

Department of Molecular Medicine and Surgery
Karolinska Institutet, Sweden

MOLECULAR ASPECTS OF
POST-CHORNOBYL AND SPORADIC
PAPILLARY THYROID CARCINOMA

Andrii Dinets M.D.



**Karolinska
Institutet**

Stockholm 2013

Faculty Opponent

Peter E. Goretzki, Professor
Department of Surgery
Heinrich-Heine University, Düsseldorf, Germany

Examination Board

Jon Tsai, Associate Professor
Department of Clinical Science, Intervention and Technology
Karolinska Institutet, Stockholm, Sweden

Harvest F. Gu, Associate Professor
Department of Molecular Medicine and Surgery
Karolinska Institutet, Stockholm, Sweden

Mikael Nilsson, Professor
Department of Medical Biochemistry and Cell Biology
University of Gothenburg, the Sahlgrenska Academy, Gothenburg, Sweden

Main Supervisor

Jan Zedenius, Associate Professor
Department of Molecular Medicine and Surgery
Karolinska Institutet, Stockholm, Sweden

Co-supervisors

Catharina Larsson, Professor
Department of Oncology-Pathology
Karolinska Institutet, Stockholm, Sweden

Anders Höög, Associate Professor
Department of Oncology-Pathology
Karolinska Institutet, Stockholm, Sweden

Anastasios Sofiadis Ph.D.
Department of Oncology-Pathology
Karolinska Institutet, Stockholm, Sweden

Mykola Hulchiy, Associate Professor
Department of Endocrine Surgery
Kyiv City Teaching Endocrinological Center, Kyiv, Ukraine

All previously published articles were reproduced with permission from the publishers.
Published by Karolinska Institutet. Printed by Larserics Digital Print AB.
Cover artwork: Illustration of the cell-cycle (XVIVO LLC/SOURCE/NEWSCOM).

Copyright © Andrii Dinets M.D., 2013
ISBN 978-91-7549-225-4

“A goal without a plan is just a wish.”
Antoine de Saint-Exupéry

To my grandmother Galina

ABSTRACT

Papillary thyroid carcinoma (PTC) exhibits various molecular abnormalities, both when sporadic and radiation-related. PTC is still diagnosed in adult individuals who were younger than 18 years at the time of the Chernobyl accident in 1986 and lived within the contaminated area. The preoperative diagnosis of PTC is based on ultrasound-guided fine needle aspiration cytology (FNAC), which is highly informative in up to 90% of biopsies. FNAC is not informative for the discrimination of follicular thyroid carcinoma (FTC) from follicular thyroid adenoma (FTA). Moreover, FNAC is often unreliable for diagnosis of cystic PTC due to its common presentation as a mural nodule in a cystic mass. In case of cystic PTC, biopsy sometimes reveals a cystic fluid containing insufficient amount of representative cells for cytology.

In this work, PTC was characterized in relation to irradiation from radioactivity at childhood. Possible preoperative diagnostic markers for discrimination between PTC and other follicular thyroid neoplasms were identified, and their validity was tested.

In **Study I** molecular, genetic and clinical characteristics in 70 post-Chernobyl PTCs were investigated. A common *BRAF* 1799T>A mutation was detected in 26 cases, overrepresentation of *RET/PTC1* in 20 whereas *RET/PTC3* was found in 4 cases. *BRAF* mutation was observed 3.5 times less frequent in the PTC accompanied by chronic lymphocytic thyroiditis (PTC/CLT) as compared to PTC only (12% vs. 44%). Greater expression of cyclin A was observed in PTC ≥ 2 cm as compared to PTC < 2 cm (1.2% vs. 0.6%). In conclusion, *BRAF* mutation and *RET/PTC1* rearrangement as well as other molecular features of adult post-Chernobyl PTC were partly overlapping with other reported PTC cohorts.

In **Study II** the SELDI-TOF mass spectrometry method was applied for PTC, FTC, FTA and normal thyroid tissue (NT). Significant overexpression of the protein S100A6 was identified in PTC as compared to FTC, FTA and NT ($p < 0.05$). This result was verified both by Western blot (WB), using the same samples, and by IHC in these and additionally in the PTC samples investigated in **Study I**. Moreover, the presence of two post-translational modifications of S100A6 was observed and verified by LC-MS/MS. S100A6 expression is strongly associated with PTC, and can therefore be tested for discrimination between follicular thyroid tumors and PTC.

In **Study III** a two dimensional gel electrophoresis followed by MALDI-TOF mass spectrometry for proteomic profiling of PTC, FTC and FTA was performed. 25 protein spots showing significantly different expression between studied groups were identified. Of these, 9 protein spots were selected for further analyses by WB using the initially studied samples and by IHC using these as well as samples from **Study I**. The findings suggest additional proteins to be deregulated in thyroid tumors, and their clinical significance can now be further studied.

In **Study IV** preoperative diagnostic markers for PTC in cystic lesions were identified by applying LC-MS/MS method. Out of all 1581 identified proteins, annexin A3 (ANXA3), carboxymethylenebutenolidase homolog (CMBL) cytokeratin 19 (CK-19) and S100A13 were selected for validation by IHC and WB. ANXA3 and CMBL showed overexpression in both controls and PTCs, whereas S100A13 and CK-19 were up-regulated in PTC only ($p < 0.05$), suggesting their possible role for discrimination between cystic PTC and benign thyroid cysts.

LIST OF PUBLICATIONS

This thesis is based on the following articles, which will be referred to in the text by their Roman numerals.

- I. Dinets A.**, Hulchiy M., Sofiadis A., Ghaderi M., Höög A., Larsson C., Zedenius J.
Clinical, genetic, and immunohistochemical characterization of 70 Ukrainian adult cases with post-Chornobyl papillary thyroid carcinoma.
European Journal of Endocrinology. 2012; 166 (6):1049-1060.
- II.** Sofiadis A., **Dinets A.**, Orre LM., Branca RM., Juhlin CC., Foukakis T., Wallin G., Höög A., Hulchiy M., Zedenius J., Larsson C., Lehtiö J.
Proteomic study of thyroid tumors reveals frequent up-regulation of the Ca^{2+} -binding protein S100A6 in papillary thyroid carcinoma.
Thyroid. 2010; 20 (10):1067-1076.
- III.** Sofiadis A., Becker S., Hellman U., Hultin-Rosenberg L., **Dinets A.**, Hulchiy M., Zedenius J., Wallin G., Foukakis T., Höög A., Auer G., Lehtiö J., Larsson C.
Proteomic profiling of follicular and papillary thyroid tumors.
European Journal of Endocrinology. 2012; 166 (4):657-667.
- IV. Dinets A.**, Pernemalm M., Eriksson H., Sviatoha V., Hulchiy M., Sofiadis A., Zedenius J., Larsson C., Lehtiö J., Höög A.
Identification of diagnostic markers for cystic papillary thyroid carcinoma by a proteomics approach.
Manuscript

CONTENTS

1	INTRODUCTION.....	10
1.1	THYROID ANATOMY, HISTOLOGY AND PHYSIOLOGY.....	10
1.2	BENIGN THYROID TUMORS.....	12
1.2.1	Follicular thyroid adenoma (FTA)	12
1.2.2	Goiter and cystic thyroid nodule	13
1.3	THYROID CANCER.....	14
1.3.1	Papillary thyroid carcinoma (PTC)	14
1.3.1.1	PTC and the Chornobyl accident	15
1.3.1.2	Clinical features and histopathology of PTC.....	15
1.3.1.3	Diagnosis of PTC.....	16
1.3.1.4	Treatment of PTC	16
1.3.1.5	Molecular genetic features of PTC	19
1.3.1.6	Prognosis of PTC.....	21
1.3.2	Follicular thyroid carcinoma (FTC)	22
1.3.3	Poorly differentiated thyroid carcinoma (PDTC) and anaplastic thyroid carcinoma (ATC).....	23
1.3.4	Medullary thyroid carcinoma (MTC).....	24
2	AIMS.....	25
3	MATERIALS AND METHODS.....	26
3.1	PATIENTS AND SAMPLES	26
3.1.1	Post-Chornobyl PTC (Studies I, II, III).....	26
3.1.2	A panel of follicular-cell derived thyroid tumors for protein expression profiling (Studies II and III)	26
3.1.3	Cystic thyroid lesions (Study IV)	27
3.2	METHODS.....	27
3.2.1	Western blot analysis.....	27
3.2.2	Immunohistochemistry (IHC)	28
3.2.3	Polymerase chain reaction (PCR)	29
3.2.3.1	Hot start PCR.....	29
3.2.3.2	Real time PCR (RT-PCR)	30
3.2.4	DNA sequencing.....	31
3.2.5	Pyrosequencing	32

3.2.6	Fluorescence <i>in situ</i> hybridization (FISH)	33
3.2.7	Mass spectrometry	34
3.2.7.1	MALDI-TOF-MS	34
3.2.7.2	SELDI-TOF-MS	35
3.2.7.3	LC-MS/MS	36
4	RESULTS AND DISCUSSION	37
4.1	STUDY I. MOLECULAR CHARACTERIZATION OF POST-CHORNOBYL PTCs.....	37
4.2	STUDY II. UP-REGULATION OF PROTEIN S100A6 IN PTC.....	40
4.3	STUDY III. PROTEIN EXPRESSION PROFILING OF THYROID TUMORS	42
4.4	STUDY IV. IDENTIFICATION OF DIAGNOSTIC MARKERS FOR CYSTIC PTC BY A PROTEOMICS APPROACH.....	43
5	CONCLUDING REMARKS	46
5.1	GENERAL CONCLUSIONS	46
5.2	FUTURE PERSPECTIVES	47
6	АБСТРАКТ (українська).....	49
7	ACKNOWLEDGMENTS	52
8	REFERENCES	57

LIST OF ABBREVIATIONS

2-DE	Two dimensional electrophoresis
ABC	Avidin-biotin complex
AFTA	Atypical follicular thyroid adenoma
ANXA3	Annexin A3
ANXA5	Annexin A5
<i>BRAF</i>	V-raf murine sarcoma viral oncogene homolog B1
bp	Base pair
CCD	Charge-coupled device
<i>CCDC6</i>	Coiled-coil domain-containing protein 6 gene
<i>CCND1</i>	Cyclin D1 gene
CDK	Cyclin-dependent kinase
cDNA	Complementary deoxyribonucleic acid
CK-19	Protein cytokeratin 19
DNA	Deoxyribonucleic acid
ESI	Electrospray ionization
FFPE	Formalin fixed paraffin embedded
FISH	Fluorescence <i>in situ</i> hybridization
FNAB	Fine needle aspiration biopsy
FNAC	Fine needle aspiration cytology
FTA	Follicular thyroid adenoma
FTC	Follicular thyroid carcinoma
HBME-1	Hector Battifora mesothelial antigen-1
H&E	Hematoxylin and eosin
IHC	Immunohistochemistry
IPG	Immobilized pH gradient
iTRAQ	Isobaric tags for relative and absolute quantitation
<i>NCOA4</i>	Nuclear receptor coactivator 4
LC-MS/MS	Liquid chromatography tandem mass spectrometry
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MIB-1	Monoclonal antibody against nuclear factor Ki-67
PCA	Principal component analysis
PCR	Polymerase chain reaction

PDIp	Protein disulfide-isomerase
PDTC	Poorly differentiated thyroid carcinoma
PLS-DA	Partial least squares discriminant analysis
PTC	Papillary thyroid carcinoma
PTC/CLT	Papillary thyroid carcinoma accompanied by chronic lymphocytic thyroiditis
PRX6	Peroxiredoxin 6
RAI	Radioactive iodine ablation therapy
<i>RET/PTC</i>	Rearrangement of <i>RET</i> proto-oncogene
RNA	Ribonucleic acid
SELDI-TOF-MS	Surface enhanced laser desorption/ionization time-of-flight mass spectrometry
SELENBP1	Selenium-binding protein 1
SNP	Single nucleotide polymorphism
TSH	Thyroid stimulating hormone
TTE	Total thyroidectomy
TR	Thyroid hormone receptor
WBS	Whole body scan with I ¹³¹

1 INTRODUCTION

The term “thyroid” originates from the Greek word “θύρεός”, meaning “shield” and the thyroid gland received its name because of its location close to the thyroid cartilage of the larynx. The first anatomical descriptions of the thyroid gland were published in medical textbooks in the 16th and 17th centuries. However, a detailed description was performed at the end of the 19th century by the Swiss surgeon Theodor Kocher, who received the Nobel Prize in 1909 for his work on the physiology, pathology and surgery of the thyroid gland [1].

1.1 THYROID ANATOMY, HISTOLOGY AND PHYSIOLOGY

The thyroid gland is the largest endocrine organ in the human body with a normal weight of 15-20 g. The gland is located next to the thyroid cartilage in anterior lateral position to the junction of larynx and trachea (Fig. 1). Typically, the thyroid gland consists of a right and a left lobe joined by the isthmus, and a pyramidal lobe which is a rudimental remnant of the thyroglossal duct. The upper part of the thyroid receives its blood supply from the two superior thyroid arteries, arising from the external carotid artery, whereas the lower parts are supplied by the inferior thyroid artery, originating from the thyrocervical trunk.

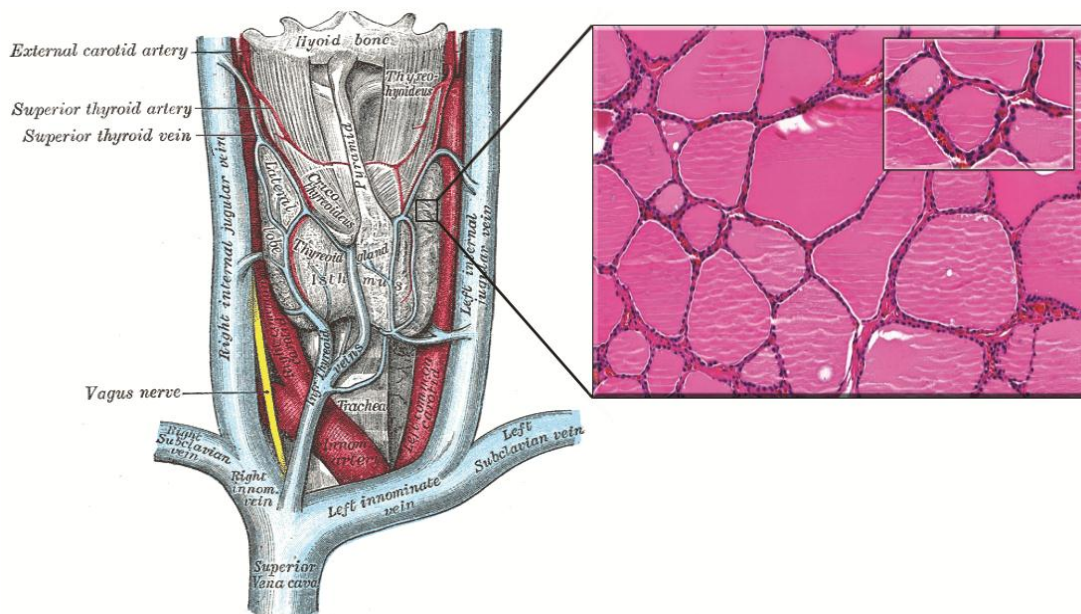


Figure 1. Anatomy of the thyroid gland (modified from Grey H, 1918 [2]). The enlargement to the right shows normal thyroid histology after hematoxylin and eosin (H&E) staining. Single layers of follicular cells constitute the thyroid follicles and secrete colloid. The case is shown in low and high magnification.

The venous blood is transported through the superior and middle veins, draining into the external jugular vein or through the brachiocephalic vein for the inferior part of the thyroid gland.

The thyroid lymphatic vessels are located under the capsule of the gland, forming multiple anastomoses in both lobes. The groups of regional lymph-nodes are mainly located along the vessels and nerves of the neck. The classification of these lymph nodes is based on the surgical anatomy and related to their location at the neck levels, or compartments [3, 4].

The main functional and structural unit of the thyroid gland is the follicle (Fig. 1). The follicle is composed of a single layer of epithelial follicular cells that secrete thyroglobulin into the lumen. Thyroglobulin is the main component of colloid, which stores the thyroid hormones. The follicular cells constitute up to 90% of thyroid cells and secrete the thyroid hormones triiodothyronine (T3) and thyroxine (T4). The remaining 10% are parafollicular cells (C-cells), that produce calcitonin [4].

The concentration of T3 and T4 is controlled by the hypothalamic-pituitary-thyroid axis, in a classical system of biological feedback, which is regulated by thyroid stimulating hormone (TSH). TSH stimulates proliferation, differentiation and functional activity of follicular thyroid cells [4]. Briefly, iodine molecules are absorbed from the gastrointestinal tract, transferred to the follicular cell, and conjugated to thyroglobulin in the colloid. This results in the formation of two types of iodotyrosines: monoiodotyrosine (MIT) or diiodotyrosine (DIT) in the presence of thyroid peroxidase. Subsequent coupling of one MIT and one DIT molecule gives rise to T3, whereas T4 is formed from reaction of two DIT [5]. The newly synthesized T3 and T4 molecules are bound to thyroglobulin and stored as colloid until release to the circulation. Iodine is crucial for T3 and T4 synthesis. Insufficient supply of iodine may disturb the regulation of the hypothalamic-pituitary-thyroid axis regulation, leading to elevated TSH production and stimulation of follicular cell activity, which can cause development of goiter.

T4, which is the inactive form of T3, constitutes the majority of newly secreted molecules by the thyroid. In the periphery T4 undergoes conversion to T3, which exhibits a higher affinity to the thyroid hormone receptor (TR) in the nuclei [6]. TR is formed by α and β units of the *TR* gene, which are located at chromosomes 17 and 3, respectively. Both units are found in different cells of the human body; however, the TR α is mainly expressed in the cells of central nervous system, whereas TR β is abundant in hepatocytes.

1.2 BENIGN THYROID TUMORS

A benign thyroid tumor is a neoplasm with distinct tumor borders within the thyroid capsule, without changes in the cells' nuclei or evidence of invasive growth patterns (*e.g.* spreading to vessels or other thyroid structures). Classification of these lesions is based on histopathological features and hormonal activity [7]. The most common type of benign thyroid neoplasm is the follicular adenoma. This may sometimes be hard to distinguish from nodules within a non-toxic colloid goiter (Fig. 2).

1.2.1 Follicular thyroid adenoma (FTA)

Follicular thyroid adenoma (FTA) is diagnosed in up to 30% of all patients with non-malignant thyroid lesions [7]. This is an encapsulated tumor exhibiting distinct evidence of differentiated follicular cells (Fig. 2) [8]. The histopathology of FTA shows a well-defined fibrous capsule, follicular cells and, in some cases, various degenerative changes such as fibrosis, hemorrhage, cystic changes and calcifications [7].

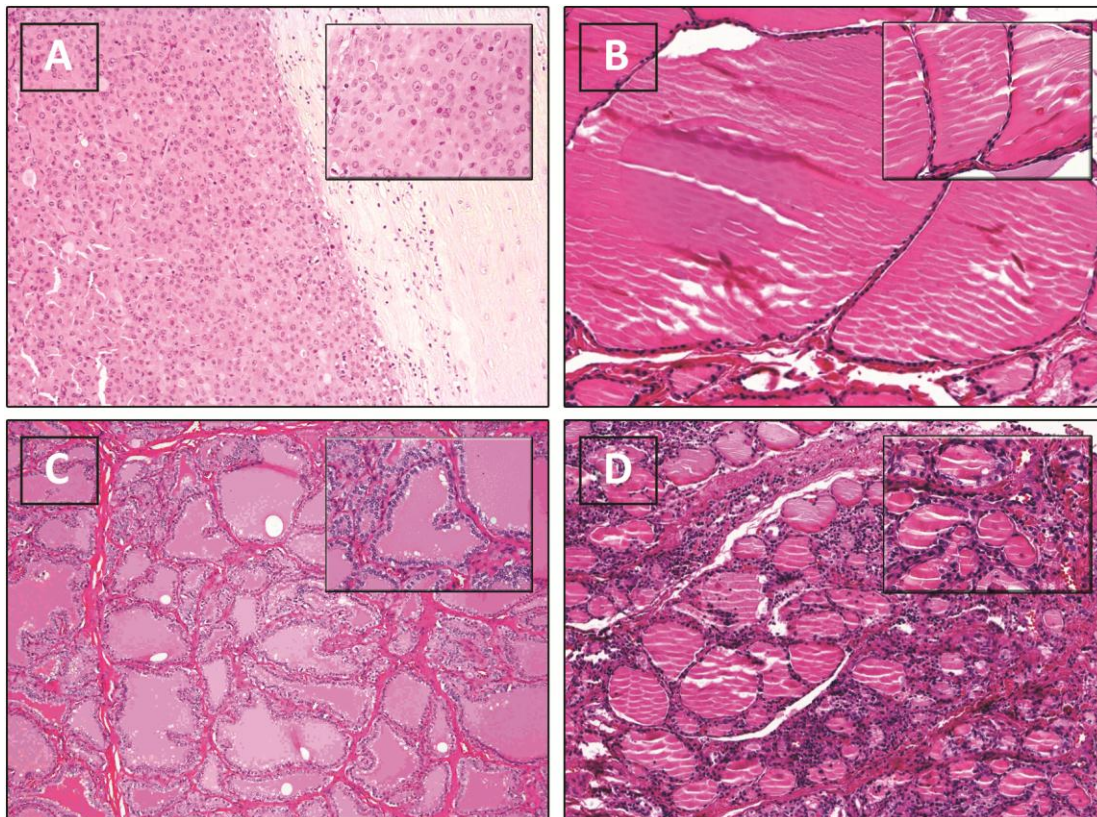


Figure 2. Histopathology of the most common benign thyroid lesions: (A) follicular thyroid adenoma (FTA), (B) non-toxic multinodular colloid goiter, (C) diffuse toxic goiter, and (D) chronic lymphocytic thyroiditis (H&E staining). Each case is shown in low and high magnification.

Invasion through all layers of the capsule and/or into vessels should be excluded in a sufficient number of tissue pieces for a reliable diagnosis of FTA.

The cells may show pleomorphic changes, mitoses, excessive cellularity or other atypical signs, and therefore fine needle aspiration cytology (FNAC) cannot distinguish FTA from its malignant counterpart, follicular thyroid carcinoma (FTC) [8]. Furthermore, there are follicular lesions described as “follicular tumors with undetermined malignant potential”, equivalent to atypical FTA (AFTA) [7, 9, 10].

The cytological diagnosis of FTA is not possible by a fine needle aspiration biopsy (FNAB). These cases should demonstrate the lack of cytological features of PTC, but cytology cannot exclude invasiveness through the capsule or into vessels, which are the criteria for FTC, resulting in low-informative FNAC reports. Therefore, the routine clinical work-up could benefit from the identification of diagnostic markers, which could be applied on FNAB specimens. Many studies have proposed molecular and genetic candidate markers for discrimination between follicular and papillary thyroid tumors, which remain to be confirmed in translational investigations [11-14]. Today patients with a FNAC showing a follicular thyroid tumor must be operated, so that a definitive diagnosis of either FTA or FTC can be established. Currently there are no tools for such a pre-operative distinction, unless there are signs of metastases, which of course is restricted to FTC.

1.2.2 Goiter and cystic thyroid nodule

The term goiter has French (*goitre*) and Latin (*guttur*) roots both meaning “throat”. Goiter is currently defined as an enlargement of the thyroid gland with nodular, multinodular or diffuse growth patterns, sometimes associated with elevated T3 and T4 levels (Fig. 2). Neither inflammatory disease nor malignancy is considered as a goiter. Non-toxic nodular and multinodular goiters are common types, affecting mainly females [15]. Although goiter is a benign entity, the condition is associated with development of papillary thyroid carcinoma (PTC) in 4-13% of cases [16-18]. These cases can be successfully identified in 80-90% of patients by physical examination followed by ultrasonographical examination and FNAC. If a cytological diagnosis of malignancy is established, surgery is of course indicated.

FNAC might be uninformative in cases of cystic thyroid nodules, which are defined as any fluid-filled nodule [17]. Such lesions account for 15-40% of all thyroid nodules, and are in most cases benign, but with evidence of cystic degeneration. Still, certain cases can be a cystic PTC, which are usually seen as mural nodules. The

diagnosis of these neoplasms is challenging, because FNAC usually reveals an abundant volume of cystic fluid, harboring a little amount of representative follicular cells resulting in low-informative reports [18]. It is worth noting that the cystic fluid is protein-rich, accumulating various compounds of epithelial products. Therefore, molecular and genetic investigations of cystic fluids could shed light on the tumorigenesis of cystic thyroid malignancies and the results of such investigations can serve as complementary tests to routine FNAC [16, 17, 19].

1.3 THYROID CANCER

Thyroid cancer comprises approximately 1% of all human malignancies [20, 21]. It is the most common endocrine malignancy constituting up to 80% of all cancers originating from endocrine organs. Thyroid cancer is classified as well-differentiated (PTC and FTC), poorly differentiated (PDTC) originating from follicular thyroid cells, and anaplastic (undifferentiated) thyroid carcinoma (ATC) in which the tumor cell origin is not always obvious. Finally, medullary thyroid carcinoma (MTC) is the result of neoplastic transformation of the C-cells (Fig. 3) [7, 22].

1.3.1 Papillary thyroid carcinoma (PTC)

Papillary thyroid carcinoma (PTC) is the most frequent type of thyroid cancer, constituting up to 80% of all thyroid malignancies. Sporadic PTC is most common in adult female patients and is rare among children, whereas radiation-induced PTC can be observed in all age groups [23-25]. Some etiological factors have been identified such as iodine excess intake, familial predisposition, radioactive and radiological exposure [7, 22, 26].

1.3.1.1 PTC and the Chornobyl accident

The accident at the Chornobyl nuclear power station in 1986 resulted in widespread contamination of large areas in Europe by various radioactive isotopes, including isotopes of iodine [27]. One of the main health consequences of exposure to radioactive iodine was a dramatic increase of PTC in the pediatric population, which later on is referred to as post-Chornobyl childhood PTC [28, 29]. Although the Chornobyl accident happened more than 25 years ago, its consequences are still seen as an elevated incidence of PTC among individuals who were younger than 18 years and lived in contaminated areas at the time of the Chornobyl accident [26, 30].

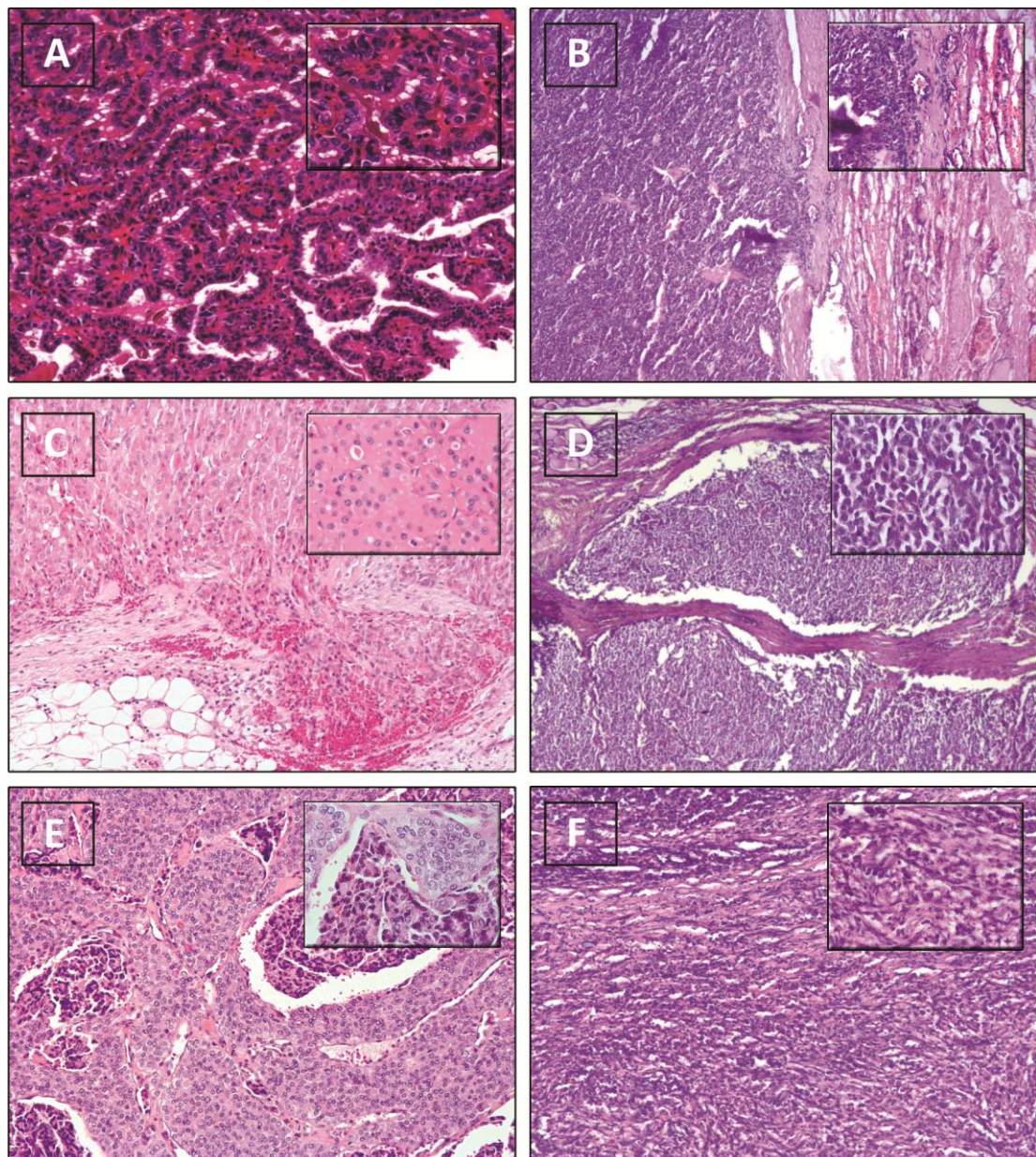


Figure 3. Microphotographs showing histopathological features of malignant thyroid neoplasms: (A) papillary thyroid carcinoma (PTC), (B) follicular thyroid carcinoma (FTC), (C) Hürthle cell carcinoma (HCC), (D) medullary thyroid carcinoma (MTC), (E) poorly differentiated thyroid carcinoma (PDTC), and (F) anaplastic thyroid carcinoma (ATC). Each case is stained by H&E and shown in low and high magnification.

1.3.1.2 Clinical features and histopathology of PTC

PTC usually presents as a palpable painless and firm nodule in the thyroid, which can be accompanied by enlarged lymph-nodes in the neck as a sign of metastatic spread. In addition to classical PTC, several histopathological growth patterns are seen such as columnar, tall cell, sclerosing, follicular, cystic [31]. Microscopically, the

classical type of PTC displays a central fibrovascular core with crowding epithelial cells forming papillary structures, psammoma bodies in the tumor stroma and typical features of the cell nucleus such as eosinophilic pseudo-inclusions, ground-glass nuclei and nuclear grooves (Fig. 3, 4) [7, 22, 31].

1.3.1.3 Diagnosis of PTC

The diagnosis of PTC is based on clinical and cytopathological investigations of a thyroid nodule (Fig. 4). PTC is typically diagnosed as a firm painless thyroid nodule, sometimes accompanied by enlarged lymph-nodes of the neck as a sign of metastatic spread. Ultrasonography usually shows a hypoechogenic neoplasm with irregular borders. Vascularity is often increased, and hyperechogenic inclusions (microcalcifications) may indicate PTC. If PTC is suspected, ultrasound-guided FNAB followed by FNAC is indicated. The cytological features of PTC are nuclear pseudoinclusions and grooves as well as enlarged crowding follicular cells. A FNAC-based diagnosis can still be a difficult task in cases of inadequate cell sampling or few findings of the PTC-specific nuclear changes [13, 32]. Moreover, there is a cystic variant of PTC developing as a mural nodule within the thyroid cystic mass, which can be misevaluated as a component of goiter. Although the cystic thyroid neoplasms are usually benign, approximately 4-13% are diagnosed as cystic PTC [16, 17]. The diagnosis of such a tumor is usually challenging, because the FNAB of the suspicious nodule within the thyroid cyst mainly reveals the cyst fluid and only small amounts of representative follicular cells that may give low-informative cytological reports [33].

1.3.1.4 Treatment of PTC

The typical scheme for treatment of a newly diagnosed PTC is total thyroidectomy (TTE) and dissection of the central neck lymph node compartment, followed by radioiodine ablation and TSH-suppressive therapy by T4 analogs.

TTE is a surgical procedure leading to removal of the thyroid lobes, isthmus and pyramidal lobe. The primary goal of TTE is total elimination of the thyroid tissue to prevent potential neoplastic transformation of follicular cells to PTC, to remove potential multifocal PTC and to facilitate follow-up with thyroglobulin that can then be applied as a tumor marker. Dissection of the central compartment (level 6) of the neck is surgical removal of all lymph nodes adjacent to the thyroid, pre-tracheal and along the tracheoesophageal groove, which is the most frequent location of local metastases [34].

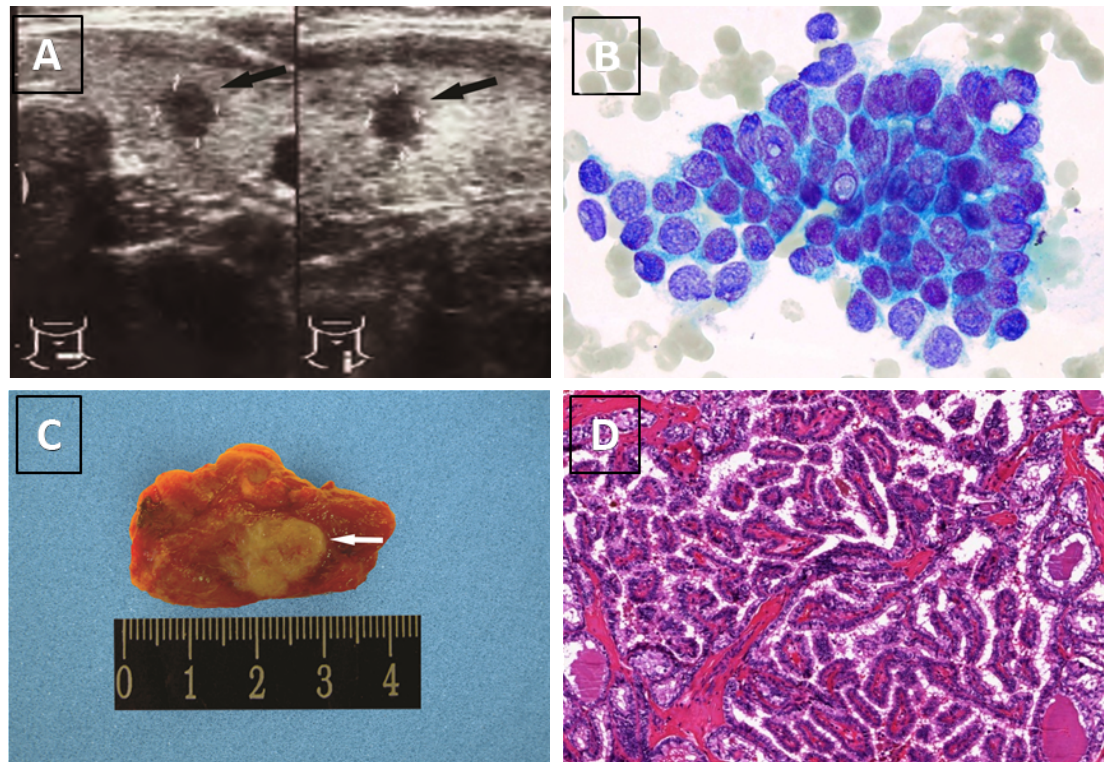


Figure 4. (A) Ultrasonography in two dimensions showing features of papillary thyroid carcinoma (PTC). A hypo-echogenic neoplasm with irregular borders is visible in the left thyroid lobe (marked by arrows). (B) Microphotograph of a cytological PTC specimen obtained by FNAC demonstrating enlarged follicular cells with nuclear grooves and inclusions (Papanicolaou staining, objective x100). (C) Photograph of a post-operative thyroid specimen with a yellowish nodule (marked by arrow) with irregular borders and invasiveness to surrounding thyroid tissue. (D) Histopathological features of PTC with papillary structures formed by crowding follicular cells attached to a fibrovascular core (H&E staining, objective x10).

However, other types of dissections are applied in cases with metastatic spread of PTC to other neck levels [4, 32, 34]. Thyroid-preserving surgery can be performed in patients with favorable prognostic features (*e.g.* PTC < 1 cm in greatest diameter, age < 45 years, no evidence of metastases, low MIB-1 index, absence of *BRAF* mutations) [35-37]. However, this approach is usually not discussed in the clinical guidelines [32, 38] or suggested during the interdisciplinary board decision making [39]. TTE is a complicated procedure because of the high level of blood supply to the thyroid gland and its relation close to the parathyroid glands and the laryngeal nerves. Thus, the most serious possible complications after TTE are bleeding, palsy of the recurrent laryngeal nerve and hypoparathyroidism due to parathyroid gland injury or removal [4, 39].

Six to eight weeks following TTE, a whole body scan (WBS) with I^{131} is indicated to the patients with PTC to identify remnant follicular cells or distant spread. If the WBS is positive, radioactive iodine ablation therapy (RAI) is indicated to destroy these remnant follicular cells [40]. RAI is performed with a dose of 30-60 mCi under a thyroid hormone withdrawal protocol or after administration of recombinant human TSH [4, 40]. In most guidelines, only patients with an “extremely low risk PTC” will not receive RAI, whereas all other patients receive ablative doses of radioiodine, and the WBS is performed shortly thereafter.

Still, there is a certain risk of PTC recurrence even after TTE and RAI. Hence, in the post-operative period virtually all patients with PTC receive TSH suppressive therapy by administration of thyroid hormone analogs, which is levothyroxine (L-T4) in most of the cases [32, 41]. TSH suppression is essential, since the PTC cells contain TSH receptors and may interact with TSH that stimulates PTC proliferation. The dose of L-T4 is individual, but considered as suppressive if the level of serum TSH is below 0.1 mU/L [41].

Chemotherapy and external radiotherapy are indicated for the treatment of cases that are RAI-refractory, with inoperable recurrence and/or who have developed distant metastases. As PTC most often displays very low proliferation, conventional chemotherapy is usually of no benefit. Selective small-molecule inhibitors of protein-kinase have been tested in several clinical trials, and BRAF inhibitors were shown as the most effective for the treatment of *BRAF* mutated metastatic melanoma [42]. This would suggest that BRAF inhibitors could also be used for PTC, in which *BRAF* mutations are common [22]. Another important therapeutic target is the PI3K/AKT pathway. Frequently showing molecular alterations in thyroid cancer, it is suggested to be targeted in the treatment schemes of therapy-resistant PTC synergistically with BRAF inhibitors [43, 44]. Also tyrosine-kinase inhibitors of various kinds have been suggested [45]. The use of molecular targets to guide selection of PTC treatment represents a novel therapeutic strategy in the management of thyroid cancer. Further translational studies of genetic and epigenetic alterations in PTC are expected to identify new targets for application of novel drugs, and eventually to fulfill the promise of molecule-based therapeutic strategy that could result in high response in cancer cells and at the same time a low toxicity [46].

1.3.1.5 Molecular genetic features of PTC

The molecular genetic profile of PTC includes various alterations such as mutations, chromosomal rearrangements and protein deregulations, affecting differentiation, proliferation and apoptosis of the follicular cell (Fig. 5). The most commonly reported abnormalities in PTC are alterations in genes and deregulations of proteins associated with tyrosine kinase signaling pathways, such as mutations of *BRAF* or rearrangements of the *RET* proto-oncogene [22].

Protein kinases regulate signaling pathways by controlling critical cellular activities. Mutated kinases, such as *BRAF*, can contribute to cancer development. The *BRAF* gene (v-Raf murine sarcoma viral oncogene homolog B1) is located in chromosomal region 7q34. The common *BRAF* mutation consists of a thymine-to-adenine transversion at position 1799 which results in substitution of valine to glutamic acid at residue 600 of the BRAF protein (V600E) [47]. *BRAF* mutation is considered as a marker of poor prognosis of PTC and is usually less abundant in patients with radiation-induced PTC [23, 48, 49].

The second most common genetic alteration in PTC is rearrangement of *RET*, a proto-oncogene encoding a transmembrane receptor of the tyrosine kinase family. Rearrangements of this gene are classified as *RET/PTC*, accounting for more than 10 different subtypes [50, 51]. *RET/PTC1* is most frequent in sporadic PTC, whereas *RET/PTC3* is associated with post-Chornobyl PTC [50, 52, 53]. Both rearrangements are paracentric inversions of chromosome 10, leading to fusion with different genes' domains (*CCDC6* for *RET/PTC1* and *NCOA4* for *RET/PTC3*). The prevalence of *RET/PTC1* and *RET/PTC3* varies from 0 to up to 87% in PTC [54]. These genetic lesions have been suggested as possible diagnostic and prognostic markers [49, 50, 52, 55].

Although mutations in the different *RAS* genes are detected mainly in FTC, mutations in *HRAS*, *KRAS* and *NRAS* genes can be identified in the follicular variant of PTC as well as in post-Chornobyl cancers [49, 56, 57]. Moreover, since *RAS* mutations (mainly *NRAS*) are also found in FTA, a possibility for progression of FTA to FTC has been proposed for such cases [58]. More recently, *RAS* mutations were also showed in post-Chornobyl PTC, suggesting its possible role in tumorigenesis of radiation-related PTC [49].

Some of the molecules that are deregulated in PTC may be applied in clinical practice. The nuclear protein Ki-67 is a marker of proliferation, frequently applied in routine clinics to assess the proliferation in different types of malignancies [35, 59].

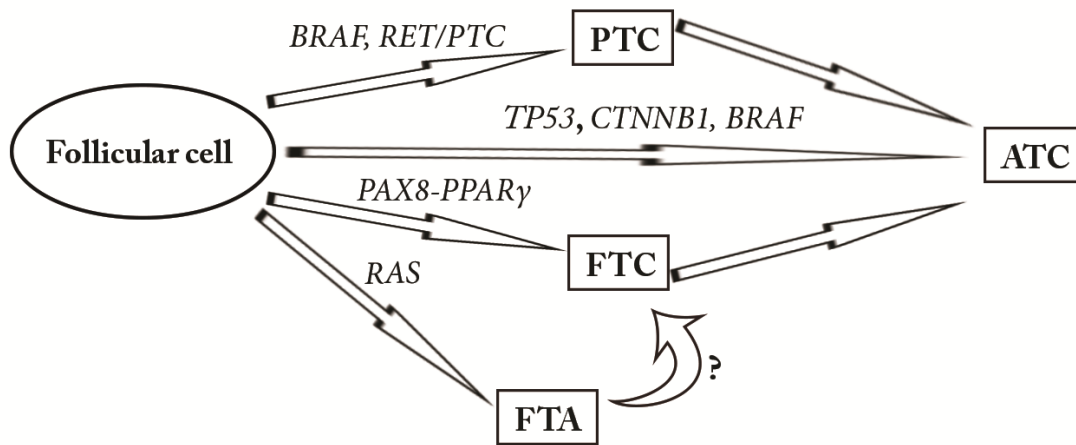


Figure 5. Schematic illustration of molecular and genetic abnormalities associated with development and progression of follicular thyroid adenoma (FTA), papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), and anaplastic thyroid carcinoma (ATC).

Ki-67 is expressed in proliferating cells and detectable during the entire cell-cycle, but switched off at the G0 phase of the cell-cycle. MIB-1 is a monoclonal antibody against Ki-67 used for immunohistochemistry, and the proportion of MIB-1 positive tumor cells is defined as the MIB-1 index. MIB-1 index is applied for FNAC specimens for diagnostic purposes, and can be used as a prognostic marker [35, 37, 60].

Cytokeratin 19 (CK-19) is frequently used as a diagnostic marker [61]. This protein belongs to the intermediate filaments, which play a role in creation of a cytoskeleton in different types of cells. CK-19 is made up of a highly complex family of polypeptides with molecular masses ranging from 40 to 68 kDa. This protein is considered to be the most sensitive protein marker for classical PTC [55]. Moreover, in routine clinical settings CK-19 is frequently applied together with Hector Battifora mesothelial antigen-1 (HBME-1), reacting, however, with unknown epitopes in PTC [61, 62].

Abnormalities of the cell-cycle regulators are commonly reported in PTC and can usually be assessed by immunohistochemical staining [35, 37, 60]. Cyclins play important roles in cell-cycle regulation through their ability to activate cyclin-dependent kinases (CDK). CDK are main regulators of cell-cycle progression, thus their up- or down-regulation might bring the cell to unscheduled proliferation. Cyclin A and cyclin D1 act to control the correct sequence of events that bring the thyroid cell through the cell-cycle phases and are expressed in thyroid neoplasms [63, 64].

Cyclin A regulates the cell-cycle through its ability to activate CDK. Cyclin A is expressed at late G1-phase, during S- and G2-phases, followed by peak expression at late G2. This type of cyclin is degraded when cells enter pro-metaphase. Cyclin A binds to CDK2 in the G1 to S transition, in the S-phase, and to CDK1 in the G2/M-phase. The specific expression of cyclin A is essential to control the correct sequence of events that bring the cell through S-phase to the G2-M checkpoint and its deregulation can cause unscheduled proliferation in PTC. Although cyclin A is well described in breast cancer, its biological role in PTC tumorigenesis remains unclear [64, 65].

Another cell-cycle regulator, cyclin D1, serves as a checkpoint in the early part of the G1 phase, allowing the progression of the cell-cycle from G1 to S phase. Expression of cyclin D1 has been described in thyroid tumors, but not in normal follicular cells [66-68].

The frequent deregulation of S100A2, S100A4, S100A6, S100A8, S100A9 and S100A10 in thyroid cancer suggests an important role for S100 proteins in PTC tumorigenesis. These proteins regulate cellular growth, differentiation, regeneration and signal transduction [69-73]. In brief, over-expression of S100A4 is associated with metastases and PTC progression [74], whereas S100A6 is suggested to discriminate between benign thyroid tumors and PTC [14, 72, 74]. Up-regulation of S100A2 and S100A10 was reported in ATC [75], which in some cases is considered to be the final stage of PTC dedifferentiation (Fig. 5) [76].

1.3.1.6 Prognosis of PTC

Prognosis of PTC is excellent for the majority of patients, showing 10-year survival rates over 90% in both sporadic and post-Chornobyl PTC [22, 77, 78]. Clinical parameters related to poor prognosis are tumor size, age > 45 years, extrathyroidal extension and metastasis to local lymph-nodes [36]. The presence of distant metastases immediately places the patient in stage 4, as suggested by the recent TNM staging system [79, 80]. In addition to the TNM system, several histopathological types of PTC have been proposed to have less favorable prognosis [81]. Columnar cell, tall cell, diffuse sclerosing and solid histopathological variants of PTC were reported to have an intrathyroidal growth pattern, multifocality as well as an elevated frequency of local and distant metastases. The solid variant was predominantly reported in post-Chornobyl childhood PTCs, whereas sporadic cases exhibit other types [81-83]. Fortunately, these tumors are relatively uncommon accounting for 0.2-12% of all PTC [81].

In addition to clinical and histopathological characteristics, molecular changes and genetic abnormalities play an important prognostic role in PTC [35, 84-87]. Thus, *BRAF* mutations, rearrangements of *RET* and elevated MIB-1 index are the most commonly reported and most frequently applied markers for prognostic purposes. *BRAF* mutations and *RET/PTC1* rearrangement are reported in sporadic PTC, whereas *RET/PTC3* is associated with post-Chornobyl childhood PTC [23, 49, 88].

1.3.2 Follicular thyroid carcinoma (FTC)

Follicular thyroid carcinoma (FTC) is a well-differentiated malignant neoplasm originating from the epithelial cells of the thyroid gland, exhibiting only follicular cell differentiation without any histopathological features of PTC [7, 22]. FTC is diagnosed in approximately 10-15% of all thyroid cancer cases, and the incidence is increased in iodine deficient areas [22]. FTC is most commonly diagnosed in female patients over 50 years of age. The tumors are often large, but local metastases to lymph-nodes are infrequent [89]. Histopathologically FTC shows well-formed follicles with solid growth patterns, invasion through the full thickness of the thyroid capsule and/or clusters of FTC cells in the vessels' lumen (Fig. 3).

Hürthle cell neoplasm is a benign or malignant thyroid tumor originating from follicular thyroid cells, exhibiting specific cytological features. They are classified as adenoma or carcinoma (HCC), equivalent to other follicular neoplasms. The histopathological examination shows polygonal cells with eosinophilic (oxyphilic) staining patterns due to elevated number of mitochondria in the cytoplasm (Fig. 3). The frequency of HCC is relatively low, constituting < 5% of all thyroid cancers [22, 90].

The diagnosis of FTC is established by final histopathology, since the tumor invasiveness cannot be evaluated by FNAC, and therefore cannot be discriminated preoperatively from FTA. Thus, investigation of the molecular profile of FTC may shed light not only on the tumor biology, but also direct the identification of diagnostic markers. Molecular and genetic profiles of FTC demonstrate various abnormalities, including up- and down-regulations, deletions and rearrangements [91]. The most commonly identified rearrangement is the translocation t(2;3)(q13;p25), resulting in the fusion of *PAX8* domains to domains of *PPAR γ* [22, 89, 92]. The treatment of the FTC is similar to other well-differentiated cancers, which is TTE followed by adjuvant RAI therapy. However post-operative RAI treatment is not always applicable to HCC, due to its resistance to I¹³¹ in up to 75% cases [90].

1.3.3 Poorly differentiated thyroid carcinoma (PDTC) and anaplastic thyroid carcinoma (ATC)

Poorly differentiated thyroid carcinoma (PDTC) has a follicular cell origin, showing histopathological features of both differentiated cancer (PTC or FTC) and anaplastic thyroid carcinoma (ATC). The incidence is 5-10% of all thyroid malignancies [22, 93]. PDTC is characterized by the presence of small cells with round nuclei and scant cytoplasm as well as diffuse solid patterns of growth, tumor necrosis and vascular invasion. However, it may contain microfollicular structures and sometimes produce thyroglobulin (Fig. 3). PDTC has a high degree of biological aggressiveness due to frequent extrathyroidal extension, as well as local and distant metastases. The prognosis of PDTC is poor; overall survival rates vary from 45 to 60% [93].

ATC is a highly malignant thyroid neoplasm exhibiting the most aggressive phenotype of all cancers in mankind [4, 40, 45]. It is considered as the final stage of dedifferentiation of well-differentiated cancers, but may arise *de novo* (Fig. 5) [94]. Clinically, ATC is manifested by dysphagia, a painful neck mass with rapid growth and evidence of invasion into local structures including trachea, esophagus and the neck vessels. Fortunately, this is a rare type of tumor which is diagnosed in less than 5% of all thyroid cancers [95]. Histopathologically ATC shows spindle and giant cells with intracellular cytoplasmic invaginations, as well as multiple mitoses and foci of necrosis (Fig. 3). The treatment is palliative for the majority of patients because of the highly progressive clinical course of ATC. External beam radiation combined with chemotherapy is used to improve the possibility of surgical removal of the primary tumor. Still, few patients survive more than 6 months after diagnosis [95].

Molecular and genetic alterations in PDTC and ATC are partly overlapping with well-differentiated thyroid malignancies [94]. Thus, *BRAF* mutation is reported in 30% of PDTC and up to 40% of ATC, which brings the question whether *BRAF* mutation may induce the progression of PTC to PDTC and ATC [96, 97]. Still, the identification of *BRAF* mutation in ATC is useful, since it gives the possibility for administration of specific BRAF inhibitors to such patients [97]. Commonly, both ATC and PDTC also show mutations of *TP53* and *CTNNB1*, a key player of the wingless (Wnt) pathway [88, 96].

The treatment of PDTC is similar to well-differentiated thyroid cancers, but can be modified depending on the clinical course of the disease. Hence, TTE and adjuvant RAI followed by TSH suppressive therapy are applicable to the majority

of patients, though higher doses of I^{131} are needed to achieve therapeutic effect as compared to PTC and FTC.

Surgical treatment is applicable for most cases of undifferentiated thyroid cancers [95]. Patients with ATC usually receive external beam radiation as well as neoadjuvant and adjuvant chemotherapy by administration of doxorubicin.

1.3.4 Medullary thyroid carcinoma (MTC)

Medullary thyroid carcinoma (MTC) is a malignancy originating from the C-cells that produce calcitonin [7]. MTC is relatively rare constituting 5-10% of all thyroid cancers [96].

This malignancy is classified as sporadic or hereditary as part of the multiple endocrine neoplasia syndrome type 2 (MEN 2) [98]. MEN 2 and familial MTC patients have germline mutations in *RET*, and sporadic MTCs often exhibit somatic mutations in the same gene [98, 99]. The somatic *RET* mutations are usually found in codon 918, and have been proposed as a poor prognostic marker [100]. Other molecular genetic alterations are relatively rare events in MTC [22, 88]. These include mutations in the glial cell line-derived neurotrophic factor receptor alpha 1 (*GFRA1*) encoding the co-receptors of RET as well as signal transducer and activator of transcription 1 (*STAT1*). In addition, DNA polymorphisms are described in aurora kinase A 1 (*AURKA1*), B-cell CLL/lymphoma 2 (*BCL-2*), cyclin-dependent kinase inhibitor 2B (*CDKN2B*), cyclin-dependent kinase 6 (*CDK6*) and catechol-O-methyltransferase (*COMT*) that are associated with RET downstream effectors, as well as in artemin (*ARTN*), glial cell derived neurotrophic factor (*GDNF*), neurturin (*NRTN*), and persephin (*PSPN*) which encode RET ligands [98].

MTC is a biologically aggressive tumor, showing metastases to neck lymph nodes in at least 50% of cases [101, 102]. Surgical treatment of MTC demonstrates good results for the majority of cases, but many patients remain with detectable disease despite surgery as judged from post-operatively elevated levels of calcitonin. MTCs do not response to RAI therapy, as they are C-cell derived. Therefore, in cases with distant metastases, chemotherapy is sometimes indicated. Similarly to PTC, the therapeutic strategy for MTC today is also molecular-orientated. Inhibitors of vascular endothelial growth factor receptor (VEGFR), the epidermal growth factor receptor (EGFR), and the RET tyrosine kinase are most commonly administrated to patients with MTC, sometimes with a striking response [103]. The prognosis of MTC is worse than for the well-differentiated cancers, with an overall survival rate of 75% [98].

2 AIMS

The overall aim of this thesis was to identify molecular changes and features in sporadic and radiation-associated papillary thyroid carcinomas and to evaluate their diagnostic role.

The specific goals of the thesis were the following:

- To characterize specific molecular features of post-Chornobyl papillary thyroid carcinomas from an Ukrainian adult cohort (Study I).
- To characterize the specific expression of S100A6 identified by proteomics and evaluate it as a diagnostic marker for papillary thyroid carcinoma (Study II).
- To identify proteins differentially expressed in follicular and papillary thyroid tumors using a proteomics approach (Study III).
- To identify and evaluate proteins expressed by the cystic variant of papillary thyroid carcinoma by protein profiling of fluid accumulated in benign and malignant thyroid cysts (Study IV).

3 MATERIALS AND METHODS

To achieve the aims of the present thesis, several cohorts of patients were identified and samples collected at institutions in Sweden and Ukraine for further molecular analyses.

3.1 PATIENTS AND SAMPLES

Clinical information and tumor specimens (formalin fixed paraffin embedded (FFPE) and/or fresh frozen) were retrieved and collected for each study.

3.1.1 Post-Chornobyl PTC (Studies I, II, III)

Totally 70 patients were identified in Kyiv City Teaching Endocrinological Center, Ukraine. These individuals were operated on for PTC during 2004-2008. All these patients were born between April 26, 1967 and January 1, 1987; they were therefore exposed to radiation due to the accident at the Chornobyl nuclear power station in Ukraine in 1986 and lived in the most heavily contaminated regions Kyiv, Chernihiv or Zhytomyr [30]. Exact data of radiation dosage were not available.

Clinical data for this cohort were retrieved from the archived medical records. FFPE tissue samples were collected for all cases. In addition, all cases were reviewed for diagnosis, tumor representativity and presence of chronic lymphocytic thyroiditis (CLT). Also, specimens of normal thyroid tissue, goiter and FTA were obtained at the same institution and included as references for immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH) analyses in Study I.

3.1.2 A panel of follicular-cell derived thyroid tumors for protein expression profiling (Studies II and III)

Fresh frozen tissue samples were obtained from patients who were operated on for thyroid neoplasms at the Department of Breast and Endocrine Surgery at the Karolinska University Hospital, Stockholm, Sweden. The tumor series included PTC (n = 10), FTC (n = 9) and FTA (n = 10). Macro- and microscopically normal thyroid specimens (n = 8) were collected from thyroid lobes of the patients who underwent radical surgical treatment for unilateral thyroid tumors. Corresponding FFPE samples were obtained for IHC. All samples were retrieved under permission of

Karolinska Biobank. Additionally anonymized FFPE samples of normal tissue of stomach, liver, pancreas, spleen and thyroid gland were used as positive and negative controls for IHC.

3.1.3 Cystic thyroid lesions (Study IV)

Fourteen samples of cystic thyroid neoplasms were collected at the Department of Pathology-Cytology at the Karolinska University Hospital during 2011-2013. After histopathological revision, 7 of these samples were classified as cystic PTCs and 7 as benign thyroid neoplasms (6 cystic nodular colloid goiters and one cystic FTA).

Clinical data were retrieved from local medical databases and from pathology reports. Fresh frozen specimens of cystic fluid as well as FFPE tissue samples for the sample group were collected for protein profiling, Western blot analysis or IHC. Additionally, cystic fluid and tissue samples from ATC and parathyroid adenoma were collected and served as controls.

3.2 METHODS

To achieve results and conclusions, different research approaches have been used, including experiments at the molecular, genetic and proteomic levels. The results were subsequently evaluated against the various clinical features of the patients included.

3.2.1 Western blot analysis

Western blot analysis is an analytical method for detection of proteins based on the reaction of an antibody with an antigen. The isolated proteins are loaded to a polyacrylamide gel for a one-dimensional electrophoresis [104, 105]. During the electrophoresis proteins that migrate are separated according to their molecular weight. The proteins are then transferred to nitrocellulose or polyvinylidene difluoride membranes with properties to bind proteins. However, such membranes bind all proteins, including antibodies; thus, the membrane must be blocked by non-fat milk solution to avoid non-specific binding. After the blocking, membranes are incubated with primary antibodies designed against the target protein. Enzyme-linked secondary antibodies are then applied which bind to species-specific portions of the primary antibody, resulting in accumulation of several molecules of the secondary antibody at

one antigen site to enhance the intensity of signal upon incubation with a chemiluminescent agent. The latter is applied to the membrane, cleaved by an enzyme (*e.g.* horseradish peroxidase) and the product of the reaction generates a luminescence signal. The signal is visualized on film or by using a scanner.

Western blot was performed in **Study II-IV** for verification of identified proteins.

3.2.2 Immunohistochemistry (IHC)

IHC is an analytical method for identification and evaluation of protein expression through an antibody-antigen reaction using a direct or indirect method (Fig. 6) [106]. Most commonly, IHC is applied to FFPE sections, but the method may also be used for fresh-frozen tissue specimens. The initial step in indirect IHC is deparaffinization of sections in 100% xylene followed by rehydration in graded series of ethanol and deionized water to replenish the fluid previously removed during the tissue fixation and embedding. The antigen retrieval is performed by incubation of sections in citrate buffer to bind calcium ions, which might distract the binding between the primary antibody and the target protein. Antigen retrieval is performed at high temperature (95-99°C) to unmask the epitopes being hidden during formalin fixation of the tissue and to increase the accessibility of target protein to the antibody. After the antigen retrieval, the sections should be pre-treated in hydrogen peroxide to

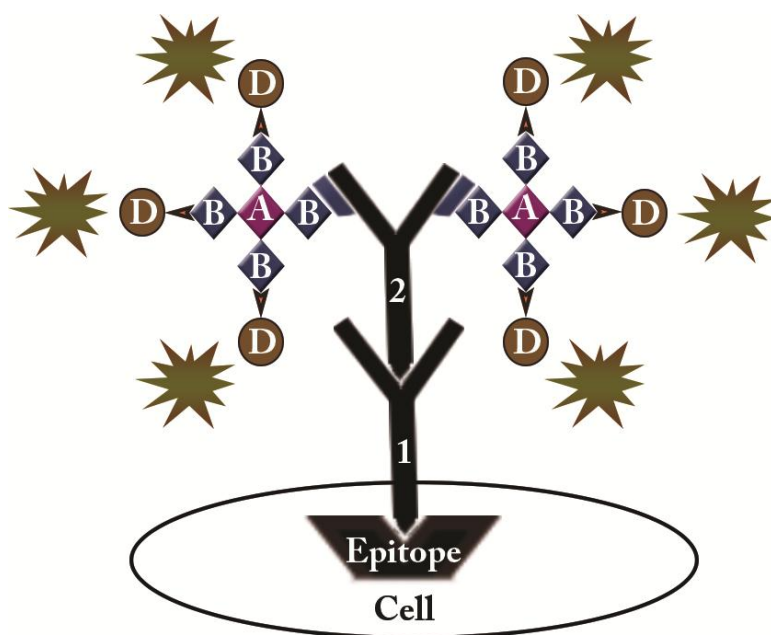


Figure 6. Illustration of the main steps of an indirect immunohistochemistry (ABC-technique). A primary antibody (1) binds to the epitope followed by binding with the secondary biotinylated antibody (2) and avidin-biotin complexes (A, B) are applied. Incubation with diaminobenzidine (D) results in the generation of brown color.

block the endogenous peroxidase, which is a potential cause of non-specific background and false positive staining. Non-specific binding of the primary or secondary antibodies is avoided by blocking sections with excessive protein solution such as bovine serum albumin (BSA). The primary antibody is added to the slides and binds to the target epitope followed by application of the secondary biotinylated antibody, exhibiting high affinity to avidin, which is a component of the avidin/biotin complex (ABC). The ABC solution is added to the section's surface to bind to the secondary antibody through interaction between free avidin molecules and biotin in the secondary antibody (Fig. 6).

It should be noted that other avidin molecules in the ABC are coupled to biotinylated horseradish peroxidase resulting in abundant concentration of enzyme at the epitope site, which increases the intensity and sensitivity of the signal upon addition of substrate. The most widely used chromogen substrate diaminobenzidine (DAB) is applied on section surface and forms a brown-colored polymeric oxidation product at the ABC site. The final steps of IHC are dehydration of sections in rising concentrations of ethanol and 100% xylene followed by counterstaining in hematoxylin.

Indirect IHC was performed in **Studies I-IV** for detection of protein expression and distribution in tumor and normal thyroid tissues.

3.2.3 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a technology based on repeated amplification of certain region(s) of DNA, resulting in generation of multiple copies of that region, which can be analyzed using various detection approaches. PCR can be performed using different methods including hot start and reverse-transcribed PCRs, which are still based on the principle of the classical PCR, using a set of forward and reverse primers.

3.2.3.1 *Hot start PCR*

Genomic DNA is used as a template that should be added to the PCR master mix solution (DNA polymerase, primers, deoxynucleotides (dNTPs) dATP, dCTP, dGTP, dTTP), Mg^{2+} , optimized PCR buffer) and loaded to the PCR machine (Fig. 7). The PCR program is started by a DNA polymerase activation step (the "hot start") which is basically 10-15 minutes incubation of samples at a high temperature (90-95°C). At the ambient temperature DNA polymerase has no catalytic activity, thus the possibility of

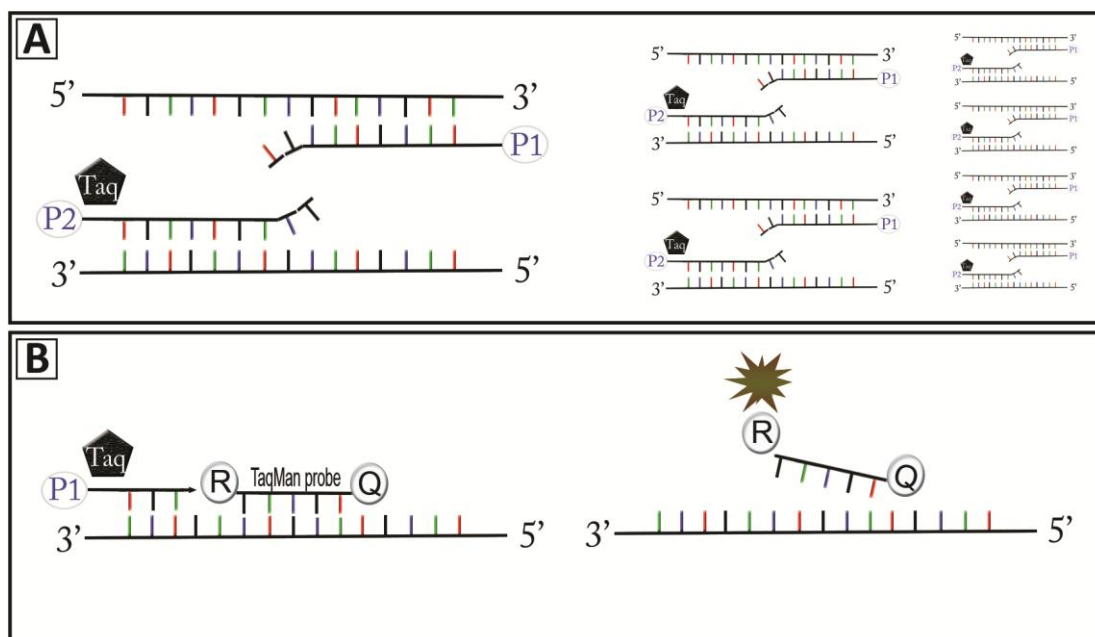


Figure 7. Scheme of polymerase chain reactions. **(A)** The main steps of hot-start PCR. A pair of primers (P1, P2) anneal to a certain region of DNA strands in the presence of DNA polymerase (Taq) resulting in building of multiple copies of DNA. **(B)** Illustration of real time PCR using a TaqMan probe. The TaqMan probe, encompassing quencher dye (Q) and fluorescent dye reporter (R) binds to the complementary part of the DNA, the TaqMan probe is then cleaved by Taq, which results in a light signal due to release of R.

non-specific amplification remains low. The first cycle begins with a denaturation step that disrupts the hydrogen bonds between DNA strands resulting in creation of single strand DNA (ssDNA). Primers anneal to the ssDNA templates in the presence of DNA polymerase catalyzing the addition of dNTPs to the complementary base at the primer-DNA template. The process is repeated for 30-35 cycles; the growth of the template generates a complementary DNA chain, which serves as a new template when the next PCR cycle starts. After elongation, the amount of complementary DNA is increased in geometric progression proportionally to the number of PCR cycles. The final elongation step is performed at 70-75°C for 5-10 minutes after the last cycle to complete the DNA elongation.

Hot start PCR was performed in **Studies I-II** as a part of Pyrosequencing and automated sequencing experiments for identification of *BRAF* mutations.

3.2.3.2 Real time PCR (RT-PCR)

Real time PCR (RT-PCR) is a method for qualitative and quantitative evaluation of certain DNA regions [107]. RT-PCR requires mRNA to be used as an initial material

which is converted to biochemically stable cDNA by using reverse transcriptase and random hexamer primers. In the current study RT-PCR was performed by using RT-PCR master mix containing a DNA polymerase, unlabeled primers, uracil-DNA glycosylase, deoxynucleotides triphosphates (dNTPs) with dUTP, optimized buffer components and a TaqMan probe (Fig. 7). The TaqMan is a molecule with a unique structure due to the presence of a reporter with fluorescent dye on the 5' end as well as non-fluorescent quencher dye on the 3' end of the probe. The reaction is started by initial denaturation of DNA polymerase at 95°C followed by primer annealing to the cDNA template, whereas the TaqMan probe hybridizes to the complementary cDNA sequence between two unlabeled primers. During PCR progression, the growing of DNA is catalyzed by DNA polymerase through extension of the unlabeled primers. The TaqMan probe is cleaved as soon as it is reached by DNA polymerase resulting in releasing of reporter from the quencher and production of a fluorescent light signal. Each cycle of RT-PCR is accompanied by the release of more reporters and by increased fluorescent intensity of the signal which is proportional to the number of released fluorophores. The produced light is detected using a charge-coupled device (CCD) camera at the RT-PCR machine and is visualized as amplification curves in the raw data output.

The RT-PCR method was used in **Study I** for identification of *RET/PTC1* and *RET/PTC3* rearrangements.

3.2.4 DNA sequencing

DNA sequencing is a technology for determination of the nucleotide sequence in a certain region of DNA or even the entire genome (Fig. 8). This method is widely used for detection of genetic alterations such as mutations, rearrangements, deletions etc. DNA sequencing is based on the chain termination chemistry developed by Frederick Sanger, which brought him the Nobel Prize for a second time [108].

A certain region of DNA is amplified by hot start PCR followed by purification of PCR products, and elongation using a single primer and DNA polymerase, initiating further incorporation of dNTP to the complementary base at the PCR amplicon. Small proportions of dideoxynucleotides (ddNTPs) are added to the reaction. These molecules exhibit similar properties as the normal dNTPs, but have a different structure due to inclusion of hydrogen at 3' position instead of a hydroxyl group and presence of fluorescent dye corresponding to one of the four bases (ddATP – green, ddCTP – blue, ddGTP – black, ddTTP – red). Therefore, the DNA chain is growing until incorporation

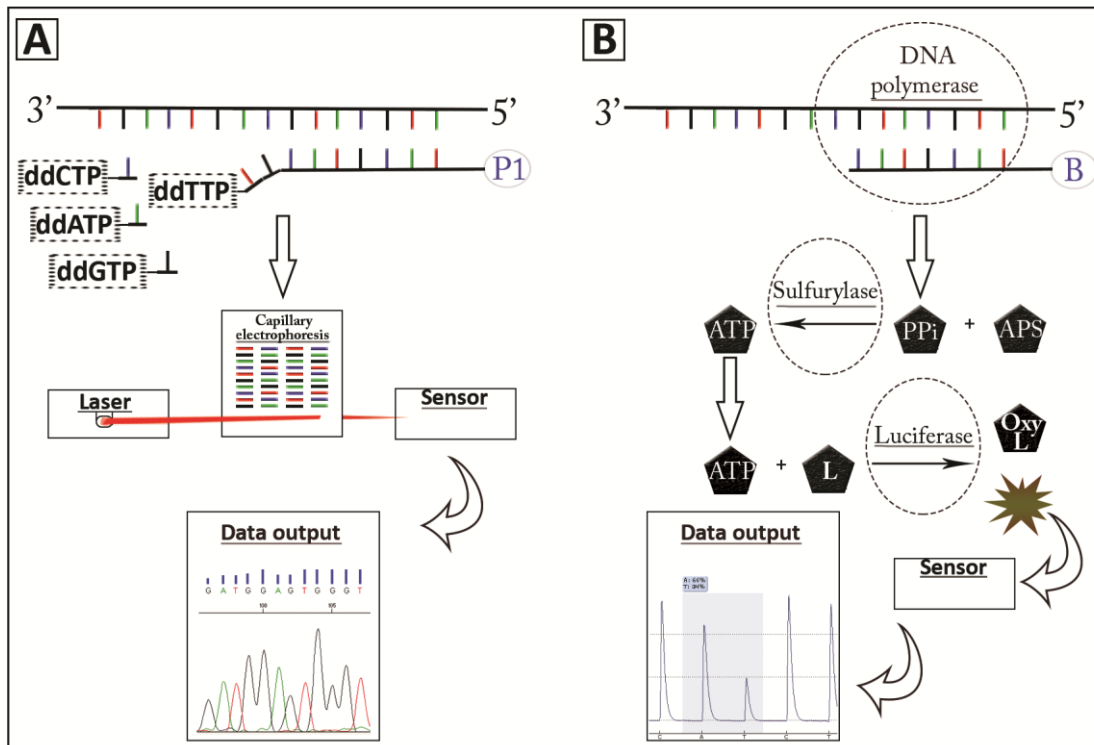


Figure 8. Illustration of main principles of (A) Sanger DNA sequencing and (B) Pyrosequencing.

of ddNTPs occurs and it is then terminated. Due to low proportion of ddNTPs compared to normal dNTPs, the chain termination occurs randomly resulting in production of fragments with different lengths. When the sequencing reaction is completed, the samples are loaded to an automated DNA sequencer for further fractionation in polyacrylamide gel by capillary electrophoresis. Due to varying lengths, the DNA amplicons migrate in strict order from shorter to longer fragments, thus fluorescent colors as well as intensity of the signal are easily recorded by the computerized system and subsequently seen as multicolored peaks in the data output.

This method was used to determine common mutations in exons 11 and 15 of *BRAF* in **Study II**

3.2.5 Pyrosequencing

Pyrosequencing is a technology based on the principle of sequencing by synthesis, which was developed at the Royal Institute of Technology, Stockholm [109]. The simplicity and high sensitivity of Pyrosequencing has been demonstrated for FFPE tissue samples in many studies, including investigations of PTC [110, 111].

The main principles of Pyrosequencing are illustrated in Figure 8. First, a FFPE tissue sample undergoes DNA isolation followed by amplification of the specific segment. One of the PCR primers is biotinylated resulting in biotinylation of the specific amplified DNA strand, which serves as template for further Pyrosequencing. The biotinylated PCR amplicon is bound to streptavidine beads (molecules with a high affinity to biotin) resulting in isolation of biotinylated DNA strands. The sequencing primer hybridizes to the single-stranded PCR amplicon followed by incubation with several enzymes including DNA polymerase. Eventually, the reaction starts when the first dNTP is added in the presence of DNA polymerase. The DNA polymerase initiates further incorporation of dNTP to the complementary base in the PCR amplicon resulting in release of pyrophosphate (PPi) in a quantity equimolar to the amount of incorporated nucleotide. Each released PPi reacts with both ATP sulfurylase and its substrate adenosine phosphosulphate (APS) resulting in PPi conversion to adenosine triphosphate (ATP). The ATP molecule acts as fuel to the luciferase-mediated luciferin-to-oxyluciferin conversion that generates light signals in amounts proportional to the number of consumed ATP. The produced light is detected by a CCD-camera followed by computerized analyses resulting in output of peak trace or a Pyrogram. The height of each peak depends on the amount of incorporated nucleotides, *e.g.*, if three dNTPs were successfully incorporated to complementary bases at ssDNA it would generate three PPi followed by generation of three ATP molecules. The triple intensity of the light signal would as a result be detected by the CCD-sensor in the end, if compared to only one dNTP molecule incorporated.

Pyrosequencing was performed in **Study I** to identify the *BRAF* 1799T>A mutation.

3.2.6 Fluorescence *in situ* hybridization (FISH)

Fluorescence *in situ* hybridization (FISH) is a molecular cytogenetic technology for identification of specific DNA sequences, applied for the detection of rearrangements, translocations, amplifications or to determine DNA copy numbers. In the current study, slides with FFPE sections were used as templates [112]. After deparaffinization and rehydration, sections underwent heating in 2-[N-morpholino]-ethanesulphonic buffer followed by protease digestion by pepsin and incubation in denaturation solution to maximize tissue permeability. The DNA probe is a fluorophore labeled molecule with homology to a certain DNA sequence. FISH was performed using a dual-color assay encompassing two types of DNA probes: one

designed to detect a target DNA sequence showing red signals, while the second was targeted to the chromosome centromere serving as a control and providing green signal. The probes were applied directly to denaturized tissue sections and were incubated overnight. After the incubation, samples underwent washing, dehydration in graded series of ethanol followed by application of fluorescence mounting solution and cover slips. Further visualization and scoring of fluorescence signals was performed by microscopy.

FISH was performed in **Study I** to evaluate *CCND1* (cyclin D1) copy numbers in post-Chornobyl PTC samples.

3.2.7 Mass spectrometry

Proteomics is the science about proteins, their expression, functions and structures. Mass spectrometry (MS) is used to identify proteins expressed in a biological substrate at a certain point of time [113, 114]. The MS data is obtained by using a mass spectrometer, which is a device composed of an ion source (*e.g.* matrix, chip array), an ion accelerator (*e.g.* electric field generator), a detector of mass-to-charge ratio (m/z) and a detector determining amount of ionized biological molecules (Fig. 9).

3.2.7.1 MALDI-TOF-MS

Matrix-assisted laser desorption/ionization (MALDI) is an ionization MS method, in which proteins or other biological analytes (*e.g.* peptides) are embedded in a matrix compound that has the ability to absorb the laser energy and subsequently ionize the analyte [114-116].

The ionized molecules undergo separation in the mass analyzer. In the current project the time-of-flight (TOF) mass analyzer was used, which is based on the principle that molecules with different mass need different time to travel a given distance under the condition that they are charged with the same kinetic energy by the electro-magnetic field (Fig. 9).

Prior to MALDI-TOF-MS, two-dimensional electrophoresis in polyacrylamide gel (2-DE) was applied in the current study. In brief, protein samples are separated by their isoelectric point followed by further separation according to molecular weight and visualization either by colorimetry or with the use of fluorescence dyes. Stained 2-DE gel is scanned and the staining intensities of all protein spots are analyzed across the study samples.

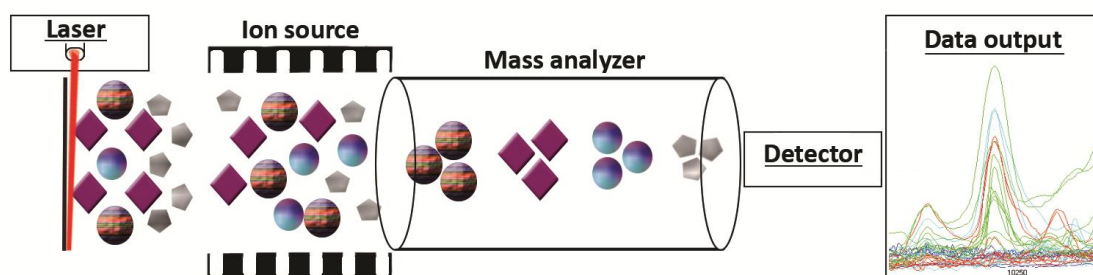


Figure 9. Schematic illustration of mass spectrometry. Biological molecules absorbed the laser energy, are ionized by ion source and subsequently separated according to their mass-to-charge ratio (m/z) at the mass analyzer. M/z is determined by the detector, generating mass-peaks of varying intensity as a data output.

By applying multivariate statistics, protein targets for identification by MS are selected [13, 116]. After excision of gel spots, destaining and trypsin digestion, the isolated peptides are combined with a matrix (*e.g.* alpha-cyano-4-hydroxycinnamic acid). This mixture is loaded to a mass spectrometer, where the peptides are ionized under the action of a laser beam. M/z is detected by the detector resulting in the final data output as a list of peptide molecular masses. This list is matched against established databases of *in silico* digested proteins (NCBI was used in this work) aiming at identifying the protein that each gel spot most probably corresponds to by matching up the theoretical peptide masses with the experimentally observed [117].

MALDI-TOF-MS was applied for protein identification in **Study III**.

3.2.7.2 SELDI-TOF-MS

Surface enhanced laser desorption/ionization time-of-flight MS (SELDI-TOF-MS) is a modification of MALDI-TOF-MS. The main distinctive feature between these two techniques is that the analyte (often consisting of proteins, but not peptides) in SELDI-TOF-MS is applied on a chip array containing chemically active surfaces exhibiting chromatographic properties (*e.g.* hydrophobic, hydrophilic, anion exchange, etc.). Depending on those properties, different analytes are bound on the chip surface. All non-specifically bound molecules, along with other undesired contaminants, can then be washed away, which is a separation step decreasing the complexity of the specimen. Besides that, this step allows the enrichment of low-abundant molecules in the analyte sample. The rest of the procedure, *i.e.* matrix application, generation and detection of ions as well as m/z analysis, is carried out according to the principles of MALDI (Fig. 9) [14, 114, 118]. A mass spectrum with “peaks” and “valleys”

constitutes the output data, which is collected and analyzed by specialized software. However, since intact proteins are analyzed, only the m/z of the proteins, but not their identity, can be evaluated.

SELDI-TOF-MS was applied in **Study II**, where MS spectra were collected using Ciphergen ProteinChip Software and peak clusters across the study groups were revealed and analyzed by Ciphergen Express software package.

3.2.7.3 LC-MS/MS

Liquid chromatography tandem mass spectrometry (LC-MS/MS) is used for identification of proteins by physical separation of peptides using chromatography and their further analysis by MS [113].

Before the LC-MS/MS procedure, all protein samples are digested using trypsin and subsequently labeled by applying isobaric tags for relative and absolute quantitation (iTRAQ) to enable quantification. iTRAQ contains reporter ions with a certain molecular weight (range 113-121 Da in **Study IV**), which can be differentiated in the MS/MS spectra. iTRAQ labeled peptide samples are pooled in a tube, undergo isoelectric focusing (IEF) in an immobilized pH gradient gel and are eluted into 72 fractions. The plate with 72 fractions of iTRAQ labeled peptides is then applied to LC-MS/MS, in which electrospray ionization (ESI) is used as the ion source. In tandem mass spectrometry (MS/MS) peptides are fragmented and generating their sequence information on the amino acid level [116]. Protein identification and peptide quantification is performed by using various software tools for LC-MS/MS data analysis [119].

LC-MS/MS was performed in **Study II** for validation of post-translational modifications of S100A6 and in **Study IV** for protein profiling of fluid from benign and malignant thyroid cysts.

4 RESULTS AND DISCUSSION

4.1 STUDY I. Molecular characterization of post-Chornobyl PTCs

In this study attempts were made to identify molecular and IHC characteristics of the cohort of PTCs from 70 Ukrainian patients with a history of irradiation as well as their correlations to clinical features.

By using Pyrosequencing and RT-PCR, 46 (66%) cases were found to carry a *BRAF* 1799T>A mutation (Fig. 10) or *RET/PTC* rearrangement (*RET/PTC1* or *RET/PTC3*). Out of these 46 PTCs, *BRAF* 1799T>A was detected in 26 cases, *RET/PTC1* in 20 cases and *RET/PTC3* in 4 cases. Moreover, *BRAF* 1799T>A mutation co-existed with *RET/PTC* rearrangements in 4 cases. Further analysis of the cohort revealed significant underrepresentation of *BRAF* 1799T>A in PTC accompanied by chronic lymphocytic thyroiditis (CLT) as compared to PTC only (12% vs. 44%). Proliferative index measured by Ki-67 using MIB-1 was low (mean 0.8%). Elevated expression of cyclin A was observed in PTC with a tumor size larger than 2 cm as compared to PTC less than or equal to 2 cm (1.2% vs. 0.6%). Immunostaining for Bcl-2 and cyclin D1 showed frequent expression for both target proteins, however no association with the clinical parameters was revealed.

In this study, a comparably large cohort of patients operated on for PTC as adults, but exposed to radiation as children or adolescents due to the Chornobyl accident in 1986, was investigated. Overall, the findings demonstrate that the clinical features were not significantly different when compared to similar features in cohorts of PTC without history of irradiation, judging by the results of other series [49, 86]. A *BRAF* 1799T>A mutation was identified in 37% of cases, which is consistent with findings in sporadic PTC [24, 120]. Moreover, a significantly lower level of *BRAF* 1799T>A in the cases of PTC/CLT as compared to PTC without CLT was found. Considering the frequent association of *BRAF* mutation with more aggressive phenotype of PTC [47, 87, 121], the absence of this molecular abnormality in PTC/CLT may indirectly indicate a better prognosis for patients with PTC accompanied by CLT, which is also in agreement with other studies [122, 123]. The rearrangement *RET/PTC1* was found in 29% and *RET/PTC3* in 6% of cases, which is in agreement with the results from other studies of *RET/PTC* [48, 85]. However, these findings are different from results of post-Chornobyl childhood PTC investigations, where a much higher frequency of *RET/PTC3* was demonstrated [23, 86]. Moreover, *RET/PTC1* was five times more

frequent than *RET/PTC3*, which is in contrast to other reported post-radiation PTC cohorts, but in agreement with studies of sporadic PTC (Table 1) [53]. Although *RET/PTC1* and *RET/PTC3* are relatively frequent molecular events, their connection to prognosis of PTC remains controversial. A worse prognosis for patients with PTC was reported in cases harboring *RET/PTC3* alone or accompanied by the *BRAF* 1799T>A mutation [83, 121]. However other studies found no correlation between *RET/PTC* and aggressive clinico-pathological features of PTC [24, 124].

The present study describes a significantly elevated expression of cyclin A in PTC larger than 2 cm in greatest diameter. Although cyclin A is a prognostic marker for breast cancer [65], its role in thyroid tumorigenesis is not fully investigated. In some other studies, this protein showed an over-expression in PDTC and ATC indicating a possible role in de-differentiation of thyroid carcinoma [125].

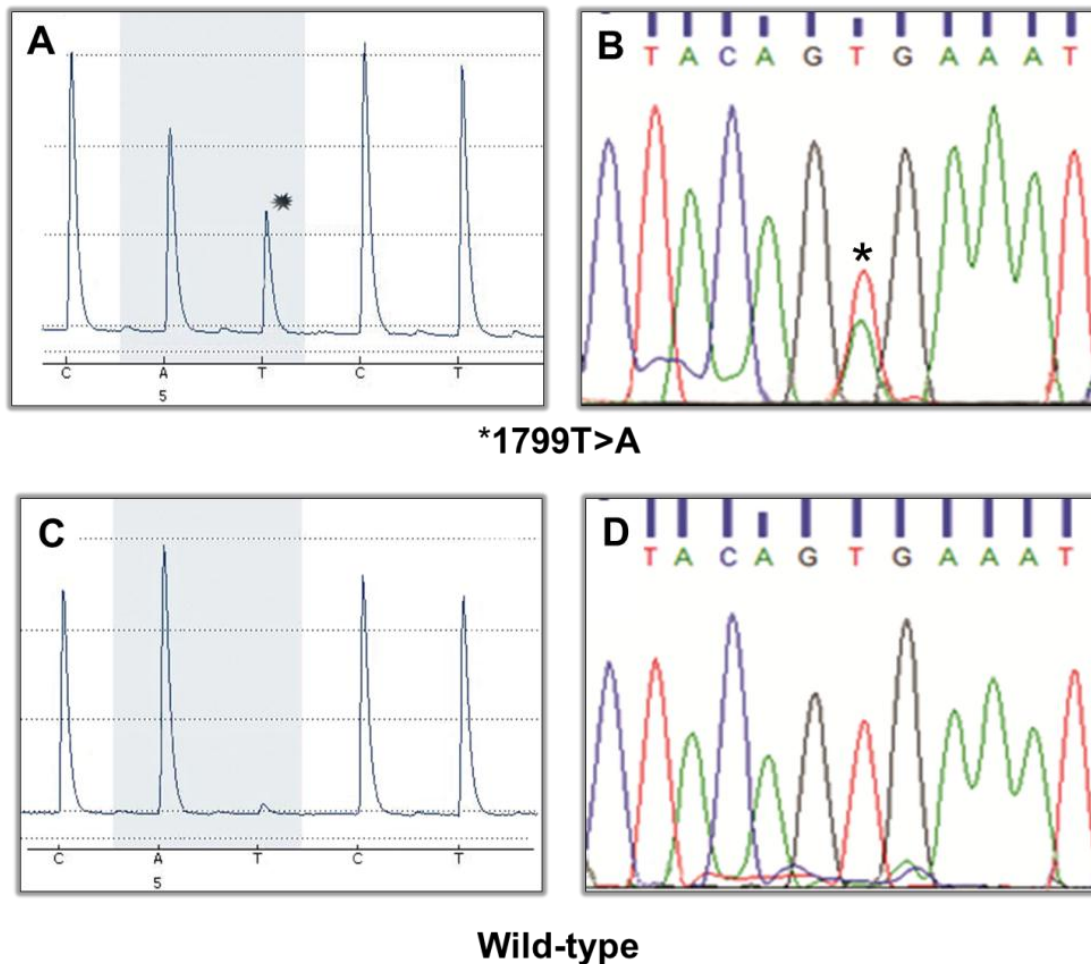


Figure 10. Demonstration of a *BRAF* 1799T>A mutation in exon 15 (marked by asterisks) by applying: (A) Pyrosequencing; (B) direct DNA sequencing. (C, D) Position 1799 of wild-type *BRAF* analyzed by the same methods.

Table 1. Comparison of molecular alterations between the published series of sporadic and radiation-induced PTC.

PTC cases no.	Area of exposure	Age at Exposure mean (range) years	Age at Surgery mean (range) years	BRAF mutation no.	RET/PTC1 no.	RET/PTC1 or 3 rearrangement RET/PTC1 RET/PTC3 no.	Total no.	MIB-1 index mean (range), % nuclei	Reference number
<i>Post-radiation PTC cases</i>									
70	Ukraine	10 (<1 - 18)	30 (19 - 39)	26 (37%)	20 (29%)	4 (6%)	24 (34%)	0.8 (0.05 - 4.5)	26*
12	France	13 (6 - 24)	38 (20 - 61)	1 (8%)	1 (8%)	2 (17%)	3 (25%)	-	126
30	USA	3 (0 - 16)	29 (10 - 59)	1 (4%)	-	-	26 (87%)	-	48
27	Ukraine	<16	14 (8 - 16)	1 (4%)	-	-	12 (45%)	-	86
55	Belarus, Ukraine	<17	- (12 - 31)	2 (4%)	6 (11%)	26 (47%)	32 (58%)	-	23
34	Ukraine	6 (1 - 17)	19 (13 - 30)	4 (12%)	5 (15%)	9 (26%)	14 (41%)	-	127
33	Ukraine	<17	24 (>15)	8 (24%)	-	-	12 (36%)	-	128
15	Ukraine	<17	14 (<15)	0	-	-	5 (33%)	-	128
<i>PTC cases without previous radiation</i>									
28	-	-	-	4 (14%)	-	-	-	-	129
107	-	-	45 (14 - 77)	31 (29%)	24 (22%)	5 (5%)	29 (27%)	-	130
55	-	-	-	16 (29%)	10 (18%)	6 (11%)	16 (29%)	-	131
60	-	-	39 (20 - 77)	24 (40%)	4 (6.5%)	5 (8%)	9 (15%)	-	24
61	-	-	54	-	1 (1.6%)	2 (3%)	3 (5%)	-	85
10	-	-	43 (25 - 97)	5 (50%)	-	-	-	-	14
54	-	-	- (<45 - >45)	42 (78%)	1 (1.8%)	4 (7%)	5 (9%)	-	121
169	-	-	- (<45 - >45)	-	40 (23.7%)	5 (3%)	45 (27%)§	-	53
18	-	-	49 (36 - 63)	-	-	-	-	1.7 (0.1 - 3.8)	132
30	-	-	62 (27 - 80)	-	-	-	-	1.9 (0.3 - 11.8)	35
185	-	-	49 (12 - 94)	-	-	-	-	2.9 (0-40)	37
108	-	-	- (<35 - >55)	-	-	-	-	(>1 - <10)	133
371	-	-	49 (17 - 83)	-	-	-	-	(<1% - >5%)	134

Comments: "- " = not analyzed, not available or not applicable; " * " – Study I; " § " – in this study 3 cases showed both RET/PTC1 and 3 rearrangements.

4.2 STUDY II. Up-regulation of protein S100A6 in PTC

In this study a proteomics approach was applied to identify and evaluate potential diagnostic markers of PTC.

SELDI-TOF-MS for cytosolic protein fractions revealed 155 peak-clusters in PTC and follicular tumors. Among these, a peak-cluster with m/z of 10.2 kDa was identified to be significantly different in PTC when compared to FTA, FTC and normal thyroid samples. An immunocapture experiment followed by SELDI-TOF-MS confirmed that the identified 10.2 kDa peak-cluster corresponded to the S100A6 protein. Moreover, cysteine and glutathione modifications of S100A6 were validated using immunoprecipitation followed by LC-MS/MS. Significant over-expression of S100A6 was confirmed by IHC and Western blot. Furthermore, 10 PTC samples were screened for mutations in *BRAF* to investigate any possible connection between this alteration and S100A6 expression levels. By using direct sequencing, *BRAF* was shown to be wild-type in exon 11 in all PTC, but mutated in exon 15 in 5 cases (Fig. 10). However, this was not correlated with S100A6 expression levels.

Significant over-expression of S100A6 was detected in PTC as compared to follicular neoplasms and normal thyroid tissue, which is consistent with studies of other malignant neoplasms [74, 135-138] and with studies describing the role of S100A6 in thyroid tumors [70, 139, 140]. S100A6 and its modifications (cysteinylation and glutathionylation) were determined in PTC by applying SELDI-TOF-MS, which has only been reported in lung tumors [136] and cultured cells [141]. The SELDI-TOF-MS findings were validated by Western blot and IHC, both showing a significantly higher expression of S100A6 in PTC as compared to other benign and malignant thyroid tumors and normal tissue (Table 2). It is worth to note that knowledge about the molecular functions and role of S100A6 in thyroid tumorigenesis was limited at the time of publication. Later on, Nipp *et al* suggested a possible role of S100A6 as a marker of PTC with metastases to lymph nodes in a proteomic investigation of PTC [72]. Similar to Matuzawa *et al* a degradation of β -catenin due to interaction of S100A6 with CacyBP was observed by Ning *et al* [71, 142]. Moreover, other studies determined a negative effect of S100A6 binding to p53-binding domain of ubiquitin E3 ligase MDM2 (an important negative regulator of p53) [73, 143]. Bao *et al* described a role for S100A6 in negative regulation of endothelial cell-cycle progression and cell senescence through the reduction of transcriptional activity of the genes encoding cyclin A and Ki-67 [144].

The results suggest that the S100A6 protein is significantly up-regulated in PTC, which indicates its role in thyroid tumorigenesis. Moreover, identification of S100A6 protein can potentially improve the discrimination between PTC and other thyroid lesions. Further investigation of S100A6 in FNAB material is needed in order to validate its utility as a diagnostic marker in routine clinical practice.

Table 2. The summary of the analyses of fresh frozen thyroid samples for Study II.

Case	Sex	Age	BRAF status	SELDI-TOF-MS peak intensity at 10.2 kDa	Western blot densitometry of S100A6	S100A6 immunohistochemistry		
						Cytosolic staining intensity	Nuclear staining intensity	Proportion stained nuclei
Normal thyroid								
N1	M	33	n/a	2.168	0.266	n/a	n/a	n/a
N2	M	38	n/a	1.918	0.135	n/a	n/a	n/a
N3	F	41	n/a	4.160	0.133	n/a	n/a	n/a
N4	M	56	n/a	4.107	0.139	n/a	n/a	n/a
N5	F	29	n/a	2.609	0.148	n/a	n/a	n/a
N6	M	26	n/a	n/a	0.143	n/a	n/a	n/a
N7	F	47	n/a	1.974	0.148	n/a	n/a	n/a
N8	F	72	n/a	n/a	n/a	0	3	>75%
N9	M	58	n/a	5.186	n/a	n/a	n/a	n/a
N10	F	51	n/a	4.474	0.142	1	2	>75%
Follicular thyroid adenoma								
FTA1	F	67	n/a	3.726	0.396	2	1	<25%
FTA2	F	88	n/a	1.199	0.194	1	2	>75%
FTA3	F	30	n/a	3.854	0.173	2	2	>75%
FTA4	M	36	n/a	1.535	0.176	1	3	>75%
FTA5	M	60	n/a	1.253	0.147	1	2	~50%
FTA6	F	41	n/a	3.580	0.130	1	2	~50%
FTA7	F	37	n/a	2.450	0.137	2	3	>75%
FTA8	F	61	n/a	1.365	0.153	1	1	<25%
FTA9	F	23	n/a	0.299	n/a	0	1	<25%
FTA10	M	41	n/a	3.508	n/a	n/a	n/a	n/a
Follicular thyroid carcinoma								
FTC1	F	52	n/a	1.254	0.161	2	1	<25%
FTC2	M	73	n/a	2.731	0.325	1	1	~50%
FTC3	M	52	n/a	1.714	0.196	2	2	~50%
FTC4	F	45	n/a	3.082	0.180	1	2	~50%
FTC5	F	52	n/a	19.656	n/a	3	2	~50%
FTC6	F	17	n/a	1.072	0.140	1	2	<25%
FTC7	M	65	n/a	1.167	0.133	1	1	~50%
FTC8	F	64	n/a	1.765	0.131	2	1	~50%
FTC9	F	14	n/a	3.771	0.167	1	2	~50%
Papillary thyroid carcinoma								
PTC1	F	38	BRAF 1799T>A	10.825	0.914	3	3	>75%
PTC2	F	68	BRAF 1799T>A	12.117	0.791	3	2	~50%
PTC3	M	48	wt	3.525	n/a	3	2	>75%
PTC4	F	39	wt	9.876	0.790	3	2	>75%
PTC5	M	33	wt	6.848	0.345	1	1	<25%
PTC6	F	97	BRAF 1799T>A	15.581	0.872	2	1	>75%
PTC7	F	45	wt	11.187	n/a	1	2	>75%
PTC8	F	48	wt	6.996	0.253	2	3	>75%
PTC9	M	25	BRAF 1799T>A	7.729	0.752	2	2	>75%
PTC10	F	32	BRAF 1799T>A	11.231	0.884	2	2	>75%

Comments: wt = wild-type BRAF; n/a = not analysed

S100A6 immunohistochemistry staining 0 = absent; 1 = weak; 2 = moderate; 3 = strong

4.3 STUDY III. Protein expression profiling of thyroid tumors

In this study proteomics analyses were performed to identify possible candidates as diagnostic markers for the discrimination between FTA and FTC as well as between FTC and PTC.

Cytosolic protein fractions partially overlapping from the sample series in Study II were used. Follicular thyroid tumors, PTC and normal thyroid tissue were investigated using gel-based proteomics coupled to MS. By using multivariate statistics (Partial Least Squares Discriminant Analysis or PLS-DA), two predictive models were built for the discrimination between FTA-FTC and FTC-PTC respectively. Out of 800 protein spots observed in 2-DE gel, 25 were included in the FTA-FTC model, while the FTC-PTC model consisted of 19 spots (all overlapping to those from the FTA-FTC model). By matching the MALDI-TOF-MS data with the NCBI database all 25 protein identities could be obtained. Nine of these were selected for further analyses: 14-3-3 (isoforms β , ϵ , and ζ), annexin A5 (ANXA5), tubulin alpha 1b (TUBA1B), peroxiredoxin 6 (PRX6), α 1-antitrypsin precursor, selenium-binding protein 1 (SELENBP1), and protein disulfide-isomerase (PDIp). Expression patterns of these proteins were validated by Western blot in four samples from each study group. Expression patterns of ANXA5 and 14-3-3 were even validated by IHC in all and additionally in the post-Chornobyl PTC samples described in Study I. In IHC experiments, cytoplasmic expression patterns of 14-3-3 were observed in 33% of FTA, 67% of FTC, and 80% of PTC. Moreover, a weak to strong staining pattern of protein 14-3-3 was detected in 65% of post-Chornobyl PTC. Evaluation of ANXA5 by IHC showed positive cytoplasmic staining in all FTA and PTC, and in 89% of FTC. IHC of ANXA5 in post-Chornobyl PTC was positive in 97% (66/68) cases demonstrating weak to strong staining patterns in 50-100% of PTC cells and negatively stained lymphocytes in PTC/CLT sections.

The findings demonstrate significantly different expression patterns of a set of protein spots identified by proteomics profiling in benign and malignant thyroid tumors. Overexpression of ANXA5 was detected in both FTC-FTA and FTC-PTC, which is consistent with findings in other proteomics studies of different malignant neoplasms [145, 146]. A decreased expression of SELENBP1 was found in PTC as compared to reference thyroid samples, which is supported by data from other reports of SELENBP1 expression in PTC as well as in colorectal, lung and ovarian cancers [139, 147]. Elevated levels of PRX6 in FTC and PTC, but low expression in FTA samples were demonstrated. Although, this enzyme of the cell's antioxidant system was

not observed in other investigations of thyroid neoplasms, overexpression of PRX6 is seen in lung, skin and endometrial cancers suggesting the possible role of PRX6 in cancer progression [148-150]. Decreased levels of PDIp in FTC and PTC were found when compared to FTA and reference thyroid. PDIp is an active β subunit of prolyl 4-hydroxylase playing a role in the regulation of cellular responses to hypoxia through the interaction with hypoxia-induced factors. Thus, the observation of low expression of PDIp in FTC and PTC indicates the ability of cancer cells to survive and proliferate in oxygen-deficient conditions due to deregulation of hypoxia-induced factors. Analysis of ATC and PTC cell-lines showed distinct up-regulation of protein 14-3-3 [151], which is involved in regulation of apoptosis, cell growth and tumor suppression in normal cells but also considered as a sign of poor prognosis [152].

4.4 STUDY IV. Identification of diagnostic markers for cystic PTC by a proteomics approach

In this study the protein content of fluid in benign thyroid cyst and cystic PTC was evaluated in order to identify and validate markers for the discrimination of these entities.

Proteomic profiling using LC-MS/MS was performed for evaluation of protein content in the fluid from benign and malignant thyroid cysts. Analysis of LC-MS/MS data resulted in identification of 1581 proteins, in which 841 contained iTRAQ labels in both pools. Of these, 10 proteins showing significantly differing expression in cystic PTC as compared to controls were identified. Furthermore, CK-19, S100A13, ANXA3 and CMBL were selected for validation by IHC and Western blot, based on the LC-MS/MS data for number of peptides and level of significance. Additionally, HBME-1 expression was evaluated by IHC, because of its overall utility for the diagnosis of PTC in routine clinical settings [55, 61].

IHC for CK-19 showed a moderate-to-strong expression pattern in 100% of cells in all cystic PTC cases, whereas controls were negative ($p < 0.05$). IHC for HBME-1 revealed higher expression in all PTC samples as compared to benign controls, showing few cells with weak immunostaining ($p < 0.05$). IHC for S100A13, CMBL and ANXA3 demonstrated similar staining patterns in all PTC and control samples. By IHC a positive staining of S100A13, CMBL and ANXA3 in normal follicular cells surrounding PTC was identified. Analysis of cyst fluid by Western blot data showed

weak expression of S100A13 in four (57%) cases of PTC, while the remaining PTC samples and all controls did not show S100A13 expression.

A novel approach for discrimination between malignant and benign thyroid cysts by proteomics profiling of cystic fluid was introduced in this study. The large number of proteins was identified by applying LC-MS/MS to the fluid component of cystic thyroid lesions. To the best of my knowledge, this is the first extensive catalog of protein content in fluid from thyroid cysts. Differences in the colors of cystic fluids in the samples were detected, and this finding indirectly suggests differences in the proteome of PTC as compared to benign lesions, which is in agreement with data from biochemical studies of thyroid cysts in a larger sample size (Table 3) [33]. Analysis of the proteomic data resulted in selection of CK-19, S100A13, CMBL and ANXA3 for further validation by IHC and Western blot. It is worth to note that CK-19 has a strong association with classical PTC [61, 62, 153], but has not previously been analyzed in thyroid fluids. Significantly up-regulated levels of CK-19 were found in PTC by LC-MS/MS, and these data were confirmed by IHC. Furthermore, negatively stained normal follicular cells surrounding PTC were observed, indicating a primary role of the PTC cells in secretion of CK-19 [61, 62]. From these data, it is hypothesized that CK-19 can be applied as a pre-operative marker for both classical and cystic PTC in addition to regular FNAC. The findings also support a role of CK-19 in discriminating between PTC and benign thyroid neoplasms.

Table 3. Details of iTRAQ labeling and fluid properties of depleted samples.

Case number	Experiment number	iTRAQ labelling	Protein (mg / ml)	Color of cystic fluid	Viscosity of cystic fluid
cPTC-1	exp 1	116	7	red	liquid
cPTC-2	exp 1	117	2.5	dark	liquid
cPTC-3	exp 2	117	4.7	dark	liquid
cPTC-4	exp 1	118	7.1	red	liquid
cPTC-5 *	exp 2	118	31.4	yellow	liquid
cPTC-6	exp 1	119	38.2	dark	liquid
cPTC-7	exp 2	119	7.4	yellow	liquid
Benign-1	exp 1	114	7.1	red	liquid
Benign-2	exp 2	114	6.7	brown	liquid
Benign-3	exp 1	115	5.5	dark	liquid
Benign-4	exp 2	115	9.8	dark	liquid
Benign-5	exp 1	113	5.1	dark	liquid
Benign-6	exp 2	113	4.3	brown	liquid
Benign-7	exp 2	116	7.2	dark	liquid

Comments: cPTC-5 was excluded from the analysis

Significant overexpression of S100A13 in cystic PTC was identified by LC-MS/MS and also observed by Western blot. However, IHC demonstrated very similar expression patterns: a slight increase with borderline significance in cystic PTC as compared to control samples was observed. Considering that S100A13 has not been investigated in thyroid cyst fluid before, the results imply that this protein can play an important role in discriminating between cystic PTC and benign thyroid cystic lesions, but further validation is warranted.

CMBL is a bioactivating hydrolase showing high expression in human liver and kidney [154], but little is known about the biological role of this protein [155]. Although significantly higher expression of CMBL was detected in PTC when compared to benign cysts by LC-MS/MS, the validation experiments did not show any significant differences. Similar figures were also observed for calcium-dependent phospholipid-binding protein ANXA3 [156]. Although LC-MS/MS data showed overexpression of ANXA3 in cystic PTC, analysis of IHC and Western blot showed similar staining patterns of ANXA3 in all studied thyroid samples, suggesting diverse biological functions of ANXA3 in thyroid cells. However, this result is not consistent with results published by Jung *et al*, who showed down-regulation of ANXA3 in classic PTC, which can indicate different pathophysiological properties between cystic thyroid lesions and classical PTC [157].

The results from **Study IV** suggest that S100A13 and CK-19 can potentially be applied as additional tests to regular FNAC, however further investigation by an alternative approach (*e.g.* ELISA) is needed to determine the distinct role of these proteins in a routine clinical settings. The clinical role of these proteins should be evaluated in a prospective study of an independent tumor material using fluid samples collected by preoperative FNAB.

5 CONCLUDING REMARKS

5.1 GENERAL CONCLUSIONS

- Post-Chornobyl PTC in adults is characterized by frequent *BRAF* 1799T>A mutation and *RET/PTC1* rearrangement, resembling the patterns of other non-radiation induced PTC cohorts rather than radiation induced childhood PTC.
- PTC accompanied by chronic lymphocytic thyroiditis has an underrepresentation of *BRAF* 1799T>A mutation indirectly suggesting different biological aggressiveness compared to PTC without this feature.
- Protein expression profiling is a powerful tool for discovering proteins deregulated in PTC as well as identification of novel diagnostic markers for malignant thyroid neoplasms.
- The protein S100A6 is up-regulated in PTC suggesting its role in discriminating PTC from other thyroid tumors.
- The proteins 14-3-3, PRX6, ANXA5, SELENBP1 and PDIp can potentially be used for the discrimination between various thyroid neoplasms of follicular cell origin.
- The proteins CK-19 and S100A13 are potential diagnostic markers in the cystic variant of PTC, which can be applied to FNAC on cystic fluid in addition to regular methods.

5.2 FUTURE PERSPECTIVES

Molecular alterations play an important role in PTC tumorigenesis and there are plenty of known genetic and epigenetic abnormalities to be associated with unfavorable clinical features. Understanding of how these alterations are involved in the tumorigenesis on a basic level is essential for identification of novel diagnostic and therapeutic targets as well as markers of poor prognosis.

Translation of findings from laboratory bench to bed side is a modern and highly effective research approach, and establishing translational studies is an important task for future investigations of thyroid cancer, not least PTC. Such translational studies may identify novel molecular targets and novel drugs could be evaluated. This could eventually fulfill the promise of a molecule-based, tailored therapeutic strategy resulting in a high response rate, but with low toxicity.

Radiation exposure is a risk factor for development of PTC. 25 years after the Chernobyl, an accident at the Fukushima nuclear plant station in Japan happened, bringing back the issue of radiation exposure and thyroid disease. This accident was local and all important measures were performed, but Fukushima was a new demonstration that nuclear fatalities are possible also today. Thus, understanding of short- and long-term consequences of radiation exposure is still an important topic of thyroid cancer research. To the best of my knowledge, post-Chernobyl PTC was and remains the best available material for basic investigations of radiation related PTC. Although the molecular alterations of childhood post-Chernobyl PTC are well investigated, it is controversial whether the radiation effect of Chernobyl accident becomes less significant nowadays, mainly due to the evidence of the elevated number of newly diagnosed PTC among adults who were children at time of radioactive exposure. Moreover, relation and consequences of the latency period to molecular profiling of post-Chernobyl PTC are not fully investigated either. Little is known about the prognostic factors for post-Chernobyl PTC. Hence, more studies of post-Chernobyl PTC are needed to correlate follow-up data with known molecular abnormalities, verifying those currently used for sporadic PTC (*e.g.* *BRAF* mutation, MIB-1) and/or identifying new prognostic markers.

The diagnostic tools for evaluation of thyroid nodules are still not efficient for all patients. The pre-operative diagnosis of PTC is based on FNAC, which in some cases is difficult to assess due to similar cytological patterns of PTC and follicular thyroid tumors. Although the pre-operative cytological discrimination between follicular thyroid tumors and PTC is highly sensitive and informative in the majority

of cases, a significant number of patients still represent a diagnostic challenge, and application of additional diagnostic markers is needed. Several molecular entities have been proposed and subsequently tested in the clinical setting, without, however, being successfully implemented as independent pre-operative diagnostic markers. Protein profiling of thyroid tumors showed excellent results for the identification of potential markers for PTC. The proteomics approach always reveals a large list of proteins, in which significantly differentially expressed molecules can be identified. Further validation is needed whether or not these proteins may become clinically useful, especially when applied on FNAC. It is a challenge to verify their sensitivity, either based on IHC or PCR methods, as compared to the proteomic methods. The number of proteins with potential value for diagnostic, prognostic and predictive reasons is constantly increasing, and it will be a challenge to design the appropriate approaches for evaluation of these in all different PTC cohorts.

6 АБСТРАКТ (українська)

Папілярний рак щитоподібної залози (ПРЩ) – це найпоширеніше злоякісне новоутворення щитоподібної залози, частка якого становить 80-85% серед інших злоякісних новоутворень даного органу. ПРЩ характеризується різноманітними молекулярно-генетичними аномаліями, що мають відмінності в спорадичних та радіаційно-індукованих карциномах. Слід зазначити, що ПРЩ демонструє високий показник захворюваності серед пацієнтів, вік яких був менше 18 років на момент аварії на Чорнобильській АЕС (ЧАЕС) в 1986 р. та які мешкали на радіоактивно забрудненій території. Діагноз ПРЩ ґрунтується на даних цитологічного дослідження аспіратів тонкогolkової аспіраційної пункційної біопсії (ТАПБ) - високочутливого та специфічного до 90% випадків методу. Проте, ТАПБ малоінформативна при диференціальній діагностиці ПРЩ та фолікулярного раку щитоподібної залози (ФРЩ) з фолікулярною аденомою щитоподібної залози (ФАЩ), через подібність цитологічної картини цих новоутворень. Також, в зразках ТАПБ визначається переважно рідинний компонент та недостатня для цитологічного аналізу кількість фолікулярних клітин при кістозному ПРЩ.

Метою дисертації було визначити молекулярні особливості спорадичного ПРЩ та ПРЩ, пов'язаного з аварією на ЧАЕС; ідентифікувати діагностичні маркери, що можуть бути застосовані на зразках ТАПБ для диференційного діагнозу ПРЩ з іншими фолікулярними новоутвореннями щитоподібної залози.

В Дослідженні I було проаналізовано молекулярні, генетичні та клінічні особливості ПРЩ, діагностованого у 70 дорослих пацієнтів, що зазнали радіоактивного опромінення в дитинстві внаслідок аварії на ЧАЕС. Для визначення мутації в 15 екзоні гена *BRAF* (*BRAF* 1799T>A) було ізольовано геномну ДНК та застосовано Піросіквенсінг. За результатами аналізу пірограм, було визначено 26 (37%) випадків з *BRAF* 1799T>A мутацією. Подальший аналіз когорти виявив, що *BRAF* 1799T>A мутація в 3.5 разів рідше визначалась в випадках ПРЩ на фоні хронічного лімфоцитарного тиреоїдиту, у порівнянні з ПРЩ без супутньої тиреоїдної патології, відповідно у 12% (2/16) та 44% (24/54) випадків, $p < 0.05$. Визначення хромосомних перестановок *RET/PTC1* та *RET/PTC3* було виконано за допомогою полімеразної ланцюгової реакції (ПЛР) в реальному часі з використанням ДНК-зондів TaqMan. Аналіз даних ПЛР виявив переважання випадків з *RET/PTC1*, у порівнянні з *RET/PTC3*, відповідно у 29%

(20/70) та 6% (4/70) випадків. Визначення експресії маркерів проліферації, апоптозу та регуляторів клітинного циклу було здійснено за допомогою імуногістохімії (ІГХ) та флюоресцентної гібридизації *in situ* (FISH) для аналізу ампліфікацій гену, що кодує циклін D1. Аналіз даних показав вищий рівень експресії цикліну A в випадках ПРЩ ≥ 2 см (1.2%), у порівнянні з ПРЩ < 2 см в найбільшому діаметрі (0.6%), $p < 0.05$. Індекс проліферації MIB-1 (Ki-67) був 0.8% та не корелював з клінічними показниками. Висновки **Дослідження I**: частота *BRAF* 1799T>A мутації та перестановки *RET/PTC1* в ПРЩ у дорослих пацієнтів частково відрізняються та частково збігається з даними досліджень інших когорт ПРЩ; довготривале спостереження дозволить визначити прогностичне значення молекулярних аномалій для даної когорти пацієнтів.

В **Дослідженні II** було застосовано поверхнево-підсилену лазерну десорбційно/іонізаційну час-польотну мас-спектрометрію (SELDI-TOF-MS) для визначення та порівняння протеому ПРЩ (n = 10), ФРЩ (n = 9), ФАЩ (n = 10) та нормальної тканини щитоподібної залози (n = 10). Аналіз даних SELDI-TOF-MS виявив мас-пик з молекулярною масою 10.2 кДа, посилена експресія якого була достовірно вища в ПРЩ ($p < 0.05$). Цей мас-пик був визначений як протеїн S100A6 за допомогою імунозахоплення та високо-ефективної рідинної тандемної мас-спектрометрії (LC MS/MS), а його підвищена експресія в ПРЩ була також підтверджена результатами вестерн блоту та ІГХ на основних зразках, а також даними ІГХ на зразках пост-Чорнобильських ПРЩ з **Дослідження I**. Висновки **Дослідження II**: S100A6 може потенційно бути застосований для диференційної діагностики ПРЩ.

В **Дослідженні III** було виконано аналіз протеому фолікулярних пухлин щитоподібної залози та ПРЩ за допомогою двомірного гелевого електрофорезу (2-DE) та матрично-активованої лазерної десорбційно/іонізаційну час-польотної мас-спектрометрії (MALDI-TOF-MS). За допомогою 2-DE були ідентифіковані 25 протеїнових плям, експресія яких достовірно відрізнялась в досліджуваних групах. Серед цих 25, для подальшого аналізу були вибрані 9 протеїнових плям, склад яких відповідав білкам: 14-3-3 (ізоформи β/α , ζ/δ та ϵ), пероксиредоксин 6 (PRX6), аннексин A5 (ANXA5), селен-зв'язуючий протеїн 1 (SELENBP1), протеїн дисульфід-ізомерази (PDIp), α -1B ланцюг тубуліну (TUBA1B), а також попередник α 1-антитрипсину (A1T1), експресія яких була підтверджена за допомогою вестерн блоту, а також з використанням ІГХ для оцінки експресії 14-3-3 та ANXA5. Імуноекспресія 14-3-3 та ANXA5 була також підтверджена на

пост-Чорнобильських зразках ПРЩ з **Дослідження I**. Висновки **Дослідження III**: визначення експресії протеїнів 14-3-3, PRX6, ANXA5, SELENBP1 та PD1p може бути застосовано для диференційної діагностики фолікулярних новоутворень щитоподібної залози, проте необхідне проведення проспективних досліджень на зразках ТАПБ для остаточного визначення діагностичної ролі цих білків.

В **Дослідженні IV** були визначені потенційні діагностичні маркери кістозного ПРЩ (кПРЩ). В якості досліджуваного матеріалу були використані зразки рідини, аспірованої з кПРЩ та доброякісних новоутворень щитоподібної залози (нетоксичного багатовузлового зобу (НБВЗ) та фолікулярної аденоми щитоподібної залози (ФАЩ). Враховуючи високу насиченість зразків кістозної рідини альбуміном та іншими протеїнами, що потенційно можуть перешкодити мас-спектрометрії, було проведено очищення зразків від таких молекул за допомогою деплеції. Деплетовані зразки були мічені за допомогою реагентів iTRAQ, після чого була виконана LC MS/MS. За допомогою даного методу було ідентифіковано 1581 протеїнів, серед яких визначено 10, що мали найвищий показник достовірності та достатню кількість пептидів. Для подальшого аналізу та верифікації експресії цитокератину 19 (СК-19), протеїну S100A13, аннексину A3 (ANXA3) та гомологу карбоксиметилбутенолідази (CMBL) були застосовані ІГХ та вестерн блот.

Аналіз ІГХ та бендів вестерн блоту виявив однакову імуноекспресію протеїнів ANXA3 та CMBL в кПРЩ та контрольних зразках доброякісних новоутворень щитоподібної залози. Також було ідентифіковано достовірно вищу експресію S100A13 в кістозній рідині кПРЩ при аналізі даних вестерн блоту та СК-19 при аналізі ІГХ тканини кПРЩ ($p < 0.05$).

Висновки **Дослідження IV**: було вперше продемонстровані результати аналізу протеому кістозної рідини кПРЩ та доброякісних новоутворень щитоподібної залози; за допомогою LC MS/MS вперше складено та проаналізовано каталог протеїнів, що акумулюються в кістозній рідині новоутворень щитоподібної залози; визначення протеїнів S100A13 та СК-19 може потенційно бути застосовано як додатковий до ТАПБ тест для диференційної діагностики кістозних новоутворень щитоподібної залози та кПРЩ.

7 ACKNOWLEDGMENTS

Apart from the efforts of person, the success of any project depends largely on the encouragement and guidelines of many others. I would like to take the opportunity and express my sincere appreciations to the people who have been supporting and helping me in a different ways during my work at Karolinska Institutet. Without you, this thesis would not have been possible.

Jan Zedenius my main supervisor, who provided an excellent supervision and introduced me to the principles of good research. With immense gratitude I thank you for continued trust and pure interest in my project, always assuming that I did a great job as well as being able to find time for meetings despite your overloaded schedule by endocrine surgery and administrative activity. Your knowledge, encouragement and guidance from the initial to the final level enabled me to develop as a researcher. You were and remain my best role model for a scientist, advisor, and teacher. I have never experienced stress with you, seeing the solutions, but not the problems. I am grateful for unforgettable discussions about science in combination with a good wine at the Molecular-Genetic Wine Seminars, as well as official and unofficial activities we had in Kyiv!

Catharina Larsson my “main” co-supervisor, who provided me the opportunity to learn the art of research and welcomed me to work in the Medical Genetics research group. Thank you for excellent support and help, for your ideas, truly expert advices and time that you spent reading and editing my manuscripts. You are very kind and honest person. You have a lively sense of humor and outstanding analytical mind. Thank you for sharing your expertise how to figure complex things out, to find the best solutions and to develop the ways of pursuing them.

Anders Höög, my co-supervisor and great pathologist who introduced me an amazing world of endocrine pathology. Thank you for enthusiastic attitude, sharing your knowledge and experience and involving me in the cystic PTC project! Despite the pile of clinical cases or vacation time, you always are able to find the time for discussions with me. You have an excellent teaching approach and always willing to help and give the best suggestions in different aspects of research, clinics and life. I thank you for your time during weekends and so many evenings that we spent evaluating IHC or discussing study results.

Anastasios Sofiadis, my co-supervisor. Thank you for excellent guidelines and sharing your experience in pipetting, performing Western Blots and other lab-related things from the first day of my work in the Medical Genetics research group. I am grateful for your patience, expert advises for my studies and introducing me proteomics and mass-spectrometry! You have a creative thinking and positive attitude. With you I learnt that successful pursuing of research project needs significance, confidence, collaboration and hard work.

Mykola Hulchiy, my co-supervisor and one of key people in development of scientific collaboration between Ukrainian and Swedish institutions since early 2006. Thank you for sharing your enthusiasm and knowledge as well as for support in organization of the international workshops in Kyiv.

Tommy Linné, "engine" of Karolinska International Research and Training committee, master of collaboration and main initiator of Swedish-Ukrainian cooperation from Karolinska Institutet side. Thank you for choosing me as a candidate to PhD study at KI in 2007, for your enthusiastic attitude, creativity and support in different situations. You are being always open for discussion and willing to help in different ways. I will keep in memory all unforgettable moments happened within the past years, especially dedicated to anniversary of Poltava battle in 2009 and Nobel Prize ceremony in 2012!

Vitalii Moskalenko, rector of National O.Bohomolets Medical University. Thank you for your interest and support for the developing of scientific collaboration between NMU and KI, your positive attitude and enthusiastic discussions during your visits to Stockholm.

Olesya Hulchiy, coordinator of collaboration between research institutions in Ukraine and Karolinska Institutet from National O.Bohomolets Medical University side. Thank you for inviting me to apply for KIRT program and for further support of my project.

Tomas Ekström, my external mentor and expert in the field of epigenetics. Thank you for excellent performance as the external mentor and nice discussions that we had during my work at CMM.

I wish to thank **Vinod Diwan** and **Göran Pershagen** for interest and support in establishing the collaboration between Karolinska Institutet and institutions in Ukraine!

I would like to show my greatest appreciation to **Janne Lehtiö** for the permission to use all required equipment and the necessary materials in your lab at the SciLifeLab to pursue the cystic PTC project! Thank you for constructive comments and warm encouragement!

Svetlana Lagercrantz, a great researcher and experienced oncologist. Thank you for being a supportive friend, sharing a lot of experiences and knowledge in different aspects of work and life. You are a wonderful and charming person with a very strong personality, spreading a very good energy! Thank you for your kindness and positive attitude!

Luqman Sulaiman, an intelligent researcher, clinician and IT-expert! Thank you for being a good friend, always willing to give a good advice and help! I am grateful for your warm encouragement and constructive comments in different issues related to our every-day work, discussions about similarities of Kurdistan and Ukraine, updates of IT-market and many other things! I wish you a very good luck in your clinical and scientific carrier and hope to see you one day becoming a chairperson of Ministry of Health of Kurdistan!

Felix Haglund thank you for cheerful attitude, stimulating chats about the computer games and amusing videos on YouTube as well as sharing your thoughts while writing the thesis parts! You are very optimistic and motivated person, demonstrating an effective and inspiring life strategy in combination with good sense of humor! **Nimrod Kiss** thank you for being friendly and supportive, for cool jokes and sharing interesting facts about Swedish lifestyle during the lunch time. Желаю тебе удачи в дальнейшем изучении русского языка! I wish to thank **Christofer Juhlin** for protocol of IHC being in use by all members of CL-group. You are creative researcher and pathologist having excellent sense of humor and producing killing jokes concerning the serious topics; **Adam Andreasson** for relaxed style and being a good team-member of CL-group. You are very talented person, who is able to combine a

research and studying at medical school with achievements in the sport; **Na Wang** for always being friendly, telling an interesting stories and facts about different aspects of life in China and suggesting me very good Asian restaurants in Stockholm.

Advice and comments given by **Weng-Onn Lui** has been a great help in my studies. You are very talented and ambitious scientist and I wish to see you one day to be a Nobel Prize winner for achievements in research of small-RNA!

David Velázquez-Fernández, an endocrine surgeon from Mexico, thank you for being always fantastic and optimistic! I owe my gratitude to **Jamileh Hashemi**, **Omid Fotouhi**, and **Janos Geli** for enthusiastic discussions about the different aspects of translational research during the lunch time and coffee-brakes! I would like to show my greatest appreciation to **Chato Taher** for your kindness and help with accommodation issues! **Pinar Akçakaya** thank you for lunch chats as well as for bringing me original spices from Turkey, and explaining me the correct name of the “rahat” lokum! **Stefano Caramuta** thank you for friendly attitude, nice talks while working in the lab and telling the stories about the life in Italy! **Deniz Özata** thank you for always being positive and optimistic, teaching me some çok güzel Turkish expressions! I am grateful to **Ming Lu** and **Hong Xie** for sharing you protocols and antibodies, as well as to **Roger Chang** and **Lin-Kiat Andrew Lee** for friendly attitude, feedbacks and encouragement, as well as for a nice working atmosphere in our lab!

I wish to thank **Göran Wallin** and **Theodoros Foukakis** for meticulous comments during the TRG-meeting as well as to **Cia Ihre Lundgren** for collaboration. I hope we'll be able to reactivate the prognostic PTC study! **Martin Bäckdahl** for support of my applications to AACR and IAES and for being a good MMK prefekt, providing an efficient support of PhD students, **Robert Branström** for friendly attitude and excellent administrative support as a study director at MMK. **Chatrin Lindahl** thank you for excellent assistance with administrative issues related to activities in Endocrine surgery group. **Erik Wennerberg** and **Andreas Lundqvist** thank you for interest to collaborate and fruitful discussions during TRG-meetings.

I'd like to thank **Jia Jing Lee (JJ)** for optimistic lifestyle and expert advises regarding sequencing of thyroid tumors, **Mohsen Karimi** for teaching me Pyrosequencing and funny chats that we used to had at CMM, **Monica Janssen** for explaining me how to do FISH. My intellectual debt is to **Mehran Ghaderi** for support, fruitful collaboration as well as excellent advices and practical help with RT-PCR and Pyrosequencing. I am grateful to **Lisa Ånfalk** for excellent assistance with retrieval and handling of tissue and fluid samples as well as for help with data collection!

My intellectual and practical debt is to **Maria Pernemalm** and **Hanna Eriksson**, thank you for guidance and help with mass spec and ELISA and very quick and highly informative response to my emails!

I wish to thank **Dawei Xu** for a smooth collaboration in *hTERT* study, **Vitalijs Sviatoha** for a contribution to cystic PTC project as well as **Anna Kwiecinska** and **Mattias Berglund** for a friendly attitude and excellent collaboration in lymphoma project. I wish also to thank **Henrik Johansson**, **Rui Branca** and **Elena Panizza** for hospitality and assistance during my work at the ScilifeLab! I appreciate the feedback offered by co-authors of articles, constituting this thesis **Lukas Orre**, **Susanne Becker**, **Ulf Hellman**, **Lina Hultin-Rosenberg** and **Gert Auer**.

I would also like to express my gratitude to **Dmytro Unukovych** for being a good friend, sharing a positive attitude and relaxed lifestyle, for splendid moments

happening while we shared an apartment in Stockholm, at the events dedicated to anniversary of Poltava Battle and while we attended ISW in Japan! **Gregory Tour**, you are very talented person, demonstrating a great achievements in music, science and clinical dentistry, thank you for hospitality, support, enthusiasm and creativity!

My deepest appreciation goes to **Mykola Kucher** for support, amiable attitude and inspiring discussions, as well as sharing your experience in horseback riding; **Vasyl Khrapach** for being a genuinely nice person, spreading a creative optimism and willing to help! **Mykola Vialiy** for being friendly, for support of my applications and for making a really great coffee! **Konstantin Svechnikov** for sense of humor and enthusiastic conversations!

I would like to acknowledge **Natalia Kassarova** and **Evgeniy Akkuratov** for invigorating discussions about the science, politics in Russia and explaining the insights into the intricate nature of life in Moscow and Toledo; **Iuliia Savchuk**, **Aram Rasul**, **Hogir Salim**, **Iryna Kolosenko** for relaxed chats, feedbacks and encouragement; **Filip Mundt**, who I always met at post-graduate courses and different research events at KI for a nice discussions about cancer research, **Mahdi Mojallal** for your optimistic attitude and chats at the KS gym and Onc-Pat Kick-off!

Anna-Maria Marino thank you for sharing a good laughs and cheerful wit chats during the coffee brakes, as well as for the best receipt of tiramisu!

I want to thank **Olena Gruzieva**, **Andrei Pyko**, **Artur Mezheyski**, **Anton Vitt** for an inspiring discussions and talks during the progress report meetings; **Tatiana Pavlova**, **Alexei Shemyakin** and **Olga Ovchinnikova** for creative optimism and sense of humor! **Andrea Armenti** “una bestia” from Roma! Thank you for introducing me the real Italy and sharing your extremely positive attitude, killing jokes and teaching me the most important Italian expressions!

I also wish to acknowledge my colleagues from the Doctoral Students association **Mélanie Thessén Hedreul**, **Mellisa Norström**, **Sofi Eriksson**, **Britta Stenson**, **Mikko Hellegren**, **Åsa Samuelsson** for an enthusiastic discussions about how to improve the KI’s regulations for doctoral studies as well as exhilarated time at the DSA parties!

I am obliged to my colleagues **Diego Iglesias Gato**, **Yin-Choy Chuan** (*trabajar!*), **Fahad Al-Zadjali**, **Carolina Gustavsson**, **Anenisia de Andrade**, **Vladana Vokojevich**, **Mattias Vesterlund**, **Monira Ahtar**, **Zahidul Khan**, **Agneta Gunnar**, **Michaela Barbro**, **Simone Picelli** for the valuable information provided by you in their respective field, for you cooperation and friendliness during the period of my assignment at CMM.

I would also like to take immense pleasure in thanking to my colleagues at CCK **Dan Grandér** for a friendly approachable attitude and talks at the KS gym, as well as to **Cecilia Krona**, **Ebba Palmberg**, **Linda Ljungblad**, **Carl Öquist**, **John Johnsen**, **Lotta Elfman**, **Per Kogner**, **Anna Kock**, **Malin Wickström**, **Cecillia Dyberg**, **Raul Calero**, **Slavica Brnjic**, **Padraig D’Arcy**, **Angelo De Milto**, **Maria Hägg Olofsson**, **Seema Jamil**, **Angelo Strambi**, **Juan Castro** for welcoming us at the ground floor of CCK and contributing to a pleasant working environment; **Bertha Brodin** for organizing a very informative sarcoma seminars and relaxed chats, **Bari Wolahan** and **Limin Ma** for being always friendly and cheerful, **Jelena Milosevic** for your optimism and numerous stimulating discussions; **Eva-Lena Halvarsson**, **Anita Endholm** and **Sören Linden** for excellent technical support.

I appreciate administrative stuff of MMK, **Therese Kindåker, Helena Nässen, Jeanette Johansen, Kerstin Florell** for the cordial support, valuable information and guidance, **Britt-Marie Witasp** for an excellent handling of financial issues, **Ann-Britt Wikström** for always willing to help administrative support during my PhD, **Lennart Helleday** and **Jan-Erik Kaarre** for excellent assistance with solving of IT-related issues.

Mariana thank you for happy moments in our life.

I owe my deepest gratitude to my family for endless love, support, keeping me harmonious and helping me putting pieces together.

Моя любимая **Мама**, спасибо тебе за твою заботу, безграничную любовь, поддержку и веру в меня, за твою сильную личность на примере которой формировались мои жизненные приоритеты!

Галя – ты очень важный человек в моей жизни, спасибо тебе за любовь и доброту, за твой огромный вклад в воспитание и развитие моего мировоззрения!

Вика, моя горячо любимая сестра! Спасибо тебе за заботу, доброту и за все радостные моменты и приколы в нашей жизни!

Дима, спасибо тебе за истинно братское отношение и поддержку, за честность, искреннюю дружбу и советы!

Таня, в тебе заложен большой потенциал, у тебя много талантов! Спасибо за понимание и заботу, а также за интересные дискуссии по поводу конного спорта и чепрачной кинологии! Я желаю тебе и впредь развивать свои способности и надеюсь, что через какое-то время увидеть твой thesis book!

Папа, спасибо за советы и поддержку в самых разных ситуациях, а также во время учебы в мединституте.

Петя, спасибо тебе за заботу и доброту, твою сильную личность и те жизненные принципы, которых ты придерживался и которые я разделяю, за твои знания и опыт, а также за все незабываемые моменты, связанные с твоим подходом к решению разнообразных задач. Очень жаль, что ты уже не прочитаешь этих строк, но память о тебе всегда жива.

I would also like to express my sincere gratitude to all **patients** for their consent to participate in my studies, academic authorities and foundations in Sweden for providing the financial means, laboratory facilities and excellent organization of work environment:

Karolinska Institutet
Karolinska Institutet Research Training Committee (KIRT)
Swedish Institutet (Visby program)
The Swedish Cancer Society (Cancerfonden)
The Swedish research Council
Radiumhemmets Forskningfonder
Karolinska Fonder
Karolinska University Hospital
Science for Life Laboratory
Center for Molecular Medicine
Cancer Center Karolinska

8 REFERENCES

1. Kocher T. A Contribution to the pathology of the thyroid gland: Being an Oration delivered before the Medical Society of London, Monday, May 21st. *Br Med J* 1906; 1 1261-1266.
2. Gray H. Anatomy of the Human Body: The thyroid gland and its relations. Philadelphia, USA: Lea & Febiger, 1918.
3. Robbins KT, Clayman G, *et al.* Neck dissection classification update: revisions proposed by the American Head and Neck Society and the American Academy of Otolaryngology-Head and Neck Surgery. *Arch Otolaryngol Head Neck Surg* 2002; 128 751-758.
4. Smith PW, Salomone LJ, *et al.* Thyroid. In *Sabiston textbook of surgery*, edn 19, ch. 38. Ed M Townsend. Canada: Saunders, an imprint of Elsevier Inc., 2012.
5. Boron WF & Boulpaep EL. Synthesis of thyroid hormones. In *Medical physiology: a cellular and molecular approach*. edn Updated, ch. 48, pp xiii, 1319 p. Philadelphia, Pa.: Elsevier Saunders, 2003.
6. Flamant F, Baxter JD, *et al.* International Union of Pharmacology. LIX. The pharmacology and classification of the nuclear receptor superfamily: thyroid hormone receptors. *Pharmacol Rev* 2006; 58 705-711.
7. DeLellis RA. *Pathology and genetics of tumours of endocrine organs*. Lyon: IARC Press, 2004.
8. McHenry CR & Phitayakorn R. Follicular adenoma and carcinoma of the thyroid gland. *Oncologist* 2011; 16 585-593.
9. Layfield LJ, Cibas ES, *et al.* Thyroid aspiration cytology: current status. *CA Cancer J Clin* 2009; 59 99-110.
10. Cibas ES & Ali SZ. The Bethesda System for Reporting Thyroid Cytopathology. *Thyroid* 2009; 19 1159-1165.
11. Giusti L, Iacconi P, *et al.* Fine-Needle Aspiration of Thyroid Nodules: Proteomic Analysis To Identify Cancer Biomarkers. *J Proteome Res* 2008; 7 4079-4088.
12. Sharma N, Martin A, *et al.* Mining the proteome: the application of tandem mass spectrometry to endocrine cancer research. *Endocr Relat Cancer* 2012; 19 R149-161.
13. Sofiadis A, Becker S, *et al.* Proteomic profiling of follicular and papillary thyroid tumors. *Eur J Endocrinol* 2012; 166 657-667.
14. Sofiadis A, Dinets A, *et al.* Proteomic study of thyroid tumors reveals frequent up-regulation of the Ca²⁺-binding protein S100A6 in papillary thyroid carcinoma. *Thyroid* 2010; 20 1067-1076.
15. Hegedus L, Bonnema SJ, *et al.* Management of simple nodular goiter: current status and future perspectives. *Endocr Rev* 2003; 24 102-132.

16. Yang GC, Stern CM, *et al.* Cystic papillary thyroid carcinoma in fine needle aspiration may represent a subset of the encapsulated variant in WHO classification. *Diagn Cytopathol* 2010; 38 721-726.
17. Lin JD, Hsuen C, *et al.* Cystic change in thyroid cancer. *ANZ J Surg* 2007; 77 450-454.
18. Garcia-Pascual L, Barahona MJ, *et al.* Complex thyroid nodules with nondiagnostic fine needle aspiration cytology: histopathologic outcomes and comparison of the cytologic variants (cystic vs. acellular). *Endocrine* 2011; 39 33-40.
19. Kristjansdottir B, Partheen K, *et al.* Ovarian cyst fluid is a rich proteome resource for detection of new tumor biomarkers. *Clin Proteomics* 2012; 9 14.
20. Fedorenko Z, Goulak L, *et al.* Cancer in Ukraine, 2011 - 2012. In *Bulletin of national cancer registry of Ukraine*. Ed I Shchepotin. Kyiv, Ukraine: National Cancer Institute of Ukraine, 2013.
21. Ericsson J, Ayoubi S, *et al.* Official statistics of Sweden. Statistics – Health and Medical Care. In *Cancer Incidence in Sweden 2011*. Ed A Åberg. Stockholm: Socialstyrelsens, 2012.
22. Xing M. Molecular pathogenesis and mechanisms of thyroid cancer. *Nat Rev Cancer* 2013; 13 184-199.
23. Nikiforova MN, Ciampi R, *et al.* Low prevalence of BRAF mutations in radiation-induced thyroid tumors in contrast to sporadic papillary carcinomas. *Cancer Lett* 2004; 209 1-6.
24. Puxeddu E, Moretti S, *et al.* BRAF(V599E) mutation is the leading genetic event in adult sporadic papillary thyroid carcinomas. *J Clin Endocrinol Metab* 2004; 89 2414-2420.
25. Socolow EL, Hashizume A, *et al.* Thyroid carcinoma in man after exposure to ionizing radiation. A summary of the findings in Hiroshima and Nagasaki. *N Engl J Med* 1963; 268 406-410.
26. Dinets A, Hulchiy M, *et al.* Clinical, genetic, and immunohistochemical characterization of 70 Ukrainian adult cases with post-Chernobyl papillary thyroid carcinoma. *Eur J Endocrinol* 2012; 166 1049-1060.
27. Avetisian IL, Gulchiy NV, *et al.* Thyroid pathology in residents of the Kiev region, Ukraine, during pre- and post-Chernobyl periods. *J Environ Pathol Toxicol Oncol* 1996; 15 233-237.
28. Stsjazhko VA, Tsyb AF, *et al.* Childhood thyroid cancer since accident at Chernobyl. *BMJ* 1995; 310 801.
29. Williams ED, Abrosimov A, *et al.* Thyroid carcinoma after Chernobyl latent period, morphology and aggressiveness. *Br J Cancer* 2004; 90 2219-2224.
30. Jacob P, Bogdanova TI, *et al.* Thyroid cancer among Ukrainians and Belarusians who were children or adolescents at the time of the Chernobyl accident. *J Radiol Prot* 2006; 26 51-67.
31. Lloyd RV, Buehler D, *et al.* Papillary thyroid carcinoma variants. *Head Neck Pathol* 2011; 5 51-56.

32. Cooper DS, Doherty GM, *et al.* Revised American Thyroid Association management guidelines for patients with thyroid nodules and differentiated thyroid cancer. *Thyroid* 2009; 19 1167-1214.
33. de los Santos ET, Keyhani-Rofagha S, *et al.* Cystic thyroid nodules. The dilemma of malignant lesions. *Arch Intern Med* 1990; 150 1422-1427.
34. Carty SE, Cooper DS, *et al.* Consensus statement on the terminology and classification of central neck dissection for thyroid cancer. *Thyroid* 2009; 19 1153-1158.
35. Kjellman P, Wallin G, *et al.* MIB-1 index in thyroid tumors: a predictor of the clinical course in papillary thyroid carcinoma. *Thyroid* 2003; 13 371-380.
36. Lundgren CI, Hall P, *et al.* Clinically significant prognostic factors for differentiated thyroid carcinoma: a population-based, nested case-control study. *Cancer* 2006; 106 524-531.
37. Sofiadis A, Tani E, *et al.* Diagnostic and prognostic potential of MIB-1 proliferation index in thyroid fine needle aspiration biopsy. *Int J Oncol* 2009; 35 369-374.
38. Watkinson JC. The British Thyroid Association guidelines for the management of thyroid cancer in adults. *Nucl Med Commun* 2004; 25 897-900.
39. Dralle H, Musholt TJ, *et al.* German Association of Endocrine Surgeons practice guideline for the surgical management of malignant thyroid tumors. *Langenbecks Arch Surg* 2013; 398 347-375.
40. Patel SS & Goldfarb M. Well-differentiated thyroid carcinoma: the role of post-operative radioactive iodine administration. *J Surg Oncol* 2013; 107 665-672.
41. Biondi B & Cooper DS. Benefits of thyrotropin suppression versus the risks of adverse effects in differentiated thyroid cancer. *Thyroid* 2010; 20 135-146.
42. Sullivan RJ, Lawrence DP, *et al.* Case records of the Massachusetts General Hospital. Case 21-2013. A 68-year-old man with metastatic melanoma. *N Engl J Med* 2013; 369 173-183.
43. Xing M. Genetic-targeted therapy of thyroid cancer: a real promise. *Thyroid* 2009; 19 805-809.
44. Kandil E, Tsumagari K, *et al.* Synergistic inhibition of thyroid cancer by suppressing MAPK/PI3K/AKT pathways. *J Surg Res* 2013.
45. Antonelli A, Fallahi P, *et al.* RET TKI: potential role in thyroid cancers. *Curr Oncol Rep* 2012; 14 97-104.
46. Xing M, Haugen BR, *et al.* Progress in molecular-based management of differentiated thyroid cancer. *Lancet* 2013; 381 1058-1069.
47. O'Neill CJ, Bullock M, *et al.* BRAF(V600E) mutation is associated with an increased risk of nodal recurrence requiring reoperative surgery in patients with papillary thyroid cancer. *Surgery* 2010; 148 1139-1145; discussion 1145-1136.
48. Collins BJ, Schneider AB, *et al.* Low frequency of BRAF mutations in adult patients with papillary thyroid cancers following childhood radiation exposure. *Thyroid* 2006; 16 61-66.

49. Leeman-Neill RJ, Brenner AV, *et al.* RET/PTC and PAX8/PPARgamma chromosomal rearrangements in post-Chernobyl thyroid cancer and their association with iodine-131 radiation dose and other characteristics. *Cancer* 2013; 119 1792-1799.
50. Thomas GA, Bunnell H, *et al.* High prevalence of RET/PTC rearrangements in Ukrainian and Belarussian post-Chernobyl thyroid papillary carcinomas: a strong correlation between RET/PTC3 and the solid-follicular variant. *J Clin Endocrinol Metab* 1999; 84 4232-4238.
51. Santoro M, Melillo RM, *et al.* RET/PTC activation in papillary thyroid carcinoma: European Journal of Endocrinology Prize Lecture. *Eur J Endocrinol* 2006; 155 645-653.
52. Unger K, Zitzelsberger H, *et al.* Heterogeneity in the distribution of RET/PTC rearrangements within individual post-Chernobyl papillary thyroid carcinomas. *J Clin Endocrinol Metab* 2004; 89 4272-4279.
53. Nakazawa T, Kondo T, *et al.* RET gene rearrangements (RET/PTC1 and RET/PTC3) in papillary thyroid carcinomas from an iodine-rich country (Japan). *Cancer* 2005; 104 943-951.
54. Marotta V, Guerra A, *et al.* RET/PTC rearrangement in benign and malignant thyroid diseases: a clinical standpoint. *Eur J Endocrinol* 2011; 165 499-507.
55. Zhu X, Sun T, *et al.* Diagnostic significance of CK19, RET, galectin-3 and HBME-1 expression for papillary thyroid carcinoma. *J Clin Pathol* 2010; 63 786-789.
56. Lee SR, Jung CK, *et al.* Molecular genotyping of follicular variant of papillary thyroid carcinoma correlates with diagnostic category of fine needle aspiration cytology: values of RAS mutation testing. *Thyroid* 2013.
57. Santarpia L, Myers JN, *et al.* Genetic alterations in the RAS/RAF/mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt signaling pathways in the follicular variant of papillary thyroid carcinoma. *Cancer* 2010; 116 2974-2983.
58. Fukahori M, Yoshida A, *et al.* The associations between RAS mutations and clinical characteristics in follicular thyroid tumors: new insights from a single center and a large patient cohort. *Thyroid* 2012; 22 683-689.
59. Leonardo E, Volante M, *et al.* Cell membrane reactivity of MIB-1 antibody to Ki67 in human tumors: fact or artifact? *Appl Immunohistochem Mol Morphol* 2007; 15 220-223.
60. Ranjbari N & Rahim F. The Ki-67/MIB-1 index level and recurrence of papillary thyroid carcinoma. *Med Hypotheses* 2013; 80 311-314.
61. de Matos LL, Del Giglio AB, *et al.* Expression of CK-19, galectin-3 and HBME-1 in the differentiation of thyroid lesions: systematic review and diagnostic meta-analysis. *Diagn Pathol* 2012; 7 97.
62. Cochand-Priollet B, Dahan H, *et al.* Immunocytochemistry with cytokeratin 19 and anti-human mesothelial cell antibody (HBME1) increases the diagnostic accuracy of thyroid fine-needle aspirations: preliminary report of 150 liquid-

- based fine-needle aspirations with histological control. *Thyroid* 2011; 21 1067-1073.
63. Lee SH, Lee JK, *et al.* Expression of cell-cycle regulators (cyclin D1, cyclin E, p27kip1, p57kip2) in papillary thyroid carcinoma. *Otolaryngol Head Neck Surg* 2010; 142 332-337.
 64. Nar A, Ozen O, *et al.* Cyclin A and cyclin B1 overexpression in differentiated thyroid carcinoma. *Med Oncol* 2012; 29 294-300.
 65. Strand C, Ahlin C, *et al.* Combination of the proliferation marker cyclin A, histological grade, and estrogen receptor status in a new variable with high prognostic impact in breast cancer. *Breast Cancer Res Treat* 2012; 131 33-40.
 66. Brzezianska E, Cyniak-Magierska A, *et al.* Assessment of cyclin D1 gene expression as a prognostic factor in benign and malignant thyroid lesions. *Neuro Endocrinol Lett* 2007; 28 341-350.
 67. Seybt TP, Ramalingam P, *et al.* Cyclin D1 expression in benign and differentiated malignant tumors of the thyroid gland: diagnostic and biologic implications. *Appl Immunohistochem Mol Morphol* 2012; 20 124-130.
 68. Troncone G, Volante M, *et al.* Cyclin D1 and D3 overexpression predicts malignant behavior in thyroid fine-needle aspirates suspicious for Hurthle cell neoplasms. *Cancer* 2009; 117 522-529.
 69. Ito Y, Arai K, *et al.* S100A8 and S100A9 expression is a crucial factor for dedifferentiation in thyroid carcinoma. *Anticancer Res* 2009; 29 4157-4161.
 70. Ito Y, Yoshida H, *et al.* Expression of S100A2 and S100A6 in thyroid carcinomas. *Histopathology* 2005; 46 569-575.
 71. Ning X, Sun S, *et al.* S100A6 protein negatively regulates CacyBP/SIP-mediated inhibition of gastric cancer cell proliferation and tumorigenesis. *PLoS One* 2012; 7 e30185.
 72. Nipp M, Elsner M, *et al.* S100-A10, thioredoxin, and S100-A6 as biomarkers of papillary thyroid carcinoma with lymph node metastasis identified by MALDI imaging. *J Mol Med (Berl)* 2012; 90 163-174.
 73. Slomnicki LP, Nawrot B, *et al.* S100A6 binds p53 and affects its activity. *Int J Biochem Cell Biol* 2009; 41 784-790.
 74. Maelandsmo GM, Florenes VA, *et al.* Differential expression patterns of S100A2, S100A4 and S100A6 during progression of human malignant melanoma. *Int J Cancer* 1997; 74 464-469.
 75. Ito Y, Arai K, *et al.* S100A10 expression in thyroid neoplasms originating from the follicular epithelium: contribution to the aggressive characteristic of anaplastic carcinoma. *Anticancer Res* 2007; 27 2679-2683.
 76. Hebrant A, Dom G, *et al.* mRNA expression in papillary and anaplastic thyroid carcinoma: molecular anatomy of a killing switch. *PLoS One* 2012; 7 e37807.
 77. Williams D. Radiation carcinogenesis: lessons from Chernobyl. *Oncogene* 2009; 27 Suppl 2 S9-18.
 78. Lundgren CI, Hall P, *et al.* Incidence and survival of Swedish patients with differentiated thyroid cancer. *Int J Cancer* 2003; 106 569-573.

79. Sobin LH, Gospodarowicz MK, *et al.* *TNM classification of malignant tumours*. Chichester, West Sussex, UK ; Hoboken, NJ: Wiley-Blackwell, 2010.
80. Ito Y, Ichihara K, *et al.* Establishment of an intraoperative staging system (iStage) by improving UICC TNM classification system for papillary thyroid carcinoma. *World J Surg* 2010; 34 2570-2580.
81. Silver CE, Owen RP, *et al.* Aggressive variants of papillary thyroid carcinoma. *Head Neck* 2011; 33 1052-1059.
82. Nikiforov YE. Radiation-induced thyroid cancer: what we have learned from Chernobyl. *Endocr Pathol* 2006; 17 307-317.
83. Romei C & Elisei R. RET/PTC Translocations and Clinico-Pathological Features in Human Papillary Thyroid Carcinoma. *Front Endocrinol (Lausanne)* 2012; 3 54.
84. Kim TH, Park YJ, *et al.* The association of the BRAF(V600E) mutation with prognostic factors and poor clinical outcome in papillary thyroid cancer: a meta-analysis. *Cancer* 2012; 118 1764-1773.
85. Kjellman P, Learoyd DL, *et al.* Expression of the RET proto-oncogene in papillary thyroid carcinoma and its correlation with clinical outcome. *Br J Surg* 2001; 88 557-563.
86. Powell N, Jeremiah S, *et al.* Frequency of BRAF T1796A mutation in papillary thyroid carcinoma relates to age of patient at diagnosis and not to radiation exposure. *J Pathol* 2005; 205 558-564.
87. Xing M, Westra WH, *et al.* BRAF mutation predicts a poorer clinical prognosis for papillary thyroid cancer. *J Clin Endocrinol Metab* 2005; 90 6373-6379.
88. Zedenius J & Foukakis T. Pathogenesis of thyroid cancer. In *Clinical Endocrine Oncology*, edn 2, ch. 15, pp 124-129. Eds J Hay & J Wass: Wiley-Blackwell, 2009.
89. Caria P & Vanni R. Cytogenetic and molecular events in adenoma and well-differentiated thyroid follicular-cell neoplasia. *Cancer Genet Cytogenet* 2010; 203 21-29.
90. Cannon J. The significance of hurthle cells in thyroid disease. *Oncologist* 2011; 16 1380-1387.
91. Foukakis T, Gusnanto A, *et al.* A PCR-based expression signature of malignancy in follicular thyroid tumors. *Endocr Relat Cancer* 2007; 14 381-391.
92. Gomez Saez JM. Diagnostic and prognostic markers in differentiated thyroid cancer. *Curr Genomics* 2011; 12 597-608.
93. Volante M, Collini P, *et al.* Poorly differentiated thyroid carcinoma: the Turin proposal for the use of uniform diagnostic criteria and an algorithmic diagnostic approach. *Am J Surg Pathol* 2007; 31 1256-1264.
94. Goyal N, Setabutr D, *et al.* Molecular and genetic markers of follicular-cell thyroid cancer: etiology and diagnostic and therapeutic opportunities. *Adv Exp Med Biol* 2013; 779 309-326.

95. Segerhammar I, Larsson C, *et al.* Anaplastic carcinoma of the thyroid gland: Treatment and outcome over 13 years at one institution. *J Surg Oncol* 2012; 106 981-986.
96. Pfeifer J. Endocrine System. In *Molecular Genetic Testing in Surgical Pathology*, ch. 12, p 240: Lippincott Williams and Wilkins, 2006.
97. Nagaiah G, Hossain A, *et al.* Anaplastic thyroid cancer: a review of epidemiology, pathogenesis, and treatment. *J Oncol* 2011; 2011 542358.
98. Figlioli G, Landi S, *et al.* Medullary thyroid carcinoma (MTC) and RET proto-oncogene: mutation spectrum in the familial cases and a meta-analysis of studies on the sporadic form. *Mutat Res* 2013; 752 36-44.
99. Zedenius J, Larsson C, *et al.* Mutations of codon 918 in the RET proto-oncogene correlate to poor prognosis in sporadic medullary thyroid carcinomas. *J Clin Endocrinol Metab* 1995; 80 3088-3090.
100. Zedenius J. Is somatic RET mutation a prognostic factor for sporadic medullary thyroid carcinoma? *Nat Clin Pract Endocrinol Metab* 2008; 4 432-433.
101. Pacini F, Castagna MG, *et al.* Medullary thyroid carcinoma. *Clin Oncol* 2010; 22 475-485.
102. Pazaitou-Panayiotou K, Chrisoulidou A, *et al.* Predictive factors that influence the course of medullary thyroid carcinoma. *Int J Clin Oncol* 2013.
103. Wells SA, Jr., Pacini F, *et al.* Multiple endocrine neoplasia type 2 and familial medullary thyroid carcinoma: an update. *J Clin Endocrinol Metab* 2013; 98 3149-3164.
104. Towbin H, Staehelin T, *et al.* Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 1979; 76 4350-4354.
105. Forsberg L, Larsson C, *et al.* Pre-fractionation of archival frozen tumours for proteomics applications. *J Biotechnol* 2006; 126 582-586.
106. Ramos-Vara JA. Technical aspects of immunohistochemistry. *Vet Pathol* 2005; 42 405-426.
107. Rhoden KJ, Johnson C, *et al.* Real-time quantitative RT-PCR identifies distinct c-RET, RET/PTC1 and RET/PTC3 expression patterns in papillary thyroid carcinoma. *Lab Invest* 2004; 84 1557-1570.
108. Sanger F, Nicklen S, *et al.* DNA sequencing with chain-terminating inhibitors. 1977. *Biotechnology* 1992; 24 104-108.
109. Ronaghi M, Uhlen M, *et al.* A sequencing method based on real-time pyrophosphate. *Science* 1998; 281 363, 365.
110. Lade-Keller J, Romer KM, *et al.* Evaluation of BRAF mutation testing methodologies in formalin-fixed, paraffin-embedded cutaneous melanomas. *J Mol Diagn* 2013; 15 70-80.
111. Kim TE, Jung ES, *et al.* DHPLC is a highly sensitive and rapid screening method to detect BRAF(V600E) mutation in papillary thyroid carcinoma. *Exp Mol Pathol* 2013; 94 203-209.

112. Summersgill BM & Shipley JM. Fluorescence in situ hybridization analysis of formalin fixed paraffin embedded tissues, including tissue microarrays. *Methods Mol Biol* 2010; 659 51-70.
113. Kang J-S. Principles and Applications of LC-MS/MS for the Quantitative Bioanalysis of Analytes in Various Biological Samples. In *Tandem Mass Spectrometry – Applications and Principles*, ch. 21, pp 441-492. Ed GR Baptista: InTech, 2012.
114. Kapp E & Schutz F. Overview of tandem mass spectrometry (MS/MS) database search algorithms. *Curr Protoc Protein Sci* 2007; Chapter 25 Unit25 22.
115. Abdallah C, Dumas-Gaudot E, *et al.* Gel-based and gel-free quantitative proteomics approaches at a glance. *Int J Plant Genomics* 2012; 2012 494572.
116. Aebersold R & Mann M. Mass spectrometry-based proteomics. *Nature* 2003; 422 198-207.
117. Pruitt KD, Tatusova T, *et al.* NCBI Reference Sequences (RefSeq): current status, new features and genome annotation policy. *Nucleic Acids Res* 2012; 40 D130-135.
118. Suriano R, Lin Y, *et al.* Pilot Study Using SELDI-TOF–MS Based Proteomic Profile for the Identification of Diagnostic Biomarkers of Thyroid Proliferative Diseases. *J Proteome Res* 2006; 5 856-861.
119. Lundgren DH, Martinez H, *et al.* Protein identification using Sorcerer 2 and SEQUEST. *Curr Protoc Bioinformatics* 2009; Chapter 13 Unit 13 13.
120. Yip L, Nikiforova MN, *et al.* Optimizing surgical treatment of papillary thyroid carcinoma associated with BRAF mutation. *Surgery* 2009; 146 1215-1223.
121. Henderson YC, Shellenberger TD, *et al.* High rate of BRAF and RET/PTC dual mutations associated with recurrent papillary thyroid carcinoma. *Clin Cancer Res* 2009; 15 485-491.
122. Kashima K, Yokoyama S, *et al.* Chronic thyroiditis as a favorable prognostic factor in papillary thyroid carcinoma. *Thyroid* 1998; 8 197-202.
123. Kim EY, Kim WG, *et al.* Coexistence of chronic lymphocytic thyroiditis is associated with lower recurrence rates in patients with papillary thyroid carcinoma. *Clin Endocrinol (Oxf)* 2009; 71 581-586.
124. Romei C, Ciampi R, *et al.* BRAFV600E mutation, but not RET/PTC rearrangements, is correlated with a lower expression of both thyroperoxidase and sodium iodide symporter genes in papillary thyroid cancer. *Endocr Relat Cancer* 2008; 15 511-520.
125. Ito Y, Yoshida H, *et al.* Expression of G2-M modulators in thyroid neoplasms: correlation of cyclin A, B1 and cdc2 with differentiation. *Pathol Res Pract* 2002; 198 397-402.
126. Ory C, Ugolin N, *et al.* Gene expression signature discriminates sporadic from post-radiotherapy-induced thyroid tumors. *Endocr Relat Cancer* 2011; 18 193-206.

127. Lima J, Trovisco V, *et al.* BRAF mutations are not a major event in post-Chernobyl childhood thyroid carcinomas. *J Clin Endocrinol Metab* 2004; 89 4267-4271.
128. Kumagai A, Namba H, *et al.* Low frequency of BRAFT1796A mutations in childhood thyroid carcinomas. *J Clin Endocrinol Metab* 2004; 89 4280-4284.
129. Sargent R, LiVolsi V, *et al.* BRAF mutation is unusual in chronic lymphocytic thyroiditis-associated papillary thyroid carcinomas and absent in non-neoplastic nuclear atypia of thyroiditis. *Endocr Pathol* 2006; 17 235-241.
130. Muzza M, Degl'Innocenti D, *et al.* The tight relationship between papillary thyroid cancer, autoimmunity and inflammation: clinical and molecular studies. *Clin Endocrinol (Oxf)* 2010; 72 702-708.
131. Frattini M, Ferrario C, *et al.* Alternative mutations of BRAF, RET and NTRK1 are associated with similar but distinct gene expression patterns in papillary thyroid cancer. *Oncogene* 2004; 23 7436-7440.
132. Lantsov D, Meirmanov S, *et al.* Cyclin D1 overexpression in thyroid papillary microcarcinoma: its association with tumour size and aberrant beta-catenin expression. *Histopathology* 2005; 47 248-256.
133. Siironen P, Nordling S, *et al.* Immunohistochemical expression of Bcl-2, Ki-67, and p21 in patients with papillary thyroid cancer. *Tumour Biol* 2005; 26 50-56.
134. Ito Y, Miyauchi A, *et al.* Prognostic significance of ki-67 labeling index in papillary thyroid carcinoma. *World J Surg* 2010; 34 3015-3021.
135. Stulik J, Osterreicher J, *et al.* Differential expression of the Ca²⁺ binding S100A6 protein in normal, preneoplastic and neoplastic colon mucosa. *Eur J Cancer* 2000; 36 1050-1059.
136. De Petris L, Orre LM, *et al.* Tumor expression of S100A6 correlates with survival of patients with stage I non-small-cell lung cancer. *Lung Cancer* 2009; 63 410-417.
137. Yang YQ, Zhang LJ, *et al.* Upregulated expression of S100A6 in human gastric cancer. *J Dig Dis* 2007; 8 186-193.
138. Kim J, Kim J, *et al.* S100A6 protein as a marker for differential diagnosis of cholangiocarcinoma from hepatocellular carcinoma. *Hepatol Res* 2002; 23 274.
139. Brown LM, Helmke SM, *et al.* Quantitative and qualitative differences in protein expression between papillary thyroid carcinoma and normal thyroid tissue. *Mol Carcinog* 2006; 45 613-626.
140. Nagy N, Decaestecker C, *et al.* Characterization of ligands for galectins, natural galactoside-binding immunoglobulin G subfractions and sarcolectin and also of the expression of calcyclin in thyroid lesions. *Histol Histopathol* 2000; 15 503-513.
141. Orre LM, Pernemalm M, *et al.* Up-regulation, modification, and translocation of S100A6 induced by exposure to ionizing radiation revealed by proteomics profiling. *Mol Cell Proteomics* 2007; 6 2122-2131.
142. Matsuzawa SI & Reed JC. Siah-1, SIP, and Ebi collaborate in a novel pathway for beta-catenin degradation linked to p53 responses. *Mol Cell* 2001; 7 915-926.

143. van Dieck J, Lum JK, *et al.* S100 proteins interact with the N-terminal domain of MDM2. *FEBS Lett* 2010; 584 3269-3274.
144. Bao L, Odell AF, *et al.* The S100A6 calcium-binding protein regulates endothelial cell-cycle progression and senescence. *FEBS J* 2012; 279 4576-4588.
145. Alfonso P, Canamero M, *et al.* Proteome analysis of membrane fractions in colorectal carcinomas by using 2D-DIGE saturation labeling. *J Proteome Res* 2008; 7 4247-4255.
146. Deng S, Wang J, *et al.* Annexin A1, A2, A4 and A5 play important roles in breast cancer, pancreatic cancer and laryngeal carcinoma, alone and/or synergistically. *Oncol Lett* 2013; 5 107-112.
147. Giusti L, Iacconi P, *et al.* A proteomic profile of washing fluid from the colorectal tract to search for potential biomarkers of colon cancer. *Mol Biosyst* 2012; 8 1088-1099.
148. Jo M, Yun HM, *et al.* Lung tumor growth promoting function of peroxiredoxin 6. *Free Radic Biol Med* 2013.
149. Rolfs F, Huber M, *et al.* Dual role of the antioxidant enzyme peroxiredoxin 6 in skin carcinogenesis. *Cancer Res* 2013.
150. Han S, Shen H, *et al.* Expression and prognostic significance of human peroxiredoxin isoforms in endometrial cancer. *Oncol Lett* 2012; 3 1275-1279.
151. Chaker S, Kashat L, *et al.* Secretome proteins as candidate biomarkers for aggressive thyroid carcinomas. *Proteomics* 2013; 13 771-787.
152. Morrison DK. The 14-3-3 proteins: integrators of diverse signaling cues that impact cell fate and cancer development. *Trends Cell Biol* 2009; 19 16-23.
153. Makki FM, Taylor SM, *et al.* Serum biomarkers of papillary thyroid cancer. *J Otolaryngol Head Neck Surg* 2013; 42 16.
154. Ishizuka T, Fujimori I, *et al.* Human carboxymethylenebutenolidase as a bioactivating hydrolase of olmesartan medoxomil in liver and intestine. *J Biol Chem* 2010; 285 11892-11902.
155. Yang X, Zou P, *et al.* Proteomic dissection of cell type-specific H2AX-interacting protein complex associated with hepatocellular carcinoma. *J Proteome Res* 2010; 9 1402-1415.
156. Wu N, Liu S, *et al.* The role of annexin A3 playing in cancers. *Clin Transl Oncol* 2013; 15 106-110.
157. Jung EJ, Moon HG, *et al.* Decreased annexin A3 expression correlates with tumor progression in papillary thyroid cancer. *Proteomics Clin Appl* 2010; 4 528-537.