1b	0	IVa
35	F	26	PTC	Classic variant	WD	4	1b	0	I
36	M	45	PTC	Classic variant	WD	4	1a	0	IVc
37	F	44	PTC	Follicular variant	WD	1	1a	0	I
38	F	68	PTC	Follicular variant	WD	3	0	0	III
39	F	39	PTC	Classic variant	WD	1	0	0	I
40	F	66	PTC	Classic variant	WD	3	1b	0	IVc
41	F	47	PTC	Classic variant	WD	3	1a	0	IVc
42	F	53	PTC	Follicular Variant	WD	1	0	0	I
43	M	55	PTC	Follicular Variant	WD	1	0	0	I
44	F	82	PTC	Insular	PD	4	0	1	IVc
45	F	35	PTC	Follicular variant, micro	WD	1	1b	0	I
46	F	43	PTC	Oncocytic	WD	1	1a	0	I
47	F	63	PTC	follicular and insular	WD	2	0	0	II
48	F	66	PTC	Follicular Variant	WD	1	0	0	I
49	F	37	PTC	Follicular Variant	WD	2	0	0	I
50	M	28	PTC	Follicular Variant	WD	3	0	0	I
51	M	52	PTC	Classic variant	WD	3	1b	0	IVc
52	F	36	PTC	Follicular variant	WD	1	0	0	I
53	F	64	PTC	Follicular variant	WD	1	0	0	I
54	F	38	PTC	Classic variant	WD	1	1a	0	I

UD: undifferentiated , PD: poorly-differentiated, WD: well-differentiated

* ALCAM patient information provided in table in main thesis text

Appendix V

Supplementary Figure S5 - Receiver operating curve analyses of protein expression in benign versus malignant thyroid tissues. Green diagonal lines represent reference lines.

Appendix VI

Supplementary Figure S6 - Detailed analyses of protein expression in benign versus malignant thyroid tissues

Protein	Nuclear Expression						Cytoplasmic Expression					
	<i>p</i> value	AUC	Sen (%)	Spe (%)	PPV (%)	NPV (%)	<i>p</i> value	AUC	Sen (%)	Spe (%)	PPV (%)	NPV (%)
<i>APLP2</i>	1.000	0.606	65	35	50	50	0.044	0.555	95	35	59.4	87.5
<i>APP</i>	<0.001	0.937	63.2	100	100	75	0.745	0.605	66.7	42.1	56	53.3
<i>AXL</i>	<0.001	0.995	100	90	90.9	100	0.001	0.788	80	73.9	72.7	81
<i>hnRNP K</i>	<0.001	0.962	100	43	54.2	80	0.002	0.816	75	69.2	55.6	84.4
<i>PGK1</i>	<0.001	0.926	100	61.9	71.4	100	<0.001	0.950	100	57.1	69	100
<i>PKM2</i>	<0.001	0.977	85.7	100	100	87	0.350	0.507	50	33.3	41.2	41.7
<i>SET</i>	<0.001	0.963	100	73.7	80	100	0.480	0.528	31.6	80	60	55.2
<i>14-3-3 zeta</i>	0.067	0.644	35	90.5	77.8	59.4	0.058	0.630	57.1	75	70.6	62.5

AUC, area-under-curve; sen, sensitivity; spe, specificity; PPV, positive predictive value; NPV, negative predictive value

IHC positivity score cut-offs are as follows:

APLP2 nuclear = 5, cytoplasmic = 5

APP nuclear = 2, cytoplasmic = 4.2

AXL nuclear = 4.7, cytoplasmic = 3

hnRNP K nuclear = 5.2, cytoplasmic = 3

PGK1 nuclear = 5.1, cytoplasmic = 5.3

PKM2 nuclear = 3.5, cytoplasmic = 3.3

SET nuclear = 5.5, cytoplasmic = 2

14-3-3 zeta nuclear = 1, cytoplasmic = 2.4

Appendix VII

Supplementary Figure S7 - Combined ALCAM and AXL sera expression in relation to clinico-pathological parameters using ELISA

Clinicopathological Parameter	Total Cases	s-AXL & ALCAM positivity, N (%)	<i>p</i>	95% CI
Pathology				
Benign	39	12 (31)	1.000	0.428 – 2.759
Cancer	43	14 (33)		
PTC Aggressiveness				
Non-aggressive	28	7 (25)	0.184	0.696 – 9.895
Aggressive	15	7 (47)		
Histotype				
Classic PTC	20	5 (25)	0.869	_____
Follicular PTC	16	6 (38)		
Tall cell PTC	2	1 (50)		
Oncocytic PTC	3	1 (33)		
Diffuse sclerosing PTC	3	2 (67)		
AJCC Stage				
I	34	10 (29)	0.547	_____
II	4	1 (25)		
III	2	1 (50)		
IV	2	2 (100)		
LN metastasis				
Absent	32	7 (22)	0.022	1.413 – 27.653
Present	11	7 (64)		
Extrathyroidal Extension				
Absent	37	11 (30)	0.373	0.411 - 13.584
Present	6	3 (50)		
Angioinvasion				
Absent	39	13 (33)	1.000	0.063 – 7.054
Present	4	1 (25)		
Multifocality				
Absent	22	7 (32)	1.000	0.299 – 3.838
Present	21	7 (26)		