Genetics, Diagnosis and Prognosis of Thyroid Tumors

Lutske Lodewijk

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Genetica, Diagnose en Prognose van Schildkliertumoren
(met een samenvatting in het Nederlands)

Proefschrift

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Lutske Lodewijk
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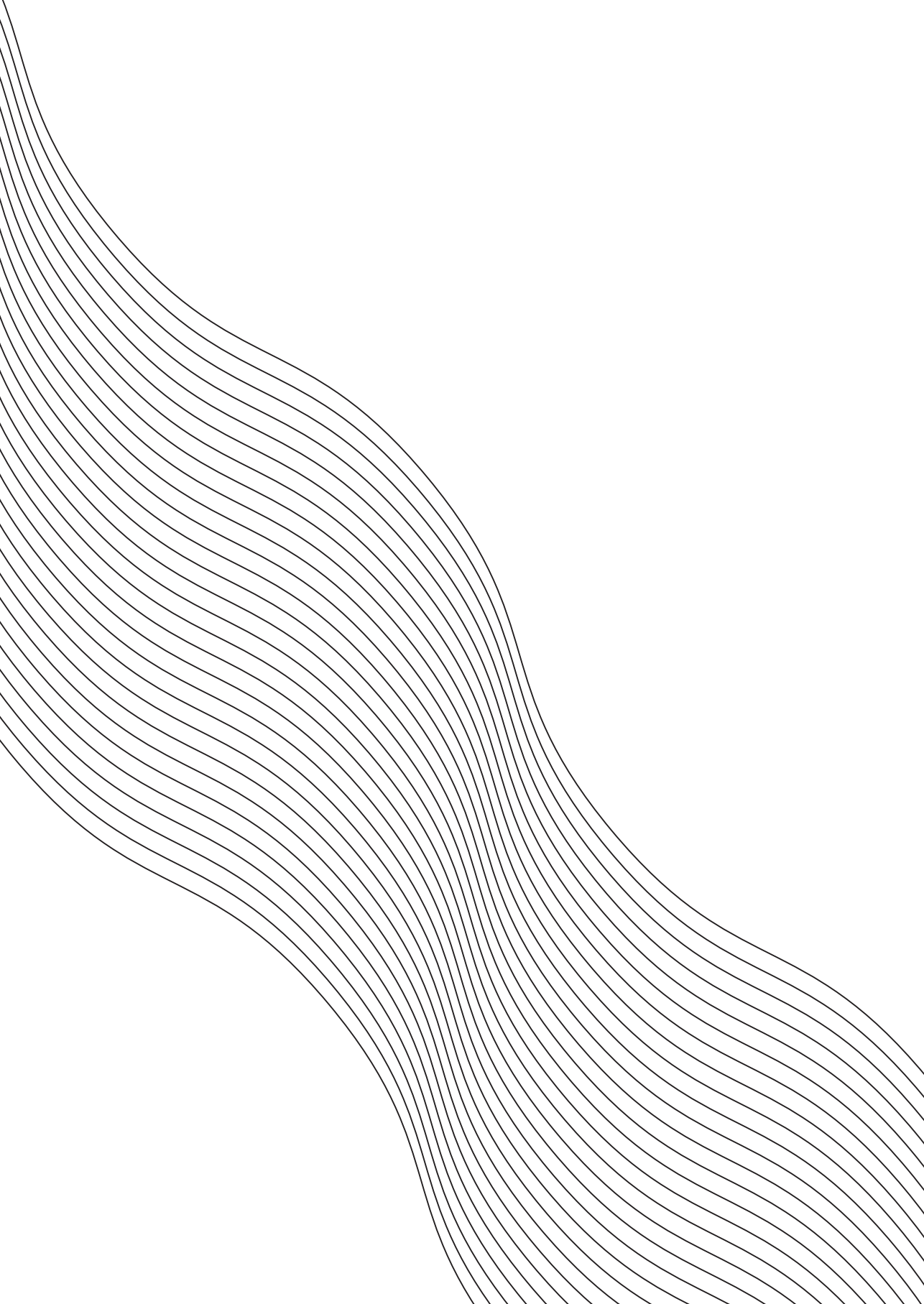
Prof. dr. M.R. Vriens & Prof. dr. I.H.M. Borel Rinkes



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Chapter 1

General introduction and outline of this thesis

Thyroid Gland and Thyroid Histology

The thyroid is a butterfly shaped organ anterior of the trachea just above the jugular notch. It contains two separate physiologic endocrine systems; one responsible for the production of the thyroid hormones thyroxine (T4) and triiodothyronine (T3), and the other responsible for the production of the hormone calcitonin. Iodine is an essential component for the production of both T4 and T3. To maintain a euthyroid state is ultimately orchestrated by the anterior pituitary gland through the secretion of thyrotropin (TSH). The physiologic actions of these thyroid hormones relate to growth, development, calorigenesis and TSH-suppression.[1]
Histologically the thyroid is predominantly composed of follicular cells, sometimes referred to as thyrocytes (Figure 1.1A). Embryologically they arise from a median endodermal mass in the region of the tongue (foramen cecum). These follicular cells are responsible for the production of thyroglobulin. They are cuboid shaped and arranged in follicles surrounding colloid; with the average size of a follicle varying from 100 to 300 μm. The colloid serves as the storeroom for thyroglobulin and thyroperoxidase, which are the precursor molecules for the production of the thyroid hormones, T4 and T3. The minority of the thyroid cells are named clear cells (or "c-cells", formerly known as parafollicular cells) that have their embryologic origin from the fourth pharyngeal pouch. C-cells are scattered between the follicles, predominantly in the middle of the thyroid lobe, and consist of only 0.1% of all thyroid cells (Figure 1.1D). They are larger than the follicular cells and show a clear cytoplasm with a H&E stain.[2]
Due to genetic alterations, these follicular cells and c-cells can become malignant and different types of cancer can arise; most notably differentiated thyroid cancer (DTC), which accounts for 95% of all thyroid cancers. DTC is a well differentiated cancer arising from the follicular cells and can be subdivided in papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC) (Figure 1.1B and C). The next type that arises from the follicular cells is anaplastic thyroid cancer (ATC). ATC is a very poorly differentiated tumor type and behaves aggressively, with a median survival of 3 months. The fourth type of thyroid cancer is medullary thyroid cancer (MTC), which arises from the c-cells (Figure 1.1D and E).[3]

Thyroid Nodules

The prevalence of palpable thyroid nodules is approximately 5% in women and 1% in men. However, when an ultrasound of the neck is performed nodules are found in 19 – 67% of patients. There is a prevalence of these nodules in women and a higher frequency in the elderly. Most of the thyroid nodules are asymptomatic and benign, only around 5% are found to be malignant.[3] In the last decades, the incidence of diagnosed thyroid nodules has increased, mostly due to a higher frequency of incidentalomas.[4, 5] Incidentalomas are thyroid nodules fortuitously detected

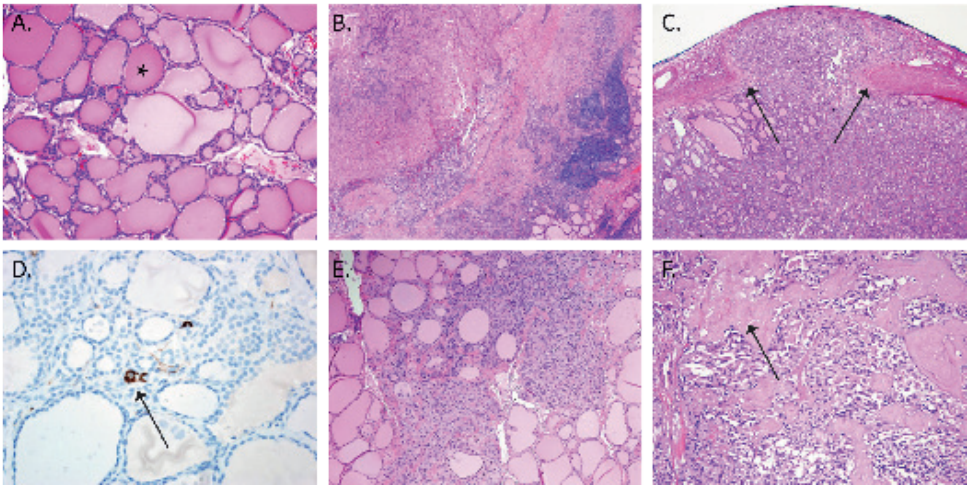

Figure 1.1: *Thyroid histology. A: normal thyroid follicles. Follicular cells are surrounding the colloid (asterisk). B: PTC. C: FTC with typical tongue of capsular invasion (between the arrows). D: normal c-cells scattered through the normal follicular structure, c-cells are indicated by calcitonin immunohistochemistry (arrow). E: MTC. F: close-up of MTC with amyloid depositions (arrow).*

during imaging studies, unrelated to the thyroid gland in an otherwise asymptomatic patient.[6] The diagnostic work-up of a thyroid nodule consists of – in short – patient history, physical examination, measurement of thyroid hormone levels and TSH, ultrasound of the thyroid and fine needle aspiration cytology (FNAC) of the thyroid nodule.[3] Thyroid FNACs should be classified using the Bethesda System for Reporting Thyroid Cytopathology, which has six diagnostic categories that have an implied cancer risk that links to an appropriate clinical management guideline (Table 1.1).[7]

Clinical management per Bethesda category is in summary as follows; Bethesda category 1 are non-diagnostic FNAC smears and are caused by an insufficient number of cells, contamination with blood or other processing issues. For these nodules a repeat FNAC should be attempted preferably with rapid onsite adequacy assessment by a cytopathologist.[7, 8] Bethesda category 2 are benign FNAC smears and for those patients follow-up is advised unless there are compressive symptoms or cosmetic concerns.[7] Bethesda category 6 are proven malignancies and management should be in accordance with the guidelines for the suspected subtype of thyroid cancer.[9] The Bethesda categories, 3, 4 and 5 pose a dilemma for the clinician, and are often referred to as indeterminate FNAC results. Often patients with indeterminate FNAC result undergo a diagnostic thyroid lobectomy, while in the majority of cases final histology proves the nodule to be benign. Thus, exposing those patients to, in hindsight, an unnecessary surgical intervention with associated morbidity and costs.[7]

In the Netherlands, the normal routine to analyze thyroid nodules often encompasses multiple visits to an out-patient clinic. Sometimes the diagnostic process can take several weeks before there is clarity about the nature of the nodule. While, in general, there is an increasing demand for rapid diagnosis for patients at risk for cancer.[10] In **chapter 2** we, therefore, aimed to evaluate the feasibility of same-day FNAC diagnosis for patients with a thyroid nodule and we were interested whether implementation of this same-day FNAC diagnosis influenced anxiety levels and patient distress.

As mentioned before, there is an increase in the detection of incidentalomas. These incidentalomas are an especially intriguing problem in patients with Multiple Endocrine Neoplasia type 1 (MEN type 1). Patients with MEN type 1 develop multiple endocrine tumors, among others hyperparathyroidism. Hyperparathyroidism is often one of the first clinical features of MEN type 1, with an average age of onset of 20 – 25 years.[11] In the diagnostic process of hyperparathyroidism an ultrasound of the neck is essential; when present, thyroid nodules are unavoidably detected.[12, 13] These thyroid incidentalomas are causing a diagnostic dilemma, since patients with MEN type 1 develop several endocrine tumors and it is under debate whether thyroid tumors are a manifestation of the disease or a coincidental finding. Therefore we describe in **chapter 3** the prevalence of thyroid tumors in patients with MEN type 1 compared to a matched non-MEN type 1 cohort and verified by immunohistochemistry whether tumorigenesis is MEN type 1 related.[14]

Table 1.1: *Bethesda classification.*

Category	Description category	Risk of malignancy (%)	Usual management
1	Non-diagnostic or unsatisfactory	1-4	Repeat FNA
2	Benign	0-3	Clinical Follow-up
3	Atypia of undetermined significance or follicular lesion of undetermined significance	5-15	Repeat FNA / Diagnostic lobectomy
4	Follicular neoplasm or suspicious for a follicular neoplasm	15-30	Diagnostic lobectomy
5	Suspicious for malignancy	60-75	Diagnostic lobectomy
6	Malignant	97-99	Total thyroidectomy

Thyroid Oncogenesis

In the past 20 years' there has been considerable progress in understanding the molecular mechanisms of thyroid cancer.[15] Genetic and epigenetic alterations in thyroid cancers are clinically relevant for several reasons: it is used to increase the value of FNAC specimen to reduce the number of indeterminate results, it is used as a prognosticator to identify those patients with more aggressive tumor behavior and it is used to develop targeted therapy for patients with metastasized disease.[16] In **chapter 4** we describe our current knowledge regarding the oncogenesis of thyroid nodules. The chapter evaluates up-to-date information on genetic and epigenetic alterations and their clinical value.

As a frequent epigenetic alteration, microRNA dysregulation is commonly seen and widely investigated. MicroRNAs are a class of non-coding RNAs, which have been demonstrated to be potential biomarkers for early cancer detection, prognostic indicators, and therapeutic targets.[17] Expression profiling of differentially expressed microRNAs has been performed in benign thyroid nodules and in the different types of thyroid cancer. From these results it has been attempted to identify a set of microRNAs that is able to discriminate between benign and malignant thyroid lesions, thereby decreasing the number of indeterminate results.[18]

In **chapter 5** we recapitulate several studies that evaluated altered microRNA expression levels in different types of thyroid cancer. We also provide an overview of validated sets of microRNAs or single microRNAs and their value in distinguishing malignant from benign lesions.

In patients with Bethesda category 3, 4 or 5 the result is called indeterminate. To retrieve certainty whether an indeterminate nodule is malignant a diagnostic lobectomy is often performed. If final histology shows the nodule to be malignant, a completion thyroidectomy is performed (2-stage procedure), and when the nodule is benign there is no indication for additional treatment (Table 1.1).[3] Up to 38% of FNAC results are indeterminate, resulting in unnecessary surgeries.[19] For this category, the understanding of genetic and epigenetic alterations is extensively investigated. Resulting in several sets of genetic mutations or altered miRNA expression levels. Among the candidate genetic markers, a somatic point mutation in the BRAF gene has been identified as the most common genetic event in papillary thyroid cancer (PTC).[20] Furthermore, this BRAF mutation occurs exclusively in carcinomas, but not in benign thyroid neoplasms, rendering it pathognomic for thyroid cancer.[21]

In **chapter 6** we describe that screening for a BRAFV600E mutation in patients with a FNAC result showing Bethesda category 5 can be a cost-effective manner to reduce the amount of 2-stage procedures.

Thyroid Cancer

Differentiated Thyroid Cancer

As DTC is curable in most cases and a chronic disease in the majority of the other cases, the essence of current literature is not prolonging survival, but -rather- to make the treatment less intensive and to prevent over-treatment, thus aiming for a more patient-tailored approach.[22] Surgery is always indicated for a malignant thyroid nodule. Nowadays, a total thyroidectomy is performed in most cases of differentiated thyroid cancer larger than 1 cm.[9] Surgery is followed by ablation therapy with radioactive iodine (RAI). Iodine is only absorbed by the follicular cells of the thyroid and thereby the radioactive iodine solely ablates the remnant thyroid cells or micrometastases. Based on the excellent overall survival of all stages of DTC, a tendency exists to de-escalate treatment. In 2009 the American Thyroid Association proposed a risk stratification system in which DTC is stratified in low-risk, intermediate-risk and high-risk subgroups, based on TNM-stage, histologic findings and results of the first posttreatment whole-body RAI scan (Table 1.2 and table 1.3).[9, 23] For patients with low-risk DTC the administered dose of radioactive iodine Surgery is always indicated for a malignant thyroid nodule. Nowadays, a total thyroidectomy is performed in most cases of differentiated thyroid cancer larger than 1 cm.[9] Surgery is followed by ablation therapy with radioactive iodine (RAI). Iodine is only absorbed by the follicular cells of the thyroid and thereby the radioactive iodine solely ablates the remnant thyroid cells or micrometastases. Based on the excellent overall

Table 1.2: *AJCC TNM-classification for thyroid cancer.*[49]

Primary Tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Tumor 2 cm or less in greatest dimension limited to the thyroid
T1a	Tumor 1 cm or less, limited to the thyroid
T1b	Tumor more than 1 cm but not more than 2 cm in greatest dimension limited to the thyroid
T2	Tumor more than 2 cm but nog more than 4 cm in greatest dimension limited to the thyroid

Primary Tumor (T)	
T3	Tumor more than 4 cm in greatest dimension limited to the thyroid or any tumor with minimal extrathyroid extension (e.g., extension to sternothyroid muscle or perithyroid soft tissue)
T4a	Moderately advanced disease Tumor of any size extending beyond the thyroid capsule to invade subcutaneous soft tissues, larynx, trachea, esophagus, or recurrent laryngeal nerve
T4b	Very advanced disease Tumor invades prevertebral fascia or encases carotid artery or mediastinal vessels
Regional Lymph Nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis
N1a	Metastasis to Level VI (pretracheal, paratracheal, and prelaryngeal/Delphian lymph nodes)
N1b	Metastasis to unilateral, bilateral, or contralateral cervical (Levels I, II, III, IV, or V) or retropharyngeal or superior mediastinal lymph nodes (Level VII)
Distant Metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis

survival of all stages of DTC, a tendency exists to de-escalate treatment. In 2009 the American Thyroid Association proposed a risk stratification system in which DTC is stratified in low-risk, intermediate-risk and high-risk subgroups, based on TNM-stage, histologic findings and results of the first posttreatment whole-body RAI scan (Table 1.2 and table 1.3).[9, 23] For patients with low-risk DTC the administered dose of radioactive iodine for the ablation therapy has been reduced and currently it is investigated whether this adjuvant therapy can be abandoned in specific patient groups without compromising survival.[24-26] In this regard, the discussion whether a total thyroidectomy or a lobectomy should be performed for patients with unifocal DTC is gaining attention.[27-29] The rationales for adhering to total thyroidectomy include the presence of contralateral carcinomas, the ability to perform RAI and the use of thyroglobulin as a follow-up marker. Supporters of total thyroidectomy argue that contralateral carcinomas could cause disease recurrence and affect survival.[30, 31] Contralateral carcinomas are reported in up to 44% of patients with DTC.[31] However, this data is mainly based on patients with DTC smaller than 1 cm while data on the incidence of contralateral carcinomas in DTC >1 cm is

Table 1.3: Stage group for DTC

DTC	T	N	M
	Under 45 years		
Stage I	Any T	c	M0
Stage II	Any T	Any N	M1
	45 years and older		
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T3	N0	M0
	T1	N1a	M0
	T2	N1a	M0
	T3	N1a	M0
Stage IVA	T4a	N0	M0
	T4a	N1a	M0
	T1	N1b	M0
	T2	N1b	M0
	T3	N1b	M0
	T4a	N1b	M0
Stage IVB	T4b	Any N	M0
Stage IVC	Any T	Any N	M1

scarce.[32, 33] To facilitate this discussion, we studied in **chapter 7**, in a descriptive manner, the prevalence of contralateral thyroid carcinomas and their histological characteristics in patients with DTC >1 cm in a large international cohort.

Medullary Thyroid Cancer

MTC is a type of thyroid cancer developing from the calcitonin producing c-cells. It occurs either as a sporadic disease or in 20 – 25% of patients in a hereditary context as a manifestation of the endocrine tumor syndrome Multiple Endocrine Neoplasia type 2 (MEN type 2). Hereditary MTC is characterized by activating mutations in the rearranged-during-transfection (RET) proto-oncogene. Sporadic MTCs harbor, in 50% of cases, an acquired mutation in the

RET proto-oncogene.[34] For prognosis, the TNM-staging system is used as shown in table 1.4. About 45% of all patients present with advanced disease, stage III-IV, which has a 10-year survival rate of 71% and 21%, respectively.[35] TNM-stage is still considered as the best prognosticator for survival in MTC patients.[36, 37] The treatment of MTC always comprises a total thyroidectomy with routine central neck lymph node dissection, and lateral neck lymph node dissection on indication. Currently, no adjuvant therapy is routinely advised.

Since calcitonin is only produced in the c-cells it is a valuable marker to diagnose MTC or to detect recurrent MTC. Therefore, calcitonin measurements are part of the diagnostic process and of the regular follow-up.[38] Patients that are considered to be cured are typically identified by undetectable calcitonin levels postoperatively. However, there is a large heterogeneity within the group of patients with TNM-stage III and IV disease and postoperatively elevated calcitonin levels; some show stable disease over years and some are rapidly progressing and metastasizing. Currently, it is not possible to identify those patients who will show rapid progression of disease.

If these patients were identifiable, the treatment strategy for adjuvant therapy could be changed to a more aggressive approach and follow-up should be intensified.

Table 1.4: *Stage group for MTC.*

MTC	T	N	M
Stage I	T1	N0	M0
Stage II	T2	N0	M1
	T3	N0	M1
Stage III	T1	N1a	M0
	T2	N1a	M0
	T3	N1a	M0
Stage IVA	T4a	N0	M0
	T4a	N1a	M0
	T1	N1b	M0
	T2	N1b	M0
	T3	N1b	M0
	T4a	N1b	M0
Stage IVB	T4b	Any N	M0
Stage IVC	Any T	Any N	M1

Currently, tyrosine kinase inhibitors that target RET proto-oncogene are under evaluation, however they seem to fail in improving overall-survival and only gain time in progression-free survival.[39, 40] By obtaining more knowledge about proteins and/or pathways that may influence prognosis of MTC, novel possible therapeutic targets might be identified. In various solid tumors, the importance of hypoxia inducible factor-1 alpha (HIF-1α) and its downstream targets has been investigated, and the correlation of high HIF-1α expression with poor prognosis has been well established.[41] In **chapter 8** we evaluated the prognostic value of HIF-1α in MTC. We show that HIF-1α is able to identify patients with a worse prognosis within TNM Stage IV patients. While calcitonin is a perfect marker to detect the presence of recurrences, the optimal imaging modality to localize these recurrences is not yet identified.

If metastatic disease is expected additional imaging procedures can be performed. Computed Tomography (CT) is the most sensitive imaging procedure to detect lung and mediastinal lymph node metastases. Three-phase contrast-enhanced multi-detector liver CT and contrast-enhanced magnetic resonance imaging (MRI) are the most sensitive methods to detect liver metastases. Axial MRI and bone scintigraphy are complementary and most sensitive to detect bone metastases. The sensitivity of these tests in localizing metastatic disease ranges between 50 – 80%, but it is likely that the sensitivity increases with raising calcitonin levels.[35, 42]

Nuclear imaging modalities such as Positron Emission Tomography – Computed Tomography (PET/CT) combined with 2-18F-fluoro-2-deoxy-D-glucose (FDG), 18F-dihydroxyphenylalanine (FDOPA) or (68)-gallium DOTATATE are under investigation but none of them are currently able to increase sensitivity significantly[43-45]. Thus, there is a need for better diagnostic imaging modalities to identify distant metastases in patients with elevated calcitonin levels without any evidence of disease on additional imaging procedures. Prostate-specific membrane antigen (PSMA) is a transmembrane protein that is gaining attention because of its applicability as a diagnostic marker for imaging and as a therapeutic target. To date, several small compounds for labelling PSMA have been developed and are currently being investigated as imaging probes for (68)Gallium-labelled PET scans [46]. For therapeutic purposes several compounds have been investigated, of which (177)Lutetium-labelled PSMA inhibitors and (225)Actinium-labelled PSMA inhibitors are promising.[47, 48] To investigate whether PSMA imaging and/or therapy might be of interest in MTC, the expression of PSMA in primary tumors and lymph node metastases was evaluated in **chapter 9**. Furthermore, **chapter 9** investigates the prognostic value of PSMA expression in MTC.

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Chapter 2

Same-day Fine Needle Aspiration Cytology diagnosis for thyroid nodules achieves rapid anxiety decrease and high diagnostic accuracy

Lutske Lodewijk, Menno R. Vriens, Wessel M.C.M. Vorselaars, Nick T.M. van der Meij, Jakob W. Kist, Maarten W. Barentsz, Helena M. Verkooijen, Inne H.M. Borel Rinkes, Gerlof D. Valk

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Abstract

Objective: The time between the moment of referral for the diagnostic workup for thyroid nodules and the outcome can be worrisome for patients. In general, patients experience high levels of anxiety during the evaluation of a lesion suspicious for cancer. Therefore, the implementation of same-day fine-needle aspiration cytology (FNAC) diagnosis is becoming standard-of-care for many solid tumors. Our aim was to assess the feasibility of same-day FNAC diagnosis for thyroid nodules and to assess patient anxiety during the diagnostic process.

Methods: For feasibility of same-day FNAC diagnosis, we assessed the proportion of patients receiving a diagnosis at the end of the visit. Accuracy was measured by comparing histology with the FNAC result. Patient anxiety was measured by the State Trait Anxiety Inventory at 6 moments during the diagnostic workup.

Results: Of the 131 included patients, 112 (86%) were female, and the mean age was 53 years. All patients, except those with a nondiagnostic FNAC result (n = 26; 20%), had a diagnosis at the end of the day. There were only two discordant results. Anxiety levels at the beginning of the day were high throughout the group, State Trait Anxiety Inventory (STAI) score 43.1 (SD 2.0) and decreased significantly more in patients with a benign FNAC result (STAI score 30.2), compared to patients with a malignant or indeterminate result (STAI score 39.6).

Conclusion: Distress of patients with a thyroid nodule undergoing same-day FNAC diagnostics was high. Same-day FNAC diagnosis is feasible and accurate for the evaluation of thyroid nodules. Therefore, same-day FNAC diagnosis seems a safer, more patient-friendly approach to diagnose thyroid nodules.

Introduction

The prevalence of palpable thyroid nodules is approximately 5% in women and 1% in men living in iodine-sufficient parts of the world.[1, 2] In the United States, the incidence of thyroid cancer has increased from 3.6 per 100,000 in 1973 to 8.7 per 100,000 in 2002, a 2.4-fold increase, which appears to continue.[3, 4] Since there were an estimated 63,000 new cases in 2014 in the U.S., and only 5 to 15% of all thyroid nodules turn out to be malignant, many patients have to undergo the diagnostic process.[1] The time between the referral for diagnostic workup and communication of the final diagnosis can be long, and this time is predominantly determined by the fine-needle aspiration cytology (FNAC) result. During the evaluation of a lesion suspicious for cancer, patients experience high levels of anxiety.[5] Uncertainty about a cancer diagnosis is stressful and can even be more stressful than waiting for more invasive and potentially high-risk treatments.[6] Stress and anxiety diminish following diagnosis; however, stressful emotions persist longer among patients in whom the diagnostic trajectory had taken longer.[7, 8]
Therefore, clinicians should make an effort to reduce the time of uncertainty about the diagnosis in patients with thyroid nodules. Same-day diagnosis is successfully implemented in the diagnostic process for other solid tumors, such as breast, colorectal, and lung cancer. For these tumors, same-day diagnosis is even becoming standard of care. A recent study on same-day diagnosis for breast cancer showed its feasibility in the vast majority of patients, without impairing diagnostic accuracy of histologic biopsies. In that study, stress and anxiety were evaluated over time by means of questionnaires during the diagnostic process.[9]
The aim of this study was to evaluate the feasibility of same-day FNAC diagnosis for thyroid cancer and patient stress and anxiety during the wait for results.

Methods

All patients referred to the University Medical Center Utrecht (UMC Utrecht) for same-day FNAC diagnosis of a thyroid nodule between October 2011 and September 2014 were included in a prospective database. This study was approved by the Medical Ethics Committee of the UMC Utrecht. Same-day FNAC diagnosis for thyroid nodules was introduced in October 2011. After referral, an endocrinologist decided whether the patient qualified for same-day FNAC diagnosis. Patients were included if they had one or more thyroid nodule(s) larger than 1 cm, with a normal thyroid function and had an indication for FNAC.
Weekly, 2 places for same-day FNAC diagnosis were available on a fixed day. Patients with unknown thyroid function (i.e., thyroid-stimulating hormone (TSH) value), underwent blood sampling prior to the appointment. Patients were first seen by a specialized nurse practitioner under supervision of a senior endocrinologist to obtain medical history, physical examination,

and interpretation of thyroid function. Subsequently, patients with nonsuppressed TSH levels underwent ultrasonography and FNAC by a radiologist. On-site cytologic examination was performed in the first 107 cases. Due to logistical difficulties, from April 2014, every patient underwent 3 FNAC needle passes to diminish nondiagnostic results without on-site evaluation. Cytology results were categorized according to the recommended diagnostic categories by the Bethesda System for Reporting Thyroid Cytopathology.[10] At the end of the day, the final diagnosis and treatment plan were discussed with the patient by the senior endocrinologist, accompanied by the nurse practitioner, and if applicable, an endocrine surgeon.
In the case of a nondiagnostic result, additional FNAC was performed after 4 to 6 weeks. After 3 nondiagnostic attempts, patients were referred for surgery or, in case the patient refused to undergo surgery, follow-up at the endocrinology outpatient clinic was offered. Patients with a benign FNAC without physical complaints either received follow-up within 6 to 12 months or returned to the initial referring physician. Patients with a benign FNAC with physical complaints were referred for surgery. When FNAC showed atypia of undetermined significance (AUS), the treatment plan (either referral for surgery or repeat FNAC) was based on preference of the endocrinologist and patient. Follicular neoplasm, suspicious for malignancy, and malignant cytology results were referred for surgery.

Data Collection

We prospectively collected information on demographic characteristics, medical history, physical examination, ultrasonography, and cytologic outcome. The outcome parameters were feasibility and impact on patient stress and anxiety levels. Feasibility was defined by the proportion of patients receiving a treatment plan at the end of the diagnostic day combined with a high diagnostic accuracy. All FNAC results, except a nondiagnostic result, were interpreted as a diagnosis and/or treatment plan. Diagnostic accuracy was evaluated by concordance between FNAC and histologic diagnosis after resection, if available.

Patient Stress and Anxiety

Between March 2013 and September 2014, stress and anxiety were measured in 42 of 63 patients. Written informed consent was obtained from each participant. Baseline worries for cancer were measured by the Cancer Worry Scale (CWS) and stress and anxiety by the 6-item State Trait Anxiety Inventory (STAI).[11, 12] Patients were asked to fill out the CWS at baseline and the STAI at 6 time points: (1) before the intake by the nurse practitioner, (2) in the radiology waiting room prior to ultrasonography and FNAC, (3) in the waiting room prior to the communication of the final results, (4) 1 day, (5) 3 days, and (6) 7 days after their visit. Patients could choose if and how (i.e., phone call, text message, or email) they preferred to be reminded to fill out the questionnaire.

Statistics

The total score of the CWS was calculated by the sum of the 4 items; for dichotomization, a cut-off value of 7 was used, which was the mean of the sum scores. STAI-scores were based on a 4-point scale, and the score was calculated as the sum scores of the 6 items. The sum scores were recalculated to a score ranging from 20 to 80 to be comparable to the 40-item STAI.[11] Changes in stress and anxiety levels over time were analyzed by a linear mixed-effects model with random intercept and slope for time to account for repeated anxiety scores nested within patients.[13] Repeated anxiety scores were considered nested within patients.

Fixed effects were added for moments in the diagnostic process. Stratified analyses were applied to examine whether time patterns in stress and anxiety levels varied by sex, age, FNAC result, dichotomized CWS, and level of education. STAI levels at baseline were compared with the Student's t test, and multiple linear regression was performed to identify risk factors that could predict high anxiety levels. P values were interpreted as significant when less than .05. Statistical analyses were performed with SPSS software, version 22.0 (IBM SPSS Statistics, Chicago, IL).

Results

Between October 2011 and September 2014, 141 patients were referred for same-day FNAC diagnosis of a thyroid nodule, and 131 patients fulfilled the inclusion criteria. Ten patients were excluded, of whom 6 did not undergo FNAC of the thyroid. Of these, in 1 patient the tumor did not originate from the thyroid, 2 patients had nodules smaller than 1 cm, 2 patients previously underwent ultrasound with benign FNAC and had stable nodule size over time, and 1 patient had an ultrasound showing signs suspicious for thyroiditis. Four patients were excluded because there was no indication for an ultrasound. Of these, 2 patients had a suppressed TSH and underwent scintigraphy, 1 patient was referred to the otolaryngology department, and 1 patient had already undergone ultrasound with FNAC in another hospital.

The included patients consisted of 112 (85.5%) female and 19 (14.5%) male patients, with a mean age of 53.3 years (SD 14.4 years; range 17 to 85 years). The majority of patients were referred because of a thyroid nodule (63 of 131, 48.1%); other reasons were multinodular goiter, positron emission tomography–positive nodule, incidentaloma, second opinion, and growing mass in the neck (Table 2.1).

Outcomes of the FNAC were divided into 6 categories according to the Bethesda System for Reporting Thyroid Cytopatholgy, respectively: nondiagnostic in 26 (19.8%), benign in 81 (61.8%), AUS in 9 (6.9%), follicular neoplasm in 4 (3.1%), suspicious for malignancy in 3 (2.3%), and malignant in 8 (6.1%) patients. The patients with a nondiagnostic FNAC underwent a second FNAC after 4 to 6 weeks. For all other patients, a treatment plan was available at the end of the

Table 2.1: Baseline characteristics of same-day diagnosis patients

	N = 131	%
Sex		
Male	19	14.5
Female	112	85.5
Age in years (mean ± SD, range)	53.3 ± 14.4 (17-85)	
Reason of referral		
Thyroid nodule	63	48.1
Multinodular goiter	33	25.2
PET-positive nodule	11	8.
Incidentaloma	18	13.7
Second opinion	6	4.6
Outcome of 1st FNAC		
I. Nondiagnostic	26	19.8
II. Benign	81	61.8
III. AUS	9	6.9
IV. Follicular neoplasm	4	3.1
V. Suspicious for malignancy	3	2.3
VI. Malignant	8	6.1
Policy after same-day diagnosis		
Referral to surgeon	39	29.8
Follow up endocrinologist	82	62.6
Discharge	4	3.1
Follow-up at other institution	6	4.6
Referred to surgeon		
Yes	49	36.6
No	82	63.4
Histology	n = 49	
Benign	34	69.4
PTC	9	18.4
FTC	3	6.1
MTC	2	4.1
ATC	1	2.0

Abbreviations: ATC = anaplastic thyroid cancer; AUS = atypia of undetermined significance; FNAC = fine-needle aspiration cytology; FTC = follicular thyroid cancer; MTC = medullary thyroid cancer; PET = positron emission tomography; PTC = papillary thyroid cancer.

diagnostic day. Eighty-two (62.6%) of the 131 patients received follow-up at the endocrinology outpatient clinic, 39 (29.8%) were referred for surgery, 4 (3.1%) were referred back to their general practitioner, and 6 (4.6%) received follow-up at another institution. For the evaluation of the feasibility of same-day FNAC diagnosis, this resulted in 80% of the patients receiving a treatment plan at the end of the day; the remaining 20% were all the nondiagnostic cases (Table 2.1).

A total of 49 of 131 (36.6%) patients were referred to the surgeon (39 after the first FNAC, 7 after the second FNAC, and 3 after the third FNAC). Thirty-four (69.4%) of the 49 patients had benign histology. Other diagnoses were 9 (18.4%) papillary thyroid carcinoma (PTC), 3 (6.1%) follicular thyroid cancer (FTC), 2 (4.1%) medullary thyroid cancer, and 1 (2.0%) anaplastic thyroid cancer (Table 2.1). In 2 of 49 (4.2%) patients, a discordant result of FNAC and final histology was found. One FNAC showed follicular neoplasm while histology revealed a 3.3 cm PTC, and 1 FNAC showed benign cytology while histology revealed a 7.5 cm FTC (Table 2.2).
For the evaluation of stress and anxiety levels of patients during the diagnostic process and the week after, we included 39 patients. Mean age was 53.1 years (SD 14.6 years; range, 19 to 80 years). The majority (36 of 39; 92.3%) of patients were female. Outcomes of the FNAC were for categories I-VI, respectively: 4 (9.5%), 29 (74.4%), 3 (7.1%), 1 (2.4%), 0 (0.0%), and 2 (5.1%). The following categories were combined into the group uncertain or malignant FNAC result: nondiagnostic, AUS, follicular neoplasm, suspicious for malignancy, and malignant.
Overall anxiety scores decreased significantly from the first measurement until the last, which was 1 week after the appointment, from 43.1 to 32.6 (P<.001). For patients with a benign FNAC result, STAI scores decreased from 42.7 to 30.2 over a period of 1 week (P<.001). In cases of an uncertain diagnosis or a malignant diagnosis, STAI scores did not decrease and remained relatively stable from 43.7 to 39.6 over a period of 1 week (P = .327). Patients with a CWS score >7 had a significantly higher STAI compared to patients with a CWS score <7 (51.2 versus 35.4; P<.001). The number of patients with a CWS score <7 decreased from 35.4 to 28.0 (P = .004), and the number of patients with a CWS score >7 decreased from 51.2 to 37.5 (P<.001).

Table 2.2: *Concordance between FNAC and histology after surgery*

	Benign	PTC	FTC	MTC	ATC
I. Nondiagnostic	5	-	1	-	-
II. Benign	20	-	**1**	-	-
III. AUS	6	-	1	-	-
IV. Follicular neoplasm	3	**1**	-	-	-
V. Suspicious for malignancy	-	3	-	-	-
VI. Malignant	-	5	-	2	1
Total	34	9	3	2	1

Abbreviations: ATC = anaplastic thyroid cancer; AUS = atypia of undetermined significance; FNAC = fine-needle aspiration cytology; FTC = follicular thyroid cancer; MTC = medullary thyroid cancer; PTC = papillary thyroid cancer.
*Discordant results are shown in bold type.

Table 2.3: Impact of same-day diagnosis on stress and anxiety as measured by the 6-Item STAI

Mean STAI scores (SE)	N	STAI 1 before intake	STAI 2 before US + FNAC	STAI 3 before diagnosis
All patients	39	43.1 (2.0)	44.0 (2.0)	42.8 (2.0)
Benign FNAC	29	42.7 (2.2)	43.1 (2.2)	42.8 (2.2)
Nondefinitive FNAC	10	43.7 (3.8)	46.8 (3.8)	42.1 (3.9)
Score CWS <7	20	35.4 (2.3)	36.8 (2.3)	37.6 (2.3)
Score CWS >7	19	51.2 (2.6)	51.5 (2.6)	47.9 (2.5)
Education: low	15	45.8 (2.5)	46.0 (2.5)	42.7 (2.7)
Education: high	16	43.3 (3.2)	45.0 (3.0)	42.6 (3.0)
Male	3	32.2 (6.3)	42.1 (7.7)	47.2 (7.5)
Female	36	44.0 (2.1)	44.0 (2.0)	42.6 (2.1)
Age ≤45 years	11	48.8 (4.0)	51.6 (4.1)	50.9 (4.1)
Age >45 years	28	40.8 (2.1)	41.0 (2.1)	39.6 (2.1)
Patients visiting the same-day diagnosis for breast lesion[9]				
All patients	127	45.2 (1.2)	42.9 (1.2)	38.4 (1.2)

Abbreviations: CWS = Cancer Worry Scale; dx = diagnosis; FNAC = fine-needle aspiration cytology; NA = not applicable; STAI = State Trait Anxiety Inventory; US = ultrasonography.

Thus, both groups showed a significant decrease over time, but patients with a high CWS score still had relatively high anxiety levels after 1 week. Female patients experienced higher levels of stress than males, and male patients experienced no significant decrease in anxiety levels over time. Patients younger than 45 years had higher anxiety levels (P = .089), and even though a significant decrease was observed, anxiety levels remained higher over time in the young patients (≤45: 37.3 versus >46: 30.6). Level of education did not influence initial STAI levels (Table 2.3).

STAI 4 1 day after dx	STAI 5 3 days after dx	STAI 6 7 days after dx	Overall P value	P value difference STAI 1
32.5 (2.0)	32.5 (2.0)	32.6 (2.2)	<.001	NA
28.1 (2.2)	30.6 (2.1)	30.2 (2.3)	<.001	.871
44.6 (3.7)	38.4 (3.8)	39.6 (4.9)	.327	
30.1 (2.3)	29.5 (2.2)	28.0 (2.4)	.004	<.001
35.3 (2.6)	35.7 (2.8)	37.5 (3.6)	<.001	
31.3 (2.6)	32.2 (2.7)	33.3 (3.6)	<.001	.599
34.3 (3.0)	32.8 (2.9)	31.8 (3.2)	<.001	
28.5 (7.4)	33.0 (7.5)	32.0 (10.2)	.546	.144
32.7 (2.1)	32.4 (2.1)	32.4 (2.3)	<.001	
35.3 (3.9)	35.7 (3.8)	37.3 (3.9)	<.001	.089
31.6 (2.2)	31.3 (2.2)	30.6 (2.7)	<.001	
32.2 (1.3)	30.7 (1.3)	30.0 (1.5)	<.001	NA

Discussion

The present study suggests that same-day FNAC diagnosis is feasible, as faster diagnostics did not interfere with diagnostic accuracy based on the observation of only 2 (4.2%) discordant findings. Eighty percent of patients received a diagnosis at the end of the day; the remaining 20% of patients had a nondiagnostic FNAC result. Furthermore, we are the first to show that anxiety levels are high in patients with a thyroid nodule, and in cases of a benign FNAC result, decrease rapidly after receiving the diagnosis. Therefore, rapid assessment of patients with a thyroid nodule should be pursued.

From our data, we conclude that same-day FNAC diagnosis is feasible with high diagnostic accuracy. The rate of patients with a nondiagnostic FNAC result might seem relatively high; however, rates in the literature range between 2 and 34%.[14-21] The rate of false-negative FNAC was 4.2% in this study, which is lower than reported in the literature.[22-24] In concordance with

previously reported literature, we found that the 2 discordant findings were in lesions larger than 3 cm.[23, 24]

Patients with a nondiagnostic FNAC result still have a lesion suspicious for cancer, and they can therefore have a high level of anxiety. Although in earlier reports it was suggested not to repeat a nondiagnositic FNAC sooner than 3 months, we performed a new FNAC after 4 to 6 weeks in case of a nondiagnostic FNAC. This is in line with what is suggested in the most recent American Thyroid Association guidelines.[25] However, in cases in which the patient history, physical examination, and ultrasound findings give a very low likelihood of a malignancy, after informing the patient, a joint decision to repeat FNAC after 8 to 12 weeks is also suitable.

On a scale ranging from 20 to 80, the average anxiety score before the intake was 42.5 (SE 1.9), which decreased significantly to 32.1 (SE 2.2) after same-day FNAC diagnosis and stabilized during the week (Table 2.3), indicating that same-day FNAC diagnosis improves patient comfort and is easily implementable.

When stratifying for benign FNAC results and uncertain or malignant FNAC, we found that anxiety levels in patients with a benign FNAC decreased rapidly, whereas patients with an uncertain or a malignant diagnosis maintained high STAI scores throughout the week.

This clearly indicates that effort should be made to alleviate this uncertainty as soon as possible by having short periods between the follow-up appointment, referral appointment with the surgeon (preferably on the same day), and the date of surgery.

Since thyroid cancer generally has a good prognosis, one can empathize that patients experience less anxiety and distress compared to patients undergoing diagnostics for other malignancies. In our institution, STAI levels were also used to evaluate anxiety during same-day diagnosis for breast cancer using similar time points.[9] Although the chance for a female patient to develop breast cancer is much higher in comparison to thyroid cancer and overall survival rates for patients with thyroid cancer are better (overall 5-year survival rate of 89% versus 98%), STAI levels were comparable throughout the diagnostic processes.

This implies that either favorable clinical outcomes of patients with thyroid cancer do not influence anxiety levels or that patients are unaware of these numbers. In another study, pain and anxiety levels prior to FNAC of the thyroid were evaluated, measured by a 20-item STAI questionnaire. The researchers found levels comparable to the general population and therefore hypothesized that this was due to a low perception of cancer risk.[26] However, in contrast to our longitudinal assessment of anxiety throughout the diagnostic process, this study only cross-sectionally measured anxiety levels of patients and is therefore not able to follow anxiety levels over time.

Eskander et al. investigated psychological morbidity of patients on the waiting list for (hemi) thyroidectomy.[27] They found that patients have mild to moderate psychological morbidity, which decreases after surgery, irrespective of the FNAC result. This is in line with our finding that shows that patients having uncertain or malignant FNAC results maintain high anxiety

levels, and it underlines that certainty about the diagnosis should be pursued as soon as possible.

Conclusion

In conclusion, we found high levels of distress in patients with a thyroid nodule undergoing same-day FNAC diagnostics, with levels comparable to patients undergoing same-day diagnostics for breast cancer. When the result of the FNAC was benign, anxiety levels decreased rapidly, showing quick relief of anxiety for this patient population. Furthermore, we showed that same-day FNAC diagnosis is feasible and accurate for the evaluation of thyroid nodules. Therefore, same-day FNAC diagnosis seems a safe, more patient-friendly approach to diagnosing thyroid nodules.

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Chapter 3

Thyroid incidentalomas in patients with Multiple Endocrine Neoplasia type 1

Lutske Lodewijk, Pim J. Bongers, Jakob W. Kist, Elfi B. Conemans, Joanne M. de Laat
Carla R.C. Pieterman, Anouk N.A. van der Horst-Schrivers, Ciska Jorna, Ad R. Hermus, Olaf M. Dekkers
Wouter W. de Herder, Madeleine L. Drent, Peter H. Bisschop, Bas Havekes, Inne H.M. Borel Rinkes
Menno R. Vriens, Gerlof D. Valk

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Abstract

Objective: Currently, little is known about the prevalence of thyroid tumors in multiple endocrine neoplasia type 1 (MEN1) patients and it is unclear whether tumorigenesis of these thyroid tumors is MEN1-related. The aim of the study was to assess the prevalence of thyroid incidentalomas in MEN1 patients compared to non-MEN1 patients and to verify whether thyroid tumorigenesis is MEN1-related.

Design: A cross-sectional study.

Methods: The study included two groups: patients with MEN1 and a matched non-MEN1 control group without known thyroid disease, who underwent ultrasound of the neck for the localization of parathyroid adenoma. Ninety-five MEN1 patients underwent ultrasound of the neck and were matched on gender and age with non-MEN1 patients. The prevalence of thyroid incidentalomas described in the ultrasound report was scored. Multinodular goiters, solitary nodes and cysts were scored as incidentalomas. Presence of nuclear menin expression was evaluated by menin immunostaining of the thyroid tumors.

Results: In the MEN1 group 43 (45%) patients had a thyroid incidentaloma compared to 48 (51%) in the non-MEN1 group, of which 14 (15%) and 16 (17%), respectively, were solitary nodes. Menin was expressed in the nuclei of all evaluated thyroid tumors.

Conclusions: MEN1 patients do not have a higher prevalence of thyroid incidentalomas compared with primary hyperparathyroidism patients without the diagnosis of MEN1. Menin was expressed in the thyroid tumors of MEN1 patients.

Introduction

Multiple Endocrine Neoplasia type 1 (MEN1) syndrome is characterized by the combined occurrence of pituitary tumors, primary hyperparathyroidism, pancreatic and duodenal neuroendocrine tumors (NET), adrenal adenomas, and NETs of stomach, lung and thymus.[1] Recently, MEN1 also turned out to be a breast cancer susceptible syndrome.[2] The syndrome is caused by an inactivating germline mutation in the MEN1 gene, wich encodes for the tumor suppressor protein menin. Tumorigenesis of MEN1-related tumors is characterized by loss of menin expression or the production of nonfunctional menin in case of missense (or in-frame) alterations of the MEN1 gene.[3] At present, little is known about the prevalence of thyroid tumors in MEN1 patients. Marx et al. found a prevalence of 12% thyroid tumors (8% follicular adenoma and 5% papillary thyroid carcinoma) in 130 MEN1 patients. These patients were screened for all types of endocrine abnormalities.[4] The recent published MEN1 guideline reports that thyroid tumors (adenomas, colloid goiters and carcinomas) occur in more than 25% of patients with MEN1. Subsequently, the guideline states that 'because of the high prevalence of thyroid abnormalities in the general population the association of thyroid abnormalities in MEN1 may be incidental and not significant'.[1] However, the lack of evidence regarding the clinical relevance of thyroid tumors might cause an extra dilemma for both endocrinologist and endocrine surgeon treating patients with MEN1.

Primary hyperparathyroidism (pHPT) occurs in 90% of the MEN1 patients. Therefore, a substantial part of this population undergoes a neck ultrasound to localize parathyroid adenomas.[5]

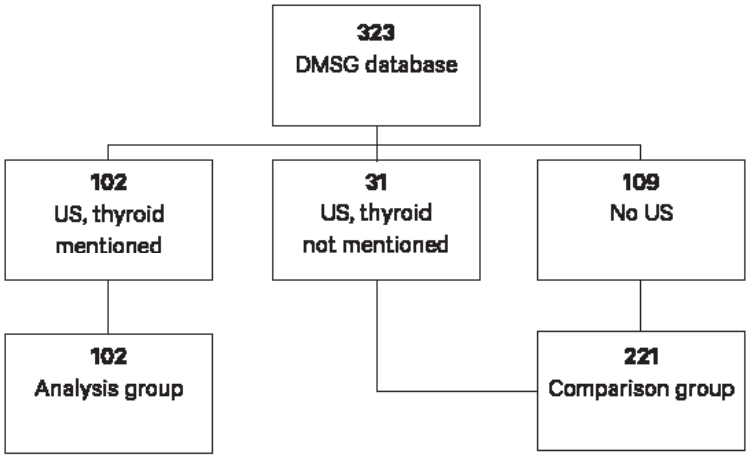

Figure 3.1: *Flowchart of patients from the DMSG database. US, neck ultrasound.*

Table 3.1: Baseline comparison between MEN1 patients with or without neck ultrasound.

	Comparison group (n= 221)	Analysis group (n= 102)	p-value
Female, n (%)	120 (54.3)	68 (66.7)	0.04
Age, mean in years (SD)	46.7 (16.5)	51.9 (14.8)	0.01
Follow-up, mean in years (SD)	10.2 (9.9)	10.5 (8.5)	0.82
pHPT, n (%)	161 (73.6)	102 (100.0)	0.00
Type of mutation, n (%)			
Clinical diagnoses*	15 (6.8)	15 (14.7)	
Nonsense	30 (13.6)	18 (17.6)	
Missense	43 (19.5)	18 (17.6)	
Frameshift	68 (30.8)	32 (31.4)	0.29
Splice	12 (5.4)	2 (2.0)	
Unclassified	2 (0.9)	0 (0.0)	
Large deletions**	48 (21.7)	15 (14.7)	
Unknown***	3 (1.4)	2 (2.0)	

*Clinical diagnosis are patients with 2 or more of the major manifestations of MEN1 without a germline mutation **Large deletions include in-frame deletions, deletions of exon 1 and 2, deletions of exon 1, 2, and 3 and deletions of the entire MEN1 gene. ***Unknown consists of patients with clinical diagnosis of MEN1 in whom either no genetic testing is performed or the exact location of the mutation is unknown. Abbreviations: SD, standard deviation; pHPT, primary hyperparathyroidism.

Because of to the anatomical relationship between thyroid and parathyroid glands it is inevitable that the thyroid is imaged during the neck ultrasound, which increases the chance of incidentally finding a thyroid tumor.

The aim of this study was to assess the prevalence of thyroid incidentalomas in the Dutch MEN1 population compared with a matched reference group of non-MEN1 patients. To support the epidemiologic findings we studied menin expression in thyroid tumors of MEN1 patients by immunohistochemistry to assess whether loss of nuclear menin was present.

Subjects and Methods

Study group

All MEN1 patients in the Dutch MEN1 Study Group (DMSG) database were identified as described previously (325 patients).[6] From this database, data regarding demographics, mutation status (according to the Human Genome Variation Society nomenclature), MEN1 manifestations, imaging, surgery, and histology reports were extracted.[7] For further analysis

patients were selected who had a neck ultrasound because of pHPT in which the thyroid was described (102 patients) (Figure 3.1). The baseline characteristics of 102 patients were compared with the other MEN1 patients to verify whether it was a representative subgroup (Table 3.1).

As a non-MEN1 reference group, 201 consecutive patients who underwent neck ultrasound between 2003 and 2012 for pHPT, not having MEN1 or known thyroid disease, were identified from the hospital radiology database of the University Medical Centers of Utrecht and Groningen in The Netherlands. This reference group will further be referred to as the non-MEN1 group. As age and gender differed significantly in the MEN1 and the non-MEN1 group, patients were matched (1:1) on these variables via the 'case-control matching' extension in SPSS. For age, a spread of 3 years was accepted for the matching. In total 95 patients could be matched. Seven MEN1 patients had to be excluded because no match was available. These consisted of five females and two men with a median age of 21 years, ranging from 15 to 33 years. Of those seven patients, two patients had a cyst.
Multinodular goiters, solitary nodes and cysts that were identified by the ultrasounds of the neck were scored. By definition these tumors are incidentalomas.

Immunohistochemistry
As proxy, for menin expression, immunohistochemistry was performed on formalin-fixed paraffin-embedded (FFPE) tissues from five thyroid samples and a negative control sample. However, certain types of the MEN1 mutations do not lead to an altered expression of menin, but due to these mutations there is a nonfunctional protein. Therefore, we listed the mutations per sample in supplementary table 3.1, see section on supplementary data given at the end of this article. All thyroid tumors were selected and evaluated by a dedicated pathologist (PJvD). As a negative control, we used a sample in which, by sequencing of the DNA, loss of heterozygosity (LOH) was proven. This sample was from a patient with infiltrative ductal carcinoma of the breast with a germline nonsense mutation (c.377G>A(p.Trp126X)).2 All tissues were sampled from surgical specimen according the standard procedure in the University Medical Center Utrecht. The slides were deparaffinized with xylene and rehydrated in decreasing ethanol dilutions. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Antigen retrieval was achieved by boiling slides in citrate buffer (pH 6.0) for 20 min. The slides were then incubated with the rabbit polyclonal antibody against menin (Menin, A300-105A, Bethyl Laboratories, Inc., Montgomery, TX, USA), dilution 1:1600, for 1 hour at room temperature.
For detection of primary antibodies, goat anti-mouse poly-HRP (Powervision, Immunologic, Immunovision Technologies, Brisbane, CA, USA) was used. All slides were developed with diaminobenzidine (DAB). The slides were counterstained with filtered hematoxylin, dehydrated

through a graded series of ethanol, immersed in xylene and mounted. Menin staining was reviewed by an experienced pathologist and compared with the negative control.

Statistical analysis

Continuous variables are expressed as means with a standard deviation S.D. if normally distributed and as median (25% and 75% percentile) if not. Categorical and dichotomous variables are expressed as absolute numbers (%). Matching was performed by the case-control-matching function available in SPSS. Student's t-test, Mann-Whitney U test, and Pearson's $\chi 2$ test were used where appropriate. Statistical significance was reached when P value was smaller than 0.05. Calculations were performed using SPSS/PC version 23.0.

Results

Baseline comparison

The presence of thyroid was mentioned in the report of the neck ultrasound in 102 patients (32%) of a total of 323 MEN1 patients. In 31 (10%) patients, an ultrasound was performed but the presence of thyroid was not mentioned in the report. No ultrasound was performed between 1990 and 2010 in 190 (59%) MEN1 patients (Figure 3.1). Patient characteristics of the groups with and without a neck ultrasound were compared with baseline characteristics (Table 3.1). The group that underwent ultrasound of the neck consisted of more female patients (68 (66.7%) vs 120 (54.3%)) and was significantly older (51.9 (14.8) vs 46.7 (16.5)). There was no difference in mean follow-up time and the type of mutation between the groups with and without a neck ultrasound.

Thyroid incidentalomas

In 43 MEN1 patients (45%) and in 48 non-MEN1 patients (51%), incidentalomas of the thyroid were found on neck ultrasound. The tumors consisted of 25 (26%) and 29 (31%)

Table 3.2: *Thyroid incidentalomas in MEN1 patients compared to a matched control group.*

	MEN1 n=95	**non-MEN1 n= 95**	**p-value**
Female, n (%)	63 (66)	63 (66)	-
Age at date of ultrasound, mean (SD)	48.3(14.3)	46.6(13.8)	-
Incidentaloma, n (%)	43 (45)	48 (51)	NS
Multinodular goitre, n (%)	25 (26)	29 (31)	NS
Solitary node, n (%)	14 (15)	16 (17)	NS
Cyst, n (%)	4 (4)	4 (4)	NS

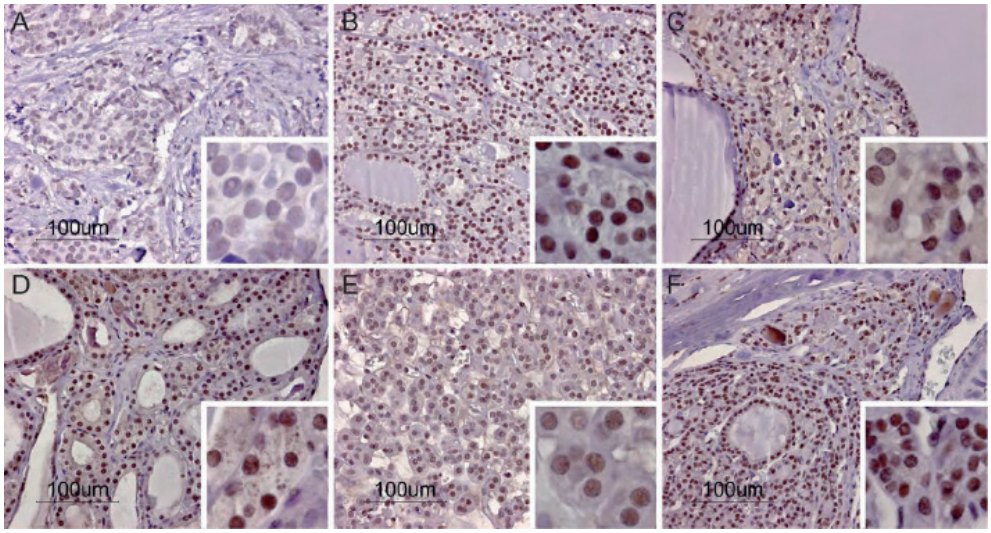

Figure 3.2: *Immunohistochemical analysis of menin protein expression in the nuclei of 5 thyroid tumors from MEN1 patients and absence of expression in one infiltrating ductal carcinoma of the breast from a MEN1 patient with proven LOH. Pictures are taken with a 20x magnification, inlays with 40x magnification. A. infiltrative ductal carcinoma of the breast; B. hyperplastic node; C. micro-invasive medullary thyroid carcinoma; D. multinodular goiter; E. follicular adenoma; F. micro-invasive follicular thyroid carcinoma.*

multinodular goiters, 14 (15%) and 16 (17%) solitary nodes, four (4%) and four (4%) cysts in the MEN1 group and the non-MEN1 group, respectively. No significant differences were found (Table 3.2). When reported, size of the solitary nodes was also analyzed. The median size of the solitary nodes was 6 mm (Interquartile range (IQR) 4.5 – 11 mm) in the MEN1 group and 8 mm (IQR 4.0 – 9.0 mm) in the non-MEN1 group it was (P value 0.94).

Thyroid histology

From 17 MEN1 patients, the histology reports of the thyroid tumors were available and the diagnoses are given in table 3.3. Follicular adenomas and nodular dysplasia were most prevalent. Immunohistochemistry was performed in a representative subset of the different types of thyroid tumors. In all thyroid tumors, we tested whether menin was present by immunohistochemical staining in the nucleus of adjacent normal and tumor tissues. In the control sample, no menin expression was found, indicating loss of heterozygosity (Figure 3.2 and Supplementary Figure 3.1, see section on supplementary data given at the end of this article).

Table 3.3: *Diagnoses of the thyroid tumors after histologic examination in MEN1 patients.*

N = 17	
Multifocal micro-invasive medullary thyroid carcinoma	1
Micro-invasive follicular thyroid carcinoma	1
Follicular adenoma	4
Multinodular goiter	2
Nodular dysplasia	5
Nodular hyperplasia	3
Lymphocytic thyroiditis	1

Discussion

The results of this study show that the prevalence of thyroid incidentalomas in patients with MEN1 is equal to a matched reference group with non-MEN1 patients. These results are in-line with the suggestion in the recently updated guideline, that the high percentage (25%) of thyroid tumors occurring in MEN1 patients is incidental and not significant.[1] These epidemiologic results are strongly supported by the immunohistochemistry, which show a positive menin staining indicating the presence of intact nuclear menin expression in a representative subset of thyroid tumors found in patients with MEN1.

The non-MEN1 patients were considered the best available control group facing the fact that a neck ultrasound was performed for the same indication as in the MEN1 patients. As MEN1 patients present with pHPT at a young age, not all MEN1 patients could be matched. Also in this young patient group a very low prevalence of thyroid incidentalomas was found which is in line with what one can expect in the general population.

In literature, prevalence rates for solitary nodes in healthy individuals are around 10% compared to 15% in our study.[8-11] Owing to the retrospective character of the study, all patients (n=31) who underwent a neck ultrasound in which the thyroid was not mentioned in the report were excluded. If we assume that there was no solitary node in those 31 neck ultrasounds, our prevalence would be similar (11%) to the prevalence rates reported in literature.

From 17 MEN1 patients, histology reports were available from thyroid tumors found by

ultrasound. Of those 17 patients, one patient had a microinvasive medullary thyroid carcinoma and one patient had a microinvasive follicular thyroid carcinoma, and the other 15 showed benign pathology. MEN1-related tumors are characterized by loss of the second allele of MEN1 gene, encoding for the protein menin, resulting in no functional copies of the gene.[12] In four case series of MEN1 patients with thyroid carcinoma, loss of heterozygositiy (LOH) was examined. The results did not show any LOH which indicates no etiological relation between the presence of MEN1 mutation and thyroid carcinoma.[13-16] We assessed loss of menin expression by immunohistochemistry in a representative subset of diagnoses; in all evaluated tissue menin was expressed throughout the tumor and adjacent normal thyroid tissue. This indicates that there is no haploinsufficiency, i.e. the intact copy of the MEN1 gene produces enough protein to bring about a WT condition.

It is a clinical challenge for both endocrinologists and surgeons to deal with thyroid incidentalomas in MEN1 patients. On the one hand, the burden of the patient needs to be as low as possible, and on the other hand, malignancies need to be identified and treated as early as possible. Our results indicate that in case of a thyroid incidentaloma in MEN1 patients, prevailing guidelines for thyroid incidentalomas in the general population can be followed.

In conclusion, our results show no difference in the prevalence of thyroid incidentalomas in MEN1 patients compared with patients with pHPT without the diagnosis of MEN1.

The epidemiologic findings were validated by menin expression in the nuclei.

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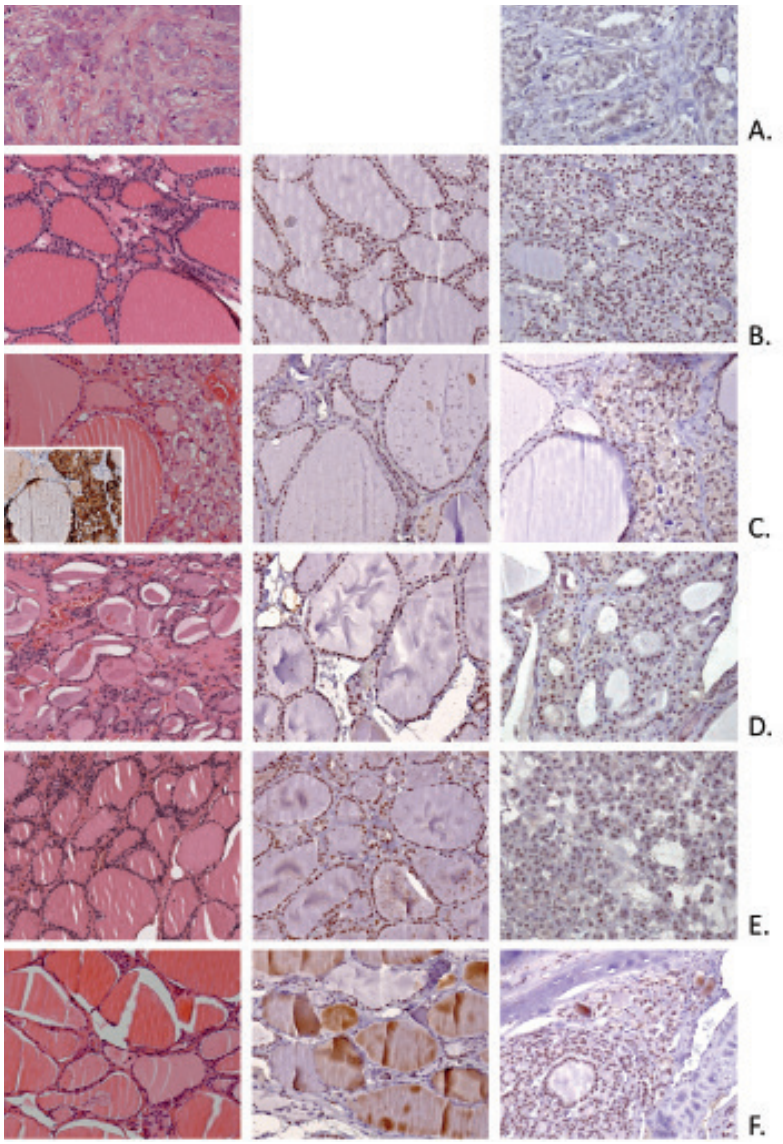
Supplementary table 3.1: *Type of germline mutation of MEN1 patients used for menin immunohistochemistry and whether altered protein expression was expected based on the mutation.*

Patient	Diagnosis	Type mutation	Expect altered protein expression	Mutation
1	Nodular hyperplasia	Missense	No	c.552G>T(p.Glu184Asp)
2	Follicular adenoma	Nonsens	Yes	c.1594C>T(p.Arg532X)
3	Micro-invasivie medullary thyroid cancer	Frameshift	Yes	c.1430dupG(p.Glu478fs)
4	Multinodular goiter	Nonsens	Yes	c.1099A>T(p.Lys367X)
5	Micro-invasive follicular thyroid carcinoma	In-frame deletion	No	c.358_360del (p.Lys-120del)
6	Invasive ductal breast carcinoma	Nonsens	Yes	c.377G>A(p.Trp126X)



Supplementary figure 3.1: *Additional immunohistochemical pictures of patients presented in figure 2. Left column shows H&E staining, middle column shows menin staining in normal thyroid tissue, right column shows menin staining in thyroid tumor. A. infiltrative ductal carcinoma of the breast; B. hyperplastic node; C. micro-invasive medullary thyroid carcinoma (inlay is calcitonin staining); D. multinodular goitre; E. follicular adenoma; F. micro-invasive follicular thyroid carcinoma.*

Chapter 4

Oncogenesis in thyroid nodules

Lutske Lodewijk, Inne H.M. Borel Rinkes, Menno R. Vriens

In: The textbook of Endocrne Surgery. 3rd ed. New Delhi: Jaypee Brothers MP; 2016. p 503-514.

Introduction

Thyroid cancer can be divided into four main subtypes: papillary, follicular, anaplastic and medullary thyroid cancer. The latter is derived from the C cells, the preceding three subtypes are derived from follicular cells. This chapter will discuss the oncogenesis of follicular derived tumors. In the last decade, this field has been fast evolving, this is mainly due to technological advances. With the wide application of more high throughput assays for genetic alterations, miRNA screens, tyrosine kinase deregulations and many more, it has become easier and cheaper. This has generated a tremendous amount of data that may be more or less relevant. This data has to be interpreted with caution, but, when interpreted in the right way it is of incredible value.[1] In general, three models of thyroid nodule oncogenesis have been proposed. These three models are briefly discussed below.

Multistep Carcinogenesis model:

This classic multistep carcinogenesis model suggests that mature thyroid follicular cells can transform into well-differentiated thyroid cancer cells, and in its turn can progress into undifferentiated thyroid cancer cells (Figure 4.1 and figure 4.3C).[2]

Risk factors, such as exposure to radiation, induce genomic instability through direct and indirect mechanisms, resulting in early genetic alterations that involve the mitogen-activated protein kinase (MAPK) signaling pathway. Oncogenic activation of MAPK signaling further increases genomic instability, leading to later genetic alterations that involve other signaling pathways, cell-cycle regulators and various adhesion molecules.[3] Based on the multistep carcinogenesis theory, follicular carcinomas are generated from follicular adenomas and papillary carcinomas from precursor cells generated from thyrocytes. Anaplastic carcinoma may develop from papillary or follicular thyroid carcinoma by dedifferentiation.[4] The various factors involved in the development and progression of follicular cell derived thyroid cancers are summarized in figure 4.1B, and will be reviewed more extensively in the next sections of this chapter.

Fetal cell Carcinogenesis model:

The fetal cell carcinogenesis model postulates that thyroid cancer cells are derived from normal stem cells or precursor cells of fetal thyroid cell origin rather than from mature thyroid follicular cells (Figure 4.2 and figure 4.3B). This model emphasizes the preexistence of a hierarchy of fetal thyroid cells within the thyroid gland that can give rise to different forms of thyroid cancer. As a consequence, the thyroid cancer cells derived by this mechanism will have a gene expression profile similar to that of fetal thyroid cells.[2] In this model three precursor cells for papillary, follicular and anaplastic thyroid cancer are identified. Prothyrocytes, thyroblasts and thyroid stem cells are identified by gene expression profiles. Two genes are essential

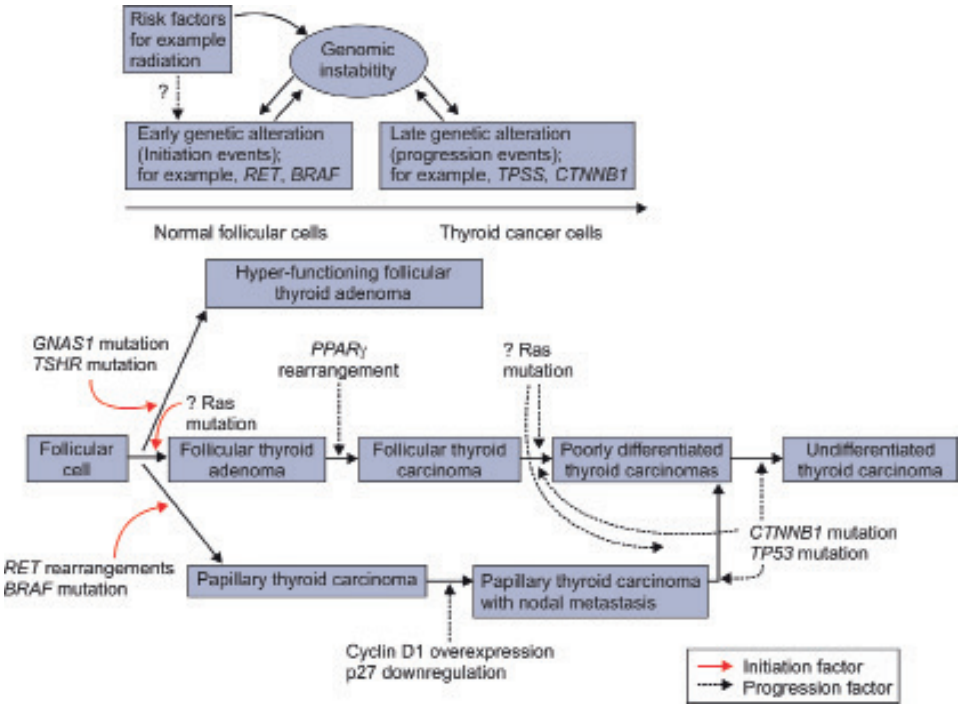


Figure 4.1 A and B: *(A) Model of multi-step carcinogenesis of thyroid neoplasms. The proposed model of thyroid carcinogenesis is based on general concepts and specific pathways. (A) Risk factors, such as exposure to radiation, induce genomic instability through direct and indirect mechanisms, resulting in early genetic alterations that involve the mitogen-activated protein kinase (MAPK) signaling pathway. Oncogenic activation of MAPK signaling further increases genomic instability, leading to later genetic alterations that involve other signaling pathways, cell-cycle regulators and various adhesion molecules. Accelerating the interactions between genomic instability and genetic alterations promotes progression from well-differentiated to undifferentiated thyroid carcinoma. (B) On the basis of clinical, histological and molecular observations, three distinct pathways are proposed for neoplastic proliferation of thyroid follicular cells, including hyper-functioning follicular thyroid adenoma (tumors that are almost always benign lesions without a propensity for progression), follicular thyroid carcinoma and papillary thyroid carcinoma. Genetic defects that result in activation of RET or BRAF represent early, frequent initiating events that can be associated with radiation exposure. Underexpression of the cyclin-dependent-kinase inhibitor p27KIP1 and overexpression of cyclin D1 are strong predictors of lymph-node metastases in papillary thyroid carcinomas. Most poorly differentiated and undifferentiated thyroid carcinomas are considered to derive from pre-existing well-differentiated thyroid carcinoma through additional genetic events, including β-catenin (which is encoded by CTNNB1) nuclear accumulation and p53 inactivation, but de novo occurrence might also occur. (GNAS1: Guanine nucleotide-binding α-subunit 1; PPARγ: Peroxisome proliferation-activated receptor-γ; TSHR: Thyroid-stimulating-hormone receptor). Adapted with permission from Kondo et al.*[3]

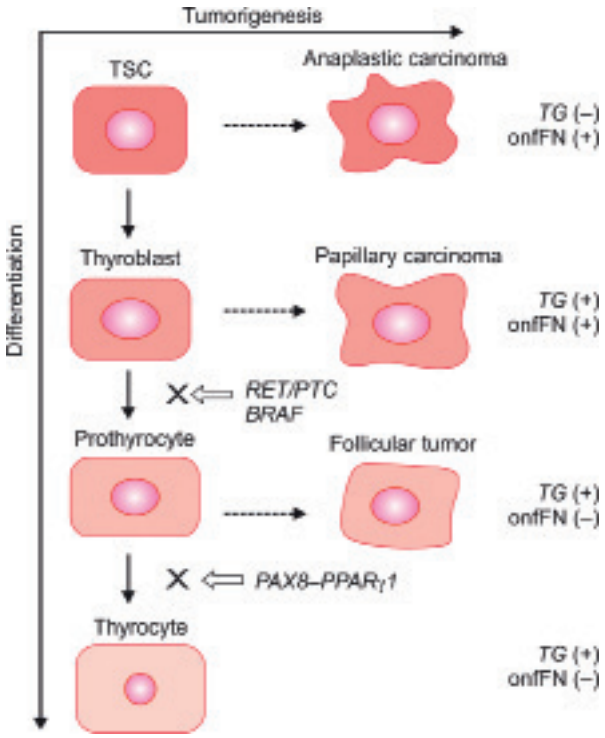


Figure 4.2: *The role of oncogenes in fetal cell thyroid carcinogenesis. Anaplastic, papillary and follicular carcinomas are derived from TSCs, thyroblasts and prothyrocytes, respectively. BRAF mutations and rearrangements of the RET (RET/PTC) prevent the differentiation of thyroblasts into prothyrocytes, resulting in the generation of papillary carcinomas, and the PAX8-PPARγ 1 rearrangement prevents differentiation of prothyrocytes into thyrocytes, resulting in the generation of follicular tumors. Adapted with permission from Takano.*[5]

thyroglobulin (Tg) and oncofetal fibronectin (onfFN). Prothyrocytes that are Tg positive and onfFN negative can transform to follicular thyroid carcinoma, thyroblasts that are Tg positive and onfFN positive can transform to papillary thyroid carcinoma and thyroid stem cells that are Tg negative and onfFN positive can transform to anaplastic thyroid carcinoma.[5] However, it must be realized that all articles published supporting this model are from the same investigators.

Thyroid Cancer Stem Cells:

The cancer stem cell model predicts that only a subset of cancer cells possess the ability to self-renew and produce progenitor cells that can reconstitute and sustain tumor growth (Figure 4.3C). Cancer stem cells can divide symmetrically or asymmetrically and are multipotent. The cancer stem cell model is, therefore, an attractive way to account for the functional diversity commonly found in thyroid cancer.[2] CD133 is used as cancer stem cell marker in many types of solid malignancies. The CD133 positive cells are believed to be cancer stem cells. In thyroid cancer, CD133 positive cells were identified in two of four anaplastic thyroid carcinoma cell lines available (ARO and KAT-4).[6, 7] The in vitro and in vivo experiments were very promising; however, in 2008 20 out of 40 cell lines were identified to be cross-contaminated with cell lines from other organs. The previously mentioned cell lines, ARO and KAT-4, were

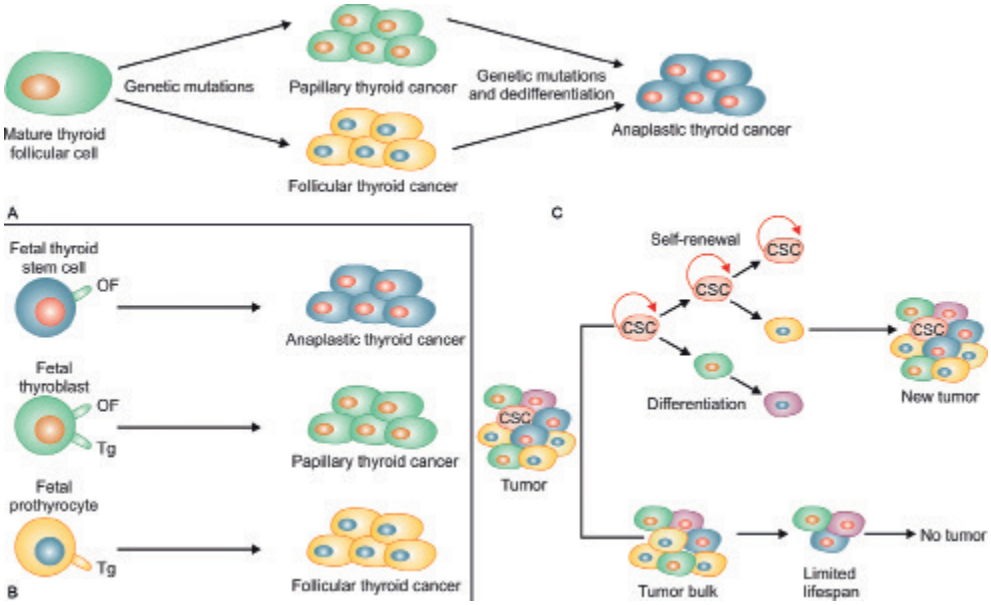


Figure 4.3: *Models of thyroid cancer development. (A) A multistep carcinogenesis model suggests that well-differentiated papillary and follicular thyroid cancer cells can transform into undifferentiated anaplastic thyroid cancer cells via multiple genetic mutations and dedifferentiation processes. (B) A fetal cell carcinogenesis model postulates that three types of precursor cells of fetal thyroid cell origin within the adult thyroid gland generate the three subtypes of thyroid cancer. (C) A cancer stem cell model predicts that tumors are organized in a hierarchy of different tumor cells and that only a subset of cancer stem cells can initiate and sustain the tumor. (CSC: Cancer stem cell; OF: Oncofetal fibronectin; Tg: Thyroglobulin).*
Adapted with permission from Lin[2]

also included in this article and seemed to be cross contaminated with a colon cancer cell line.[8] New side populations were identified that expressed stem cell markers such as Oct-4, HES1, JAG1, MYC, JUN or FZD5. Nevertheless, in in vivo experiment both populations were able to form subcutaneous tumors; this suggests that stem cell properties are not restricted to these markers. Thyroid cancer tissue obtained at the time of thyroidectomy from patients with follicular, papillary and anaplastic thyroid cancer contain a small population of ALDH positive cells. When these ALDH positive cells are cultured in vitro and in vivo they form tumors with features comparable to that seen in patients.[9] The existence of thyroid cancer stem cells has important implications in understanding whether and how they drive disease, and whether they facilitate tumorigenesis and metastasis. This knowledge will help us to develop improved assays for drug discovery and toxicity, to prevent metastasis, and to design new intervention

strategies for the disease.[2] Whether one or a combination of the models discussed is correct, is at this point unclear. However, for the concept of tumor formation this should be kept in mind and in the future more effort should be made to clarify this.

Genetics

BRAF:

BRAF is a serine-threonine kinase that is translocated to the cell membrane after being bound and activated by RAS, which results in the phosphorylation and activation of MAPK kinase and other downstream targets of the MAPK signaling pathway.[10] Gain of function BRAF mutation provides an alternative route for the aberrant activation or ERK signaling that is implicated in the tumorigenesis of several human cancers.[3] The most common alteration in sporadic papillary carcinoma is the BRAF(V600E) mutation. In follicular variant papillary carcinomas, the BRAF(K601E) mutation is regularly found and in a solid variant of papillary carcinoma the in frame VK600-1E deletion (BRAF(VK600-1E)) has been detected. This indicates a possible genotype-phenotype correlation.

The clinical impact of the presence of a BRAF mutation is well reviewed. In a meta-analysis, the associations between BRAF mutation and race, age, gender, size, histologic subtype, extrathyroidal growth, metastasis and stage have been studied. This study indicated that BRAF mutation in PTC is associated with histological subtype, extrathyroidal extension and advanced clinical stage, but is not correlated with race, patient age, gender or tumor size in patients with PTC. This suggests that the presence of a BRAF mutation is an important and useful prognostic molecular marker.[11] In another report BRAF mutation was an independent predictor of tumor recurrence, even in patients with stage 1 and 2 disease.[12] Importantly, BRAF mutations have also been associated with the decreased ability to trap I-131 and treatment failure in recurrent disease.[12-14] In PTC, a BRAF mutation is present in approximately 40-45% of tumors, but it has been reported in up to 80% of tumors.[15] However, in anaplastic thyroid carcinoma, the frequency is remarkably lower than in PTC, around 20-40% (Table 4.1).[10]

In light of the “multistep carcinogenesis model” this is a notable finding, since anaplastic thyroid carcinoma is thought to be a dedifferentiated form of PTC and BRAF mutation is thought to be an event in carcinogenesis. Proponents of the other alternative theories, use this as one argument against the “multistep carcinogenesis model”. BRAF(V600E) testing on fine-needle aspiration (FNA) cytology has been evaluated in several studies and in a metareview. The consensus is that testing for BRAF mutation alone in FNA specimens is unlikely to solve the dilemma of indeterminate cytology. When all studies are taken together only 17% of all indeterminate cytology results that turned out to be cancer by histology can be diagnosed by BRAF mutational analysis.[16]

Table 4.1: Genetic defects in thyroid cancer.

Genetic alteration	Well-differentiated thyroid carcinoma		Poorly differentiated thyroid carcinoma	Undifferentiated thyroid carcinoma	Post-chernobyl childhood thyroid cancer
	Papillary thyroid carcinoma	Follicular thyroid carcinoma			
RET rearrangement	13 - 43%	0%	0 - 13%	0%	50 - 90%
BRAF mutation	29 - 69%	0%	0 - 13%	10 - 35%	0 - 12%
BRAF rearrangement	1%	Unknown	Unknown	Unknown	11%
NTRK1 rearrangement	5 - 13%	Unknown	Unkown	Unknown	3%
Ras mutation	0 - 21%	40 - 53%	18 - 27%	20 - 60%	0%
PPARG rearrangement	0%	25 - 63%	0%	0%	Unknown
CTNNB1 mutation	0%	0%	0 - 25%	66%	Unknown
TP53 mutation	0 - 5%	0 - 9%	17 - 38%	67 - 88%	Unknown

CTNNB1: B-catenin; NTRK1: Neurotrophic tyrosine kinase receptor, type 1; PPARG: Peroxisome-proliferator-activated-receptor-gamma.

RAS:

Human HRAS, KRAS and NRAS genes encode highly related G-proteins that reside in the inner surface of the cell membrane and transmit signals arising from cell membrane receptor tyrosine kinases and G-protein coupled receptors along the MAPK, PI3K-AKT and other signaling pathways.[10] They are signal-switch molecules, which regulates cell fate by coupling receptor activation to downstream effector pathways that control diverse cellular response such as proliferation, differentiation and survival.[17] Activating point mutations in NRAS in codon 61 and HRAS in codon 61 are most common. RAS mutations are found in a variety of thyroid tumors, including 10-20% of PTC, 40-50% of follicular carcinomas and 20-40% of poorly differentiated and anaplastic carcinomas (Table 4.1).[10] The mutation is also found in 20-40% of benign follicular adenomas. RAS mutations are more common in iodine-sufficient areas and in

follicular variant of PTC than in typical PTC. Interestingly, activating RAS mutations are rare in radiation-induced thyroid cancers cases from the Chernobyl reactor meltdown. The role of RAS mutations in thyroid lesions is controversial; its presence in adenomas might suggest that it is a precursor for RAS-positive follicular carcinomas and follicular variant of PTC. However, the high degree of observer variation in the diagnosis of follicular adenoma and follicular variant PTC may explain this finding as well. The presence of a RAS mutation might also define a subset of tumors with a more aggressive phenotype. There is a close relationship between oncogenic RAS and the loss of histologic features that characterize well-differentiated thyroid tumor phenotypes. RAS mutations are associated with poor prognosis among differentiated carcinomas independent of tumor stage and of whether the tumor is subclassified morphologically as well or poorly differentiated, papillary or follicular.[18]

PAX8/PPARγrearrangement:

The peroxisome proliferator-activated receptor-(PPARγ) is the subject of a unique and interesting rearrangement in follicular thyroid carcinoma. PPARγ is a member of the steroid nuclear-hormone-receptor superfamily that forms heterodimers with retinoid X receptor.[3] The rearrangement leads to the fusion between a portion of the PAX8 gene, which encodes a paired domain transcription factor, and the PPARγ gene. The fusion results in a strong overexpression of the chimeric PAX8/PPARγ protein. It is a prototypical alteration found in follicular thyroid carcinoma, where it occurs with a frequency of 30-35%. The rearrangement is also seen in 2-13% of follicular adenomas, and in 1-5% of follicular variant of PTC (Table 4.1). The presence of the rearrangement is also associated with a vascular-invasive phenotype. While RAS mutations and PAX8/PPARγ rearrangements are predominantly present in follicular tumors it is interesting to see that overlap between the two mutations is hardly seen, indicating that these conventional follicular thyroid carcinomas consist of at least two groups of tumors developing through distinct molecular mechanisms.[19, 20] The signaling pathways involved in the PAX8/PPARγ rearrangement are not fully clarified yet, several gene expression profiling studies have been performed, and it seems that angiopoietin-like 4, aquaporin 7, enolase 3 and placental growth factor are involved.[20, 21]

RET/PTC rearrangement:

The RET/PTC rearrangement results when a portion of the RET gene is fused to one of several possible partner genes. All chimeric genes contain RET, which encodes for the intact tyrosine kinase domain of the RET protein fused to an active promoter of another gene that drives the expression and ligand-independent dimerization of the RET/PTC protein with constitutive tyrosine kinase activity.[10] RET/PTC is a classic oncoprotein that activates the MAPK and the PI3K-AKT pathways.[14] The prevalence of RET/PTC rearrangements is high in tumors associated with a history of radiation exposure. This includes those subjected to either accidental (mostly

radioiodine) irradiation or therapeutic (mostly external beam) irradiation, as 50-80% of these PTC harbor RET/PTC rearrangements (Table 4.1).[22] The prevalence and specificity of RET/PTC rearrangements for PTC varies dramatically. Mostly, this variation exists because of the heterogeneous distribution of this rearrangement within the tumor and the various sensitivities of the detection methods used. RET/PTC rearrangements can be present in a large proportion of the tumor cells and detected by multiple methods (clonal RET/PTC) or occur in a small fraction or single cells within the lesion and be detectable only by ultrasensitive detection techniques (nonclonal RET/PTC).[23, 24] RET/PTC is formed by fusion of the 3' portion of the RET gene, coding for the receptor tyrosine kinase, and the 5' portion of various unrelated genes. The two most common rearrangement types, RET/PTC1 and RET/PTC3, are paracentric inversions since both RET and its respective fusion partner, H4 or NCOA4, reside on the long arm of chromosome 10. The other known RET/PTC rearrangements are all interchromosomal translocations.[22] The partner genes share common characteristics: they are expressed in thyroid follicular cells and therefore provide an active promoter for the RET tyrosine kinase domain, and they contribute dimerization domains that are essential for dimerization and ligand-independent activation of the truncated RET protein. Virtually all breakpoints in the RET gene occur within intron 11, leaving intact the tyrosine kinase domain of the receptor and enabling the RET/PTC oncoprotein to bind SHC via Y1062 and activate the RAS-RAF-MAPK cascade.[25, 26] RET/PTC1 rearrangements are more common in younger patients, are less commonly associated with aggressive disease, and are not associated with loss of sodium-iodide symporter expression. The clonal RET/PTC rearrangement is, just as BRAF mutations, 100% specific for PTC.[27]

TRK:

The neurotrophic receptor-tyrosine kinase (NTRK1) is another, less prevalent, chromosomal rearrangement occurring in PTC. The NTRK1 gene resides on chromosome 1q22 and can be fused to at least three different partner genes located on the same or different chromosomes, i.e. TPM3, TPR and TFG. The activated receptor initiates several signal-transduction cascades, including the ERK, PI3K and the phospholipase-Cγ pathways. It occurs in only 5-15% of sporadic PTC; however, it seems to differ between different geographical areas (Table 4.1).[3, 10]

PTEN:

The protein product of the gene for phosphatase and tensin homolog deleted on chromosome ten (PTEN) is a prominent negative regulator of the PI3K/AKT signaling pathway. PTEN is a phosphatase that dephosphorylates PIP3 and thus inhibits signaling of the PI3K/AKT pathway. Somatic PTEN mutations also occur in thyroid cancers, particularly in follicular thyroid carcinoma and anaplastic thyroid carcinoma, with a low prevalence of <10% and 5-15%, respectively.[28] Despite the rarity of this mutation, the function of PTEN is important in the carcinogenesis of thyroid tumors. This will be discussed further in the section on epigenetics.

TP53:

The TP53 gene encodes the cell-cycle regulator p53. Activation of wild-type p53 can lead to G1 cell-cycle arrest through p21CIP1 and apoptotic cell death, preventing replication of cells with damaged DNA. Conversely, loss of function mutation of TP53 induces genomic instability, owing to weakened DNA repair systems, and subsequent cancer progression. Point mutations are found in 50-80% of cases of anaplastic thyroid carcinoma, are less frequently seen in poorly differentiated thyroid carcinoma and are rarely seen in well-differentiated thyroid carcinoma (Table 4.1).[3, 10] The increased prevalence of TP53 mutations with thyroid cancer progression (well to poorly differentiated) is therefore seen as supportive of the multistep carcinogenesis model.

CTNNB1:

CTNNB1 is the gene encoding for β-catenin that is involved in cell-adhesion and a downstream effector of the Wnt signaling pathway. Membrane β-catenin expression is progressively reduced with loss of tumor differentiation, resulting in tumor invasiveness, and increasing metastatic potential.[29] Point mutations in exon 3 of the gene are found in up to 60% of anaplastic carcinomas and in 10-20% of poorly differentiated carcinomas (Table 4.1).
The analysis of β-catenin expression or mutation status may be useful to objectively subtype thyroid carcinomas and more accurately predict outcomes.[29]

MicroRNAs

Biogenesis and function of miRNAs:

MicroRNAs (miRNAs) are a class of small, non-coding RNAs that posttranscriptionally control the translation and stability of mRNAs.[30] Their biogenesis and biological networks are complex; they are first synthesized as large RNA precursors, processed in the nucleus into pre-miRNAs, folded into imperfect stem-loop structures, transported to the cytoplasm, whereupon it is further cleaved by Dicer1 into a double-stranded miRNA. After the double strand separation, mature miRNA forms, in combination with Argonaute proteins, the RNA-induced silencing complex (RISC), whereas the passenger strand is usually degraded. The mature strand is important for specific-target mRNA recognition and its consequent incorporation into RISC. The expression of the target mRNAs is silenced by miRNAs, either by mRNA cleavage or by translational repression (Figure 4.4). In addition, miRNAs have a number of unexpected functions including the targeting of DNA, ribonucleoproteins or increasing the expression of a target mRNA.[31, 32] Although hundreds of miRNAs are known to have dysregulated expression in cancer, key studies evaluating their biological and molecular roles, and their potential therapeutic applications, are still rare. Yet understanding the function of miRNAs is crucial

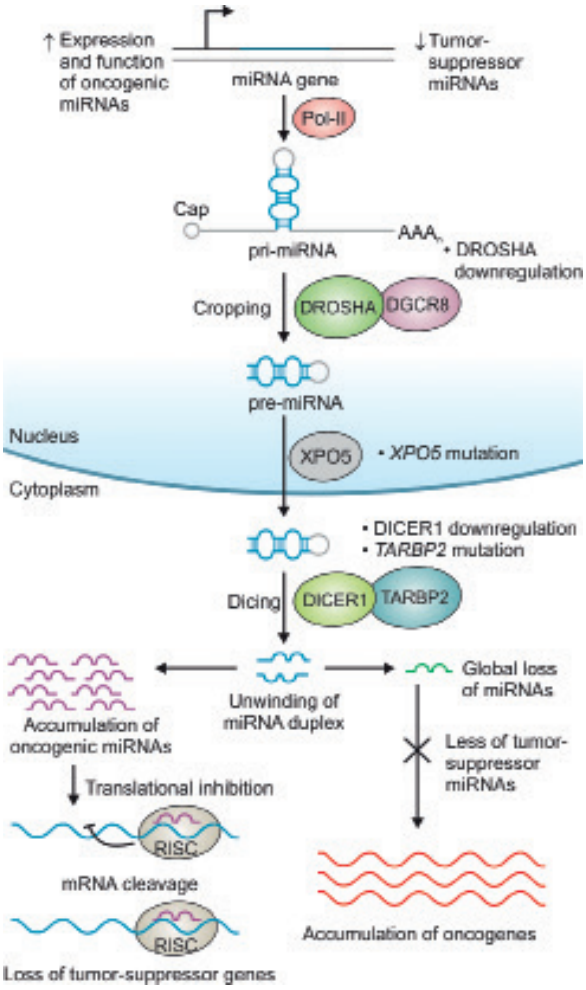


Figure 4.4: *Cancer cells present global downregulation of miRNAs, loss of tumor-suppressor miRNAs and specific accumulation of oncogenic miRNAs. The alteration in miRNA expression patterns leads to the accumulation of oncogenes and downregulation of tumor-suppressor genes, which leads to the promotion of cancer development. (A) The expression and function of oncogenic miRNAs is increased by genomic amplification, activating mutations, loss of epigenetic silencing and transcriptional activation. By contrast, tumor-suppressor miRNAs are lost by genomic deletion, inactivating mutations, epigenetic silencing or transcriptional repression. (B) After transcription, global levels of miRNAs can be reduced by impaired miRNA biogenesis. Inactivating mutations and reduced expression have been described for almost all the members of the miRNA processing machinery. If there is a downregulation of DROSHA this can lead to a decrease in the cropping of primary miRNA (pri-miRNA) to precursor miRNA (pre-miRNA). In the case of XPO5 mutation, pre-miRNAs are prevented from being exported to the cytoplasm. Mutation of TARBP2 or downregulation of DICER1 results in a decrease in mature miRNA levels. (Pol II: RNA polymerase II; RISC: RNA-induced silencing complex). Adapted with permission from Lujambio and Lowe.*[31]

if we hope to uncover the roles of this form of gene regulation in cancer and to harness this knowledge for therapeutic benefit.[30] miRNA deregulation is extensively investigated in thyroid cancer. For thyroid cancer it is of specific interest since it is a potential diagnostic tool, to improve uncertain FNA cytology diagnosis, it may also discriminate between more aggressive subtypes of differentiated thyroid cancer and may be a potential therapeutic target.

miRNAs as diagnostic markers:

The FNA in thyroid nodules is indeterminate in up to 30% of cases which makes the demand for more accurate markers high. Preferably, this should focus on identifying true negative samples. To reduce the amount of unnecessary diagnostic hemithyroidectomies safely, a diagnostic test needs to assure that a thyroid malignancy is never indicated as a benign lesion by the test. This makes the negative predictive value (NPV) and the sensitivity the most important statistical parameters. Recent reports have found sets of miRNAs that have a 100% sensitivity and/or NPV, in these report sets of 2 to 7 miRNAs were tested on FNA samples to discriminate between benign and malignant lesions.[33] Most promising miRNAs in this regard are; miR-221, miR-222, and miR-146b.

miRNAs as prognostic markers:

The miRNAs are used within PTC to distinguish a more aggressive subtype. MiR-146b, miR-222, MiR-34 and MiR-130b are differentially expressed in aggressive compared with non-aggressive PTC. Among tumors that harbor a BRAF(V600E) mutation, overexpression of MiR-146b was associated with aggressive behavior, suggesting that it may further refine the prognostic importance of BRAF.[34] In anaplastic thyroid carcinoma, four miRNAs were found to be downregulated, these miRNAs may target proteins that are involved in the transformation of thyrocytes. This might initiate a more undifferentiated phenotype.[35]

miRNAs as therapeutic targets:

The miRNAs can be oncogenes or tumor suppressors depending on the cellular context in which they are expressed, which means that defining their precise contribution to cancer can be a challenge. In general, cancer cells present global downregulation of miRNAs; however, in thyroid carcinoma the opposite is seen. Alteration in miRNA expression could lead to increased expression of growth promoting genes or oncogenes, and or decrease expression of tumor-suppressor genes, which could lead to cancer initiation and progression. However, the role of miRNAs in cancer is complex as some miRNAs may repress several positive components of a pathway, whereas others can target both positive and negative regulators. In cancer cells, this can mean that some miRNAs could simultaneously target oncogenes and tumor-suppressor genes. In addition, combinations of miRNAs can cooperate to regulate one or several pathways, which increases the flexibility of regulation but complicates therapeutic possibilities.[31] Currently, the effects of the most promising individual miRNA-based therapeutics are tested in mouse models, it is too early to draw a conclusion, but preliminary results look promising.[30]

Epigenetics

Epigenetics refers to study of heritable changes in gene expression that occur without any alteration in the primary DNA sequence. Epigenetic changes can act in concert with genetic changes, they play a role in the earliest steps of tumorigenesis, suggested by the growing list of tumor suppressor genes that are often epigenetically silenced but rarely genetically mutated in the preinvasive stages of many cancers.[36]

Epigenetics is used to describe the study of chromatin biology (Figure 4.5). Chromatin is the macromolecular complex of DNA and histone proteins. It provides the scaffold for the packaging of our entire genome and contains the heritable material of eukaryotic cells.

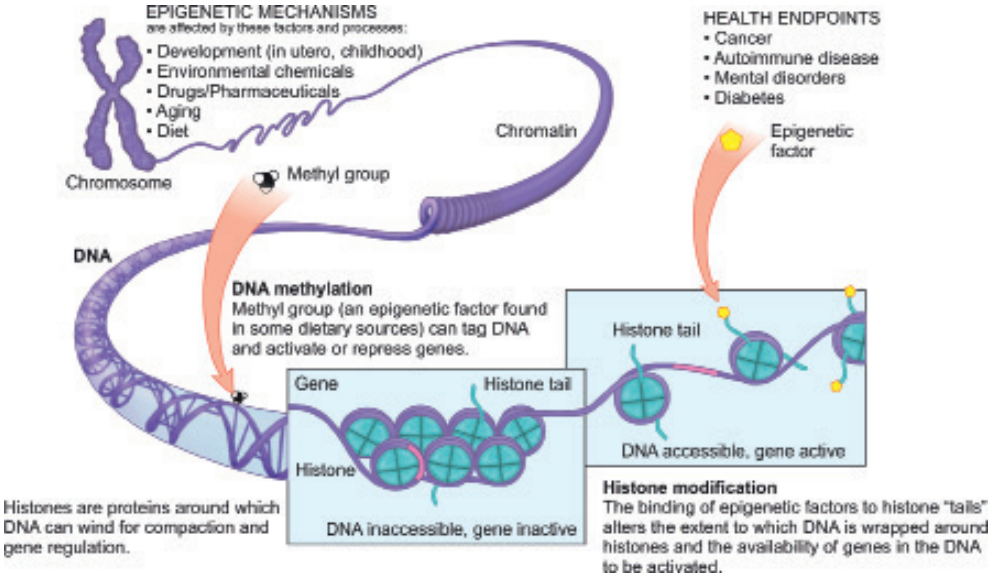


Figure 4.5: *An overview of the macromolecular complex of DNA and histone proteins. Adapted with permission from the National institute of Health (NIH gov). Public domain.*

The basic functional unit of chromatin is the nucleosome, around which the DNA is wrapped. Nucleosomes compact and package DNA in a dynamic and highly controlled fashion that caters to the multitude of DNA-based processes. Consecutive nucleosomes are separated by unwrapped "linker" DNA. Wrapped nucleosomal DNA is inherently less accessible than linker DNA, thus the genomic positioning and compaction of nucleosomes strongly influences the ability of proteins to bind target sequences within DNA and to carry out their function.[37] In cells, chromatin can exist in either an open or a closed configuration with respect to its accessibility for nuclear proteins. Regulation of this involves what has been called "epigenetic" mechanisms,

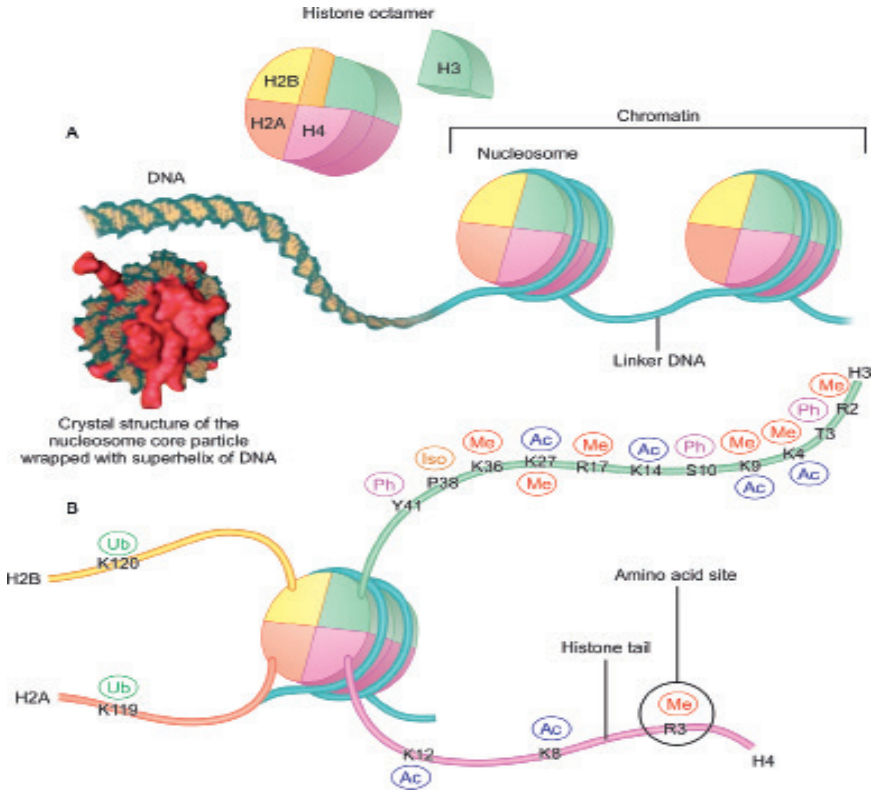


Figure 4.6 A and B: *The nucleosome. The basic functional unit of chromatin is the nucleosome (A), which is composed of a histone octamer around which DNA is wrapped. Octamers are separated by linker DNA. The histone octamer is assembled from a histone H3:H4 tetramer and two H2A:H2B dimers. The histone tails of all four core histones are subject to a variety of post-translational modifications (B). These include methylation (Me), acetylation (Ac), phosphorylation (Ph), ubiquitylation (Ub), and proline isomerization (Iso), all of which occur at the site of a specific amino acid, such as K4 and K9 on the histone H3 tail. The same histone amino acid may be subject to different post-translational modifications, which may facilitate different biologic outcomes. Adapted with permission from Dawson et al.[32] Copyright © 2012. Massachusetts Medical Society. Reprinted with permission from Massachusetts Medical Society.*

including DNA-methylation, post-translational histone modifications, nucleosome remodeling and noncoding RNAs (Figure 4.6).[38]

DNA methylation:

The DNA methylation occurs at CpG dinucleotides and commonly in CpG-rich DNA stretches referred to as “CpG-islands”. The majority of human gene promoters (50-70%) are

embedded within CpG islands, and methylation of such islands correlates with transcriptional repression. CpG methylation patterns are frequently altered in tumor cells and an increased methylation contributes to promoter inactivation of tumor suppressor genes. In thyroid cancer, several alterations in DNA methylation are known. Cyclin-dependent kinases (CDK) have an important role in regulating cell cycle, transcription and mRNA processing. Cyclin-dependent kinase inhibitors are proteins that block CDK kinase activity. Cyclin-dependent kinase inhibitors are often downregulated in thyroid neoplasms. For example, the inhibitor p16INK4A is downregulated and hypermethylated in up to 30% of thyroid neoplasms.
The tumor-suppressive Ras effector, RAS association domain family 1, splicing isoform A (RASSF1A) contains a Ras association domain, and plays a role in the regulation of cell cycle and apoptosis. In the thyroid, there is a high frequency of RASSF1A hypermethylation, it is seen in 33-44% of follicular adenomas and increases to 70-100% in follicular thyroid carcinomas, this suggests that epigenetically silencing of RASSF1A is an early step in thyroid tumorigenesis. PTEN, an important tumor suppressor gene inhibiting the PI3K/AKT signaling pathway, has been found aberrantly methylated in about 50% of PTC and almost 100% of follicular carcinomas and adenomas. A close association between BRAF mutation and aberrant methylation of several tumor-suppressor genes in PTC, including the genes for tissue inhibitor of matrix metalloproteinase-3 (TIMP3), death associated protein kinase (DAPK), and retinoic acid receptor β2 (RARβ2) has been reported. This association correlated with high-risk clinicopathological characteristics of PTC, including extrathyroidal invasion, lymph node metastasis, and advanced disease stages.[36]

Histone modifications:

Posttranslational modifications of the tails of histones can lead to either gene activation or repression, depending on which residues are modified and the type of modification. Overall, histone modifications affect chromatin confirmation and consequently influence gene transcription, DNA repair and replication, and cell cycle checkpoints. Unfortunately, little information about the histone modifications present in thyroid tumors and the relationship between such modifications and thyroid cancer behavior is at present available. However, it is shown that levels of acetylated H3 at residue K18 are lower in anaplastic thyroid carcinoma with respect to differentiated thyroid carcinoma, suggesting that acetylation is switched off in the thyroid tumor progression. Furthermore, it has been demonstrated that the enhancer of zeste homolog 2 (EZH2), is specifically overexpressed in ATC, and it directly contributes to transcriptional silencing of PAX8 gene and anaplastic thyroid carcinoma differentiation.[36]

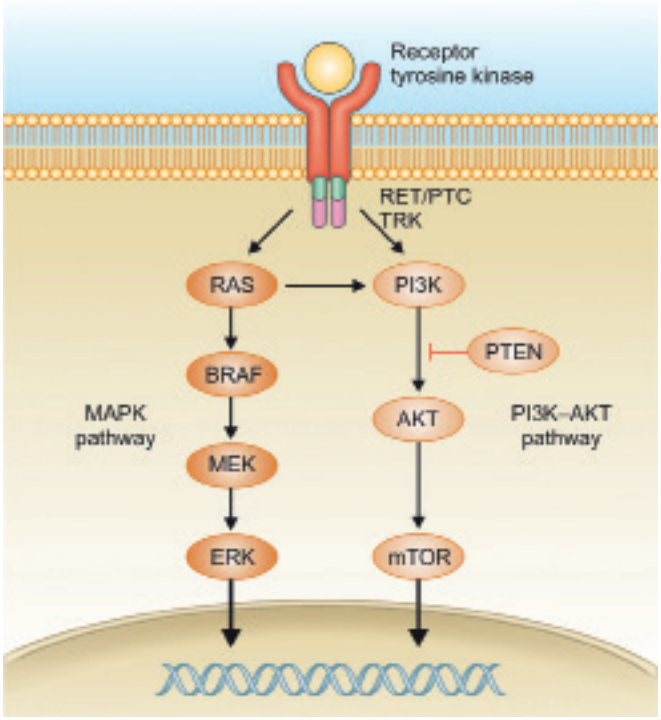


Figure 4.7: *The main signaling pathways involved in thyroid carcinogenesis are the MAPK and PI3K–AKT pathways. These pathways are involved in propagation of signals from various cell membrane receptor tyrosine kinases into the nucleus, and they regulate multiple cell processes including proliferation, differentiation and survival. Activation of the MAPK pathway by oncogenic stimuli such as mutated BRAF, RAS or the chimeric fusion proteins RET/PTC and TRK is a common tumor initiating event in well differentiated papillary carcinoma and in some follicular carcinomas. Mutations involving the effectors of the PI3K–AKT pathway such as the PI3K subunit PIK3CA, AKT1 and PTEN are found more frequently in follicular carcinomas and in less differentiated types of thyroid cancer. Adapted with permission from Nikiforov and Nikiforova.*[10]

Signaling pathway deregulation

MAPK/ERK signaling pathway:

The MAPK/ERK pathway has a fundamental role in thyroid tumorigenesis in the regulation of cell proliferation and survival. In thyroid carcinoma, the MAPK/ERK pathway is driven by activating mutations, such as BRAF and RAS mutations and RET/PTC rearrangements.
The classical MAPK/ERK pathway is activated by an extracellular mitogenic stimuli that activates a receptor tyrosine kinase in the cell membrane then RAS, RAF, MEK and ERK. ERK is activated by phosphorylation and enters the nucleus where it upregulates tumor-promoting genes and downregulates tumor suppressor genes.[14] Recent studies have identified several negative regulators of the MAPK/ERK signaling pathway and their action have been analyzed. Sprouty, Spred and Sef act as conserved inhibitors and engage on different levels of the MAPK/ERK signaling pathway. These regulators could control the duration, magnitude and/or

subcellular compartmentalization of ERK activity.[39] The MAPK/ERK signaling pathway is now a highly promising target for the development of mechanism-based anticancer drugs. However, specific blockade of this pathway alone is mostly cytostatic rather than cytotoxic, which limits the therapeutic efficacy of MEK-inhibitors when administered alone.[40]

PI3K-AKT signaling pathway:

In thyroid cancer PI3K-AKT signaling can be constitutively activated by inactivating mutations in PTEN, and activating mutations in RAS, RET/PTC rearrangement and mutations in PI3KCA and AKT1. PI3K's represent a family of kinases that phosphorylate the 3-hydroxyl group in phosphatidylinositol inositides (PtdIns). PI3k's bind to, and are activated by many tyrosine kinase receptors either through direct interactions or indirectly to adapter molecules. Activation of PI3K's lead to an increase in the production of PtdIns, these PtdIns bind to the plextrin homology (PH) domains of a number of PH-domain containing proteins, including AKT isoforms.[41] This results in a conformational change in AKT that exposes two crucial amino acid residues for phosphorylation: T308 by PDK-1 and S473 by mTOR complex 2. AKT is activated by phosphorylation, and it phosphorylates many other proteins involved in protein synthesis, cell survival, proliferation, and metabolism. PI3K-, AKT-, and mTOR-inhibitors or a combination of those have been evaluated or are currently under evaluation, the results so far show some activity. For further improvement in response, molecular correlates that can be used for patient selection need to be determined.[42]

Conclusion

Due to ongoing dedication, improved techniques, development of all sorts of high throughput technologies, openness in research and international collaborations, our understanding of carcinogenesis is fast evolving. In thyroid cancer, this is no different, on one hand it opens up dozens of possible targets for therapy and opportunities for improved diagnostics. This chapter gives an overview of the key genetic and epigenetic events involved in thyroid cancer initiation and progression.

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Chapter 5

The value of miRNA in diagnosing thyroid cancer: A systematic review

Lutske Lodewijk, Anne M. Prins, Jakob W. Kist, Gerlof D. Valk, Onno Kranenburg, Inne H.M. Borel Rinkes, Menno R. Vriens

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Abstract

Thyroid cancer is the most common endocrine neoplasm accounting for approximately 1,7% of total cancer diagnoses. The gold standard for evaluation of thyroid nodules is cytology from fine needle aspiration. In 30% of biopsies there is no conclusive diagnosis and patients undergo a diagnostic hemithyroidectomy. Somatic mutations occur frequently in thyroid cancer, the value of testing FNA biopsies on different mutation is analyzed, it improves accuracy, but their sensitivity is low. Another class of molecules with potential diagnostic value are miRNAs (miRNA, miR). MiRNAs function as gene regulators thereby controlling many cellular processes including cell growth, differentiation, proliferation, and apoptosis. Several studies have analyzed the expression of miRNAs in thyroid cancer, either by performing microarray analyses or validating a set of miRNAs. Recent reports focused on the diagnostic value of miRNAs in indeterminate FNA biopsies. In this systematic review we will provide an overview of all miRNAs found to be up- or downregulated in the different types of thyroid carcinomas, give an overview of the value of validated sets of microRNAs or single microRNAs in distinguishing malignant from benign lesions and conclude with a clinical view on future study strategies.

Introduction

Thyroid cancer is the most common endocrine neoplasm accounting for approximately 1.7% of total cancer diagnoses.[1] The incidence has increased in the last decades; however, mortality has been stable over the years with a rate of 0.5 per 100.000 people.[2-5] Thyroid nodules are diagnosed in over 5% of the adult population and can be benign adenomas or malignant lesions. Carcinomas are derived from two types of hormone-producing cells; follicular cells and parafollicular C-cells. More than 95% of the thyroid carcinomas originate from follicular cells and can be subdivided into three main categories; papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC) and anaplastic thyroid carcinoma (ATC). The latter is an undifferentiated carcinoma and has a distinct clinical pattern, is highly aggressive, has a median survival of 3–9 months and accounts for 14–39% of thyroid cancer deaths. Patients with these tumors present with a tumor mass in the neck and invasive features.[6] PTC and FTC are both well differentiated tumors and have an excellent 5- and 10-year survival rate of 98.7% and 98.2% for PTC and a 5-year survival of 95.6% for FTC. Medullary thyroid carcinomas (MTC) are derived from the parafollicular C-cells, they account for a minority (5%) of thyroid carcinomas. The 5-year survival rate for patients with MTC is 82.1%.

The gold standard for the diagnostic evaluation of thyroid nodules is cytology from fine needle aspiration (FNA). Based on the FNA biopsies the lesion may be diagnosed as; benign (60%–70%), malignant (5%–7%), suspicious for malignancy (< 10%), follicular lesion or neoplasm of unknown significance (10%), follicular lesion or neoplasm (10%–20%), non-diagnostic (5%–15%)[7]. In 30% of biopsies there is no conclusive diagnosis and it remains unclear whether the nodule is malignant or benign, a hemithyroidectomy is indicated for definitive histological diagnosis.[8, 9] In order to differentiate between benign follicular adenoma (FA) and FTC a histological analysis is required, since the presence of capsular or vascular invasion is the determining feature.[10] Other procedures to diagnose thyroid nodules have to be developed to reduce the number of diagnostic hemithyroidectomies.

Somatic mutations in thyroid carcinomas occur frequently, in PTC > 70% of patients have such a mutation and in FTC this is > 60%.[11, 12] Common mutational mechanisms are point mutations such as BRAFV600E and KRAS, or chromosomal rearrangements, such as RET/PTC and PAX8/PPARG.[11, 13-15] In the pursue for diagnostic markers to improve the accuracy of FNA cytology, mutational analyses are performed on indeterminate FNA biopsies. A review of the studies performed so far reported a specificity of 95–100% and a sensitivity of 38–86%.[16] The fact that some patients have a thyroid malignancy without any mutations makes mutational analyses impractical for routine check-up. Further effort should be on tests with a higher sensitivity that effectively identify benign lesions.[11, 16-21]

Another class of molecules with potential diagnostic value are the miRNAs (miRNA, miR), a class of gene regulators of approximately 22 nucleotides in length in their active form.

MiRNAs function as gene regulators thereby controlling many cellular processes including cell growth, differentiation, proliferation, and apoptosis.[22] Specific miRNAs can act as oncogenes or tumor suppressors.[23] The diagnostic potential of miRNAs has been demonstrated in, for instance lung cancer and colorectal cancer.[24-26]

Table 5.1: *Overview of studies that performed miRNA microarray analyses.*

Study	Analyzed samples	Methods	Tumor upregulated miRNAs	Tumor downregulated miRNAs
He H. et al. 2005	15 PTC, 10 adjacent tissue	460 miRNA microarray in fresh frozen tissue	**PTC vs. adjacent:** miR-146, 221, 222, 21, 220, 181a, 181c, 181, 155	**PTC vs. adjacent:** -
Pallante P. et al. 2007	30 PTC tissues, 10 tissues other lobe	368 miRNA microarray in fresh frozen tissue	**PTC vs. other lobe:** miR-222, 221, 181b, 220, 213	**PTC vs. other lobe:** -
Weber F. et al. 2006	12 FTC, 8 FA	460 miRNA microarray in unspecified tissue	**FTC vs. FA:** miR-197, 346, 192	**FTC vs. FA:** -
Visone R. et al. 2007	10 ATC, 10 NT	368 miRNA microarray in fresh frozen tissue	**ATC vs. NT**: -	**ATC vs. NT:** miR-30-d, 125b-1, 26a, 125-2, 30a-5p, 224, 092-2, 138-1, 25, 125a
Tetzlaff M.T. et al. 2007	10 PTC, 10 MNG	754 miRNA microarray in FFPE tissue	**PTC vs. MNG:** miR-221, 222, 21, 31, 172, 34a, 213, 181b, 223	**PTC vs. MNG:** miR-218, 300, 292, 345
Nikiforova M.N. et al. 2008	9 PTC, 5 FTC, 2 pd-PTC, 2 ATC, 4 FA, 5 NT, 5 HN	158 miRNA microarray in fresh frozen tissue	**PTC vs. NT:** miR-187, 221, 222, 146b, 155, 122a, 31, 205, 22 **c-FTC vs. NT:** miR-187, 224, 155, 222, 221, 146b **o-FTC vs. NT:** miR-187, 221, 339, 183, 222, 197 **PDTC vs. NT:** miR-187, 221, 129, 222, 146b, 339, 183 **ATC vs. NT:** miR-302c, 205, 137, 187, 214, 155, 224, 222, 221	**Not tested**

Study	Analyzed samples	Methods	Tumor upregulated miRNAs	Tumor downregulated miRNAs
Braun J. et al.2010	3 ATC, 3 NT	~776 miRNA microarray in unspecified tissue	**ATC vs. NT:** miR-21	**ATC vs. NT:** miR-200c, 30a, 30b, 30d, 30c, 141, 26a, 99a, 138, 200b, 29c, 200a, 26b, 125a, 130a, 30a-3p, 7, let-7g, 30e, let-7f, 125b, let-7i, 19b, 29b, 331-3p, 99b
Yip L. et al. 2011	12 PTC, 4 NT	319 miRNA microarray in fresh frozen tissue	**PTC vs. NT:** miR-146b, 222, 155, 221, 31	**PTC vs. NT:** miR-138, 1, 130b
Reddi H.V. et al. 2011	12 FTC, 7 NT, 10 FA	1200 miRNA microarray in fresh frozen tissue	**FTC vs. NT:** miR-122, 1248, 375 **FTC vs. FA:** miR-122, 221, 375	**FTC vs. NT:** miR-31, solexa-8048-104, miR-923 **FTC vs. FA:** miR-624, 29
Kitano M. et al. 2011	8 HCC, 6 PTC, 6 fv-PTC, 6 FTC, 6 FA, 8 MNG, 7 HA	1263 miRNA microarray in fresh frozen tissue	**Malignant vs. benign:** miR-146b-5p, 222	**Malignant vs. benign:** miR-451, 7, 144
Vriens M.R. et al. 2012	12 fv-PTC, 8 c-PTC, 15 FA, 12 FTC, 12 HCC, 4 ATC, 7 NT, 14 HN	~850 miRNA microarray in fresh frozen tissue	**Malignant vs. benign:** miR-584, 635, 564, 550, 628-3p	**Malignant vs. benign:** miR-149, 100, 138, 125b, 768-3p
Rossing M. et al. 2012	12 FTC, 12 FA, 10 NT	1269 miRNA microarray in fresh frozen tissue	**FC vs. FA:** miR-637, 631 **FC vs. NT:** miR-221, 96, 182, 597, 222	**FC vs. FA:** miR-512-3p, 886-5p, 450a, 301b, 429 **FC vs. NT:** miR-199b-5p, 144*, 199b-3p, 199a-5p, 144

MicroRNAs with at least a 2-fold change in expression reported by the authors, only significant miRNA expression differences are reported when specified by the authors. ATC anaplastic thyroid carcinoma; FA follicular adenoma; FFPE formalin-fixed paraffin-embedded; FTC follicular thyroid carcinoma; c-FTC conventional variant of FTC; o-FTC oncogenic variant of FTC; HA Hürtle cell adenoma; HCC Hürtle cell carcinoma; HN hyperplastic nodules; GD Grave's Disease; MNG multinodular goiter; NT normal thyroid; PTC papillary thyroid carcinoma; c-PTC conventional PTC; fv-PTC follicular variant of PTC; pd-PTC poorly differentiated PTC; tc-PTC tall cell PTC

Table 5.2: Overview of studies that selected sets of microRNAs.

Study	Analyzed samples	Methods	Tumor upregulated miRNAs	Tumor downregulated miRNAs
Chen Y.T. et al. 2008	32 PTC, 24 FA, 11 HN, 2 FTC, 5 NT	6 selected miRNAs in FFPE tissue	**PTC vs. benign:** miR-146b, 222	**PTC vs. benign:** -
Sheu S.Y. et al. 2009	50 c-PTC, 71 tc-PTC, 56 fv-PTC, 44 micro-PTC, adjacent tissue	5 selected miRNAs in FFPE tissue	**c-PTC vs. adjacent:** miR-146b, 221, 222 **micro-PTC vs. adjacent:** miR-146b, 221, 222, 181b, 21 **tc-PTC vs. adjacent:** miR-146b, 221, 222, 21 **fv-PTC vs. adjacent:** miR-146b, 222, 221	**any tumor vs. adjacent:** -
Schwertheim S. et al. 2009	15 PDTC, 9 PTC, 9 ATC, 4 NT	10 selected miRNAs in FFPE	**PTC vs. NT:** 146b, 222, 221, 21, 181b, 30d, 125d, 26a, 304-5p, let-7c **PDTC vs. NT:** - **ATC vs. NT:** 21, 222, 221, 146b, 181b	**PTC vs. NT:** - **PDTC vs. NT:** 26a, 125d, let-7c **ATC vs. NT:** let-7c, 30d, 26a, 304-5p, 125d
Sheu S.Y. et al. 2010	10 PTC, 10 adjacent tissue	5 selected miRNAs in FFPE tissue	**PTC vs. adjacent:** miR-146b, 221, 222, 21, 181b	**PTC vs. adjacent:** -
Sheu S.Y. et al. 2010	50 PTC (10 c-PTC, 10 tc-PTC, 30 fv-PTC), 21 FTC, 10 FA, 10 MNG	5 selected miRNAs in FFPE tissue	**PTC vs. adjacent:** miR-146b, 221, 222, 21 **fv-PTC vs. adjacent:** miR-146b, 221, 222, 21 **FTC vs. adjacent:** -	**PTC vs. adjacent:** - **fv-PTC vs. adjacent:** - **FTC vs. adjacent:** miR-221, 21, 222, 146b
Chou et al. 2010	100 PTC, 16 NT	3 selected miRNAs in fresh frozen	**PTC vs. NT:** miR-146b, 221, 222	**PTC vs. NT:** -
Mazeh H. et al. 2011	20 PTC, 20 adjacent tissue, 2 MNG, 3 GD, 2 TA	6 selected miRNAs in fresh frozen FNA samples	**PTC vs. adjacent:** miR-146b, 221, 222, 187, 31, 21	**PTC vs. adjacent:** -
Keutgen X.M. et al. 2012	12 c-PTC, 1 pd-PTC, 16 fv-PTC, 6 FTC, 1 HCC, 22 FA, 43 HN, 22 TH	6 selected miRNAs in fresh frozen FNA samples	**malignant vs. benign:** -	**malignant vs. benign:** miR-197, 328

Study	Analyzed samples	Methods	Tumor upregulated miRNAs	Tumor downregulated miRNAs
Keutgen X.M. et al. 2012	12 c-PTC, 1 pd-PTC, 16 fv-PTC, 6 FTC, 1 HCC, 22 FA, 43 HN, 22 TH	6 selected miRNAs in fresh frozen FNA samples	**malignant vs. benign:** -	**malignant vs. benign:** miR-197, 328
Shen R. et al. 2012	3 ATC, 5 FTC, 32 PTC, 4 fv-PTC, 6 FA, 1 HA, 8 NH, 1 NNG	8 selected miRNAs in fresh frozen FNA samples	**malignant vs. benign:** -	**malignant vs. benign:** miR-146b, 221
Mian C. et al. not yet published	34 sMTC, 6 hMTC, 8 other lobe	9 selected miRNAs in fresh frozen tissue	**sMTC and hMTC vs. other lobe:** miR-21, 127, 154, 224, 323, 370, 183, 375, 9*	**sMTC and hMTC vs. other lobe:** -

MicroRNAs with at least a 2-fold change in expression reported by the authors, only significant miRNA expression differences are reported when specified by the authors. ATC anaplastic thyroid carcinoma; FA follicular adenoma; FFPE formalin-fixed paraffin-embedded; FTC follicular thyroid carcinoma; c-FTC conventional variant of FTC; o-FTC oncogenic variant of FTC; HA Hürtle cell adenoma; HCC Hürtle cell carcinoma; HN hyperplastic nodules; GD Grave's Disease; MNG multinodular goiter; NT normal thyroid; PTC papillary thyroid carcinoma; c-PTC conventional PTC; fv-PTC follicular variant of PTC; pd-PTC poorly differentiated PTC; tc-PTC tall cell PTC

MiRNA expression may help distinguishing between benign and malignant lesions and in assessing the degree of aggressiveness to direct therapy.[27-29] In this systematic review, we will provide an overview of all miRNAs found to be up- or downregulated in the different types of thyroid carcinomas and combine the results so far. Furthermore, we will give an overview of validated sets of microRNAs or single microRNAs and their value in distinguishing malignant from benign lesions. The incentive is to create direction for future work and create an overview of relevant miRNA literature.

Interpretation of miRNA studies

To determine miRNA expression levels in tissues or FNA biopsies different preservation procedures are used; fresh frozen tissue or formalin-fixed paraffin- embedded (FFPE) tissue. Zhang et al. show convincing evidence that FFPE- and fresh frozen tissue have comparable quality in miRNA preservation. MiRNA studies are either performed in cell lines or in tissue.[30, 31] Notable is the finding that there is little overlap between deregulated miRNAs found in cell lines and in tissue.[32-34] Possible explanations are changing miRNA levels because of DNA damage caused by culturing, a selection bias because not all tumors produce a viable cell line in culture

and comparing a cell line derived from a single patient to the average miRNA expression levels from larger cohorts gives greater importance to outliers.[30, 35] However, cell lines may be used to evaluate the biological function of miRNAs with functional experiments.[36-39]
Thirteen studies included in this review performed microarray analyses (Table 5.1). Another 9 studies either chose a set of microRNAs from their previous work or based their choice on a literature search (Table 5.2).

MiRNA expression in PTC

As PTC is the most common thyroid cancer, most studies included tissue of PTC patients. Five groups used a miRNA microarray and seven tested a selected set of miRNAs based on literature. By using microarray analysis, He et al. showed a more than 2-fold overexpression of miR-146, 221, 222, 21, 220, 181a, 181c, 181, and 155 when compared to unaffected thyroid tissue from the same patients. In particular miR-146, 221 and 222 were more than 10-fold overexpressed. No miRNAs were found that were more than 2-fold downregulated.[40] In 2006, Pallante et al. reported on 5 miRNAs (miR-222, 221, 181b, 220, 213) that were more than 2-fold upregulated in the tumors, but found no downregulated miRNAs, using the other thyroid lobe as control tissue. In contrast to the previous studies the expression levels were only 4 times higher in PTC tissue.[41] Tetzlaff et al. tested PTC tissue versus multinodular goiter (MNG) of other patients in 2007. A more than 2-fold upregulation was found for miR-221, 222, 21, 31, 172, 34a, 213, 181b, and 223 and a more than 2-fold downregulation was found for miR-218, 300, 292, and 345. They used FFPE tissue instead of fresh frozen and concluded that this tissue is a suitable resource for miRNA analysis.[42] In a subanalysis of Nikiforova et al. a small number of PTC samples (n = 9) were compared with normal thyroid tissue (NT). MiR-187, 221, 222, 146b, 155, 122a, 31, 205, and 224 were highly upregulated with no mention of downregulated miRNAs.[43] In 2011, Yip et al. compared aggressive and non-aggressive PTC tumors to NT, but mainly focused on the difference between aggressive and non-aggressive tumors. Of prognostic value might be the finding of a 2-fold upregulation miR-146b, 221, 222, 31 and a 2-fold downregulation for miR-1, 34b, 138, 130b comparing the two types of PTC.[29]
In conclusion, the miRNAs reported to be upregulated in PTC versus benign tissue (normal tissue, follicular adenoma, or nodular goiter) are miR-221 and 222 in all five miRNA microarray studies, miR-181b in four, and three studies found an overexpression of miR-146, 181a, 31, 224, 34a, 155, and 213.29, 40-42, 44 The most profound overexpression was found for miR-222 and 221 in most studies, together with miR-146 in two studies. Four groups reported on downregulated miRNAs in PTC. Despite the fact that the results are less consistent between the different studies and the fold changes are smaller, downregulation of miR-138, 345, and 130b were reported in several studies.[29, 40-42]

MiRNA expression in FTC

In 2006, Weber et al. identified with microarray analysis 4 miRNAs (miR-192, 197, 328, 348) that were significantly upregulated in FTC versus FA. However, after validation only miR-197 showed an overexpression of more than two times.[45] Nikiforova et al. divided their FTC samples into two histological types: the conventional and Hürthle cell type.[43] Combining both types the most overexpressed miRNAs were miR-187, followed by miR-221 and 222. When both FTC subtypes are analyzed separately upregulated miRNAs include miR-224, 155 and 146b in conventional FTC and miR-339, 183 and 197 in Hürthle cell FTC compared to NT.[44] Reddi et al. performed a microarray and compared tumors with and without PAX8/PPARG-rearrangement and compared the results to both NT and FA. When all tumor tissue was compared to benign tissue, miR-122 and 375 were upregulated. MiR-221 was only upregulated compared to FA, and miR-1248 only compared to NT[46] In 2012, Rossing et al. found more down- than upregulated miRNAs in a microarray performed on FTC versus FA and NT samples, especially miR-199b-5p and miR-144 were almost undetectable in the carcinomas. Other downregulated miRNAs were miR-512-3p, 886-5p, 450a, 301b and 429 versus FA and miR-199b-5p, 144*, 199b-3p, 199a-5p and 144 versus NT. They confirmed upregulation of the miRNAs reported by Weber and Nikiforova.[47] Sheu et al. were the only group performing a miRNA analysis with a selected subset of 5 miRNAs (miR-146b, 181b, 21, 221, and 222). In contradiction to the microarray studies, where miR-146b, 221 and 222 were upregulated; they report a 3-fold downregulation for miR-146b and 21 compared with FA.[48]

MiRNA expression in ATC

Visone et al. found in the more aggressive ATC mainly downregulation of miRNAs and no miRNAs were upregulated.[49, 50] The miRNAs that were downregulated more than 2-fold were miR-30-d, 125b-1, 26a, 125-2, 30a-5p, 224, 092-2, 138-1, 25, and 125a.[50] Interestingly, miRNAs that are commonly upregulated in PTC are not deregulated in ATC, their expression seems to decrease in the process that a PTC dedifferentiates to ATC. Nikiforova et al. only searched for upregulation and found a fold change of more than 9 times for miR-302c, 205, 137, 187, 214, 155, 224, 222, and 221, with the most dramatic overexpression found for the first 3 miRNAs with expression levels more than 60 times higher than in NT.[43, 44]

Braun et al. describe 62 down- and 21 upregulated miRNAs in their small sample set of 3 ATCs and 3 NT samples. The most dramatic downregulation was found for miR-30d, 125b, 26a, 30a, 138, 125a, and let-7c.51 Schwertheim et al. confirmed in a set of 10 selected miRNAs the downregulation of five of those miRNAs. They found in contrast with the report of Visone et al. upregulation of miR-21, 221, 222, and 146b.[50, 52, 5]

Table 5.3: Overview of studies that analyzed the diagnostic value of miRNAs or sets of miRNAs.

Study	Material	Analyzed samples	Statistical analysis	Comparison
Nikiforova M.N. et al. 2008	Indeterminate FNAs	Derivation group: n = 41 Validation group: n = 62	LDA	Malignant vs. benign
Mazeh H. et al. 2011	FNAs of PTC and contralateral lobe, FNAs from benign tissue	PTC-group: n = 20 Benign group: n = 27	Every miRNA was analyzed seperately	PTC vs. contralateral lobe
Vriens M.R. et al. 2012	Indeterminate FNAs	78 benign, 37 malignant	Every miRNA was analyzed seperately	Malignant vs. benign
Rossing M et al. 2012	Snap frozen tissue	12 FTC, 12 FA, 6 NT	SVM and LOOCV	FTC vs. FA
Kitano M. et al. 2012	FNAs	Derivation group: n = 95 Validation group: n = 59	Logistic regression model	Malignant vs. benign

MicroRNAs analysed	Accuracy (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
miR-187, 221, 222, 146b, 155, 224, 197					
If ≥ 1 miR is upregulated	95	100	94	-	-
If ≥ 3 miRs are upregulated	98	88	100	-	-
miR-21	79	50	100	100	73
miR-31	77	45	100	100	71
miR-146b	91	80	100	100	87
miR-187	79	50	100	100	73
miR-221	98	90	100	100	93
miR-222	96	90	100	100	93
	75	-	-	-	81
miR-19a, 501-3p, 17, 335, 106b, 15a, 16, 374a, 542-5p, 503, 320a, 326, 330-5p, let-7i.	-	-	-	100	92
miR-7, 126	76	100	29	36	100

Table 5.3: Overview of studies that analyzed the diagnostic value of miRNAs or sets of miRNAs.

Study	Material	Analyzed samples	Statistical analysis	Comparison
Keutgen X.M. et al. 2012	indeterminate FNAs	Derivation group: n = 29 Validation group: n = 72	SVM-RBF	Malignant vs. benign
Shen R. et al. 2012	FNAs	Derivation group: n = 60 Validation group: n = 68	LDA, multivariate logistic regression classification model	Malignant vs. benign

Only articles that calculated sensitivity, specificity, accuracy, NPV, and/or PPV are included. Subanalyses are not included in this table. FA follicular adenoma; FTC follicular thyroid carcinoma; FNA Fine Needle Aspiration; PTC papillary thyroid carcinoma; LDA Linear Discriminant Analysis; SVM-RBF Support Vector Model with radial basis kernel.

MiRNA expression in MTC

In our search only one article came up testing medullary thyroid carcinoma versus any benign tissue, other groups compared sporadic versus hereditary MTC.[54] Mian et al. included these both types of MTC and selected 9 miRNAs testing 42 pathological tissue samples and 8 samples of NT. All nine miRNAs (miR-9*, 375, 154, 183, 127, 224, 370, 323, 21) were found to be upregulated with nearly no overlap between pathological and normal specimens, furthermore no difference was detected between sporadic MTC, hereditary MTC or C-cell hyperplasia.[55]

MiRNA expression in malignant tissue

Two of the larger studies in this systematic review identified the miRNA expression profiles of various malignant thyroid tissues versus benign tissue with a microarray. In 2011, Kitano et al. grouped papillary and follicular thyroid carcinoma and Vriens et al. also included anaplastic and Hürthle cell carcinoma.[56, 57] Results from the microarray by Kitano et al. revealed 34 miRNAs that were significantly expressed; of which miR-146-5p and 222 were upregulated and miR-451, 7, and 144 were downregulated more than 2-fold. Vriens et al. used a cut off value of >5-fold difference in expression and with this limit 10 miRNAs were assessed for validation. Four miRNAs were significantly downregulated in malignant versus benign tissue; miR-100, 125b, 138, and 768-3p.

MicroRNAs analysed	Accuracy (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
miR-222, 328, 197, 21	90	100	86	-	-
miR-146b, 221, 187, 30d	85	89	78	89	78

Keutgen et al. and Shen et al. performed a validation of selected miRNAs in combined malignant tissue versus benign tissue, both published in 2012. No upregulated miRNAs were found, however both groups found two miRNAs to be silenced in malignant thyroid tissue; miR-197 and 328 (Keutgen et al.), and surprisingly miR-146b and 221 were downregulated in the samples of Shen et al., while they are generally associated with upregulation in PTC tissue.[58, 59]

MiRNA as a diagnostic marker

The ability to examine miRNAs in FFPE samples makes miRNAs a very attractive biomarker.[26, 28] In contrast to mRNA, miRNA is not significantly affected by fixation, and can be readily extracted from FFPE samples due to their small sizes and remarkable stability.[31] Additionally, miRNA extraction from fine needle aspirations is possible, though the yield of miRNA is smaller and the quality is poorer. Nevertheless, final results were comparable with the control FFPE samples.[60]

Since the discovery of miRNAs their diagnostic value has been an area of interest. Unraveling the different expression patterns in tumors is an essential step. FNA of thyroid nodules is indeterminate in up to 30% of cases which makes the demand for more accurate markers high.[7] Preferably this search should focus on identifying true negative samples. To reduce the amount of unnecessary diagnostic hemithyroidectomies safely, a diagnostic test needs to assure that a thyroid malignancy is never indicated as a benign lesion by the test. This makes the negative predictive value (NPV) and the sensitivity the most important statistical parameters.

So far seven articles have been published that validated a single or a set of microRNAs for their accuracy, sensitivity, specificity, PPV and/or NPV (Table 5.3). Of those, 3 papers report a sensitivity or a NPV of 100%, their study design was comparable; with a derivation group, a validation group and they all tested a set of miRNAs.[7, 43, 58, 61] Kitano et al. only found a specificity of 29%, in contrast to a specificity of 86% for Keutgen et al. and 94% for Nikiforova et al., reflected by an accuracy of 76% for Kitano et al. versus 90% and 95% for Keutgen et al. Nikiforova et al. respectively.[43, 58, 61]

Mazeh et al. analyzed single microRNAs and compared a FNA biopsy from a proven malignant nodule with one from the contralateral lobe. Their predictive values were surprisingly high; however, they neglected the heterogeneity between patients, which is a highly complicating factor in composing a signature that differentiates between benign and malignant nodules.[62] The studies of Shen et al. and Vriens et al. analyzed a set of microRNAs with a sensitivity and/or NPV of below 100%, which compromises clinical utility.[57, 59] Rossing et al. compared FA from FTC, but the material used was snap frozen tissue which gives a higher microRNA yield and better quality than FNA samples evaluated in clinical practice.[47]

MiRNA targets

As most miRNAs have over 5000 target genes not all have been identified for the deregulated miRNAs in thyroid cancer. However, to underline the importance of miRNAs in tumor biology we would like to highlight the function of some. Mir-21 is one of the best evaluated miRNAs so far as is it upregulated in many cancer types. It plays a role in tumorigenesis, progression and metastasis as well as anticancer drug resistance by inhibiting PTEN and PDCD4, both tumor suppressor proteins.[63, 64] MiR-146b is often overexpressed in thyroid carcinoma derived from follicular cells, while it is rarely deregulated in other cancer types. It plays an oncogenic role by binding to the 3' untranslated region of SMAD4, an important member of the transforming growth factor ß signaling pathway.[65] MiR-221 and MiR-222 have a direct role on the transcription of p27Kip1 mRNA, which has a function in cell cycle progression to drive quiescent cells into the S-phase.[49] The identification of target genes leads to better understanding of their function in essential cellular processes. However, it is beyond the scope of this review to clarify the function of all of them since we focus mainly on miRNAs as a diagnostic marker.

Future perspectives

Publications on miRNA are increasing and the search for a set of miRNAs that can distinguish between malignant and benign tumors has started. From the results so far we can conclude that final consensus has not been reached yet and the focus should be on well-designed

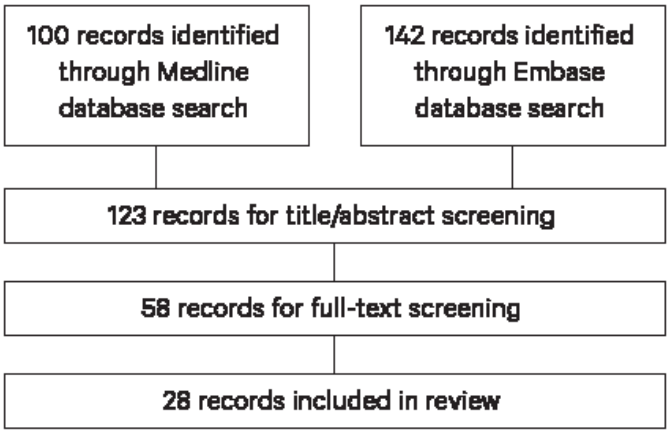
studies, where FNA samples of a derivation group and a validation group are prospectively tested. The statistical focus should be on identifying the true negative population, since this will have an important clinical impact. Reducing the number of surgeries will make the test rapidly cost-effective.

The differences in expression between the different types of thyroid tumors make it appealing to search for a set of miRNAs that is not only designed to differentiate benign from malignant, but will also distinguish the type of tumor. Nevertheless, caution is needed since this will probably decrease statistical accuracy and will have little clinical relevance since every malignant nodule has to be removed and will be histologically evaluated. The ongoing search to evaluate the effect of the miRNAs on the target genes will be essential before using miRNAs as a therapeutic target.

Supplementary box 5.1: *Search methodology*

Box 5.1	**Search methodology**
Literature search	Terms: thyroid neoplasm and miRNA and all conceivable synonyms. Search machines: Medline and Embase
Exclusion criteria	title/abstract scanning: 1) case reports 2) non-English papers 3) conference supplements 4) tested less than 3 miRNAs 5) used non-human tissue or cell lines full-text scanning: 1) did not report results as either fold change or P-value 2) did not report fold changes in malignant versus any benign/normal tissue
Critical appraisal	Appraisal tool for diagnostic testing of critical appraisal skills program (CAPS)

Supplementary box 5.2: *Flowchart included articles*



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Chapter 6

Preoperative BRAF(V600E) mutation screening is unlikely to alter initial surgical treatment of patients with indeterminate thyroid nodules; a prospective case series of 960 patients.

Lutske Lodewijk, Jakob W. Kist, Gerlof D. Valk, Menno R. Vriens, Inne H.M. Borel Rinkes

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With great interest we read the article of Kleiman et al. addressing the putative added value of preoperative BRAF(V600E) analysis on the initial surgical strategy.[1] As the basic surgical strategy for indeterminate thyroid nodules differs between many US centers and Europe, we propose that the data of Kleiman et al. might lead to an opposite conclusion regarding the use of preoperative BRAF testing in the European setting. To substantiate this hypothesis, we have used data from the Netherlands Cancer Registry.

As pointed out by Kleiman et al., the standard surgical approach to indeterminate thyroid nodules in the USA is to perform a total thyroidectomy on all patients with Bethesda category V and on all nodules with "worrisome cytologic features", eg. nuclear grooves and pseudoinclusions. As 12 of the 13 BRAF mutants had already undergone total thyroidectomy as the initial procedure based on the cytology, routine preoperative BRAF testing would have altered surgery in only one patient.

In contrast to the situation in many US centers, in the Netherlands, as well as in large parts of Europe, a diagnostic hemithyroidectomy is performed routinely on all patients with indeterminate FNA results. When the final histology reveals malignancy a completion thyroidectomy is done as second stage operation.

In the Netherlands 459 patients were diagnosed with DTC in 2011. In their flowchart Kleiman et al did not differentiate between Bethesda category III/IV and Bethesda category V. Nonetheless, we could extract this from the text and tables. In short, 36 (12%) of the indeterminate results were Bethesda category V, of those 36, 26 (72%) were malignant, and of those 26, 11 (31%) were BRAF positive. When we apply these results to our nationwide data, using the same distribution of relative ratios as found by Kleiman, we would have had 42 patients with Bethesda category V. Of those 42 patients, 30 (72%) would have had a malignancy, 13 of which (31%) would comprise a BRAF(V600E) mutation. Hence, by performing preoperative BRAF(V600E) analysis in the group of patients with Bethesda category V, these 13/42 patients (31%) would have benefited as their initial surgical treatment would have been changed to total thyroidectomy, and a two-stage procedure could have been avoided. This would involve a serious benefit, including a shortened period of uncertainty for the patient, avoidance of a second operation, and a significant reduction of the time between diagnosis and final treatment. Of course, we realize that our analysis is not statistically flawless. For practical reasons, we have relied on the assumption that cytology scores in our country are not significantly different from those in the USA. This assumption is supported by two other studies, describing comparable ratios of indeterminate cytology results and BRAF(V600E) positivity[2, 3]

Subsequently, we performed a rough estimate as to whether the BRAF(V600E) mutation analysis could be cost-effective. If only nodules with Bethesda category V are tested, 42 BRAF(V600E) tests have to be performed to detect 13 BRAF(V600E) mutations. A calculation based on the costs of hemi- and total thyroidectomy in our institution showed that this would not lead to a significant increase in costs.

Based on the notion that the Dutch approach is comparable to the strategy of large parts of Europe the introduction of preoperative BRAF testing on Bethesda category V nodules could make a difference: a considerable number of patients might be spared unnecessary two stage surgery without increasing the total costs of the treatment. We therefore propose that the impact of preoperative BRAF(V600E) testing of indeterminate thyroid nodules on initial surgical management is predominantly dependent on the routine initial surgical strategy adhered to.

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Chapter 7

Characteristics of contralateral carcinomas in patients with differentiated thyroid cancer larger than 1 cm.

Lutske Lodewijk, Wouter P. Kluijfhout, Jakob W. Kist, Inge Stegeman, John T.M. Plukker
Els J. Nieveen van Dijkum, H. Jaap Bonjer, Nicole D. Bouvy, Abbey Schepers, Johannes H.W. de Wilt
Romana T. Netea-Maier, Jos A. van der Hage, Jacobus W.A. Burger, Gavin Ho, Wayne S. Lee, Wen T. Shen
Anna Aronova, Rasa Zarnegar, Cassandre Benay, Elliot J. Mitmaker, Mark S. Sywak, Ahmad M. Aniss
Schelto Kruijff, Benjamin James, Raymon H. Grogan, Laurent Brunaud, Guillaume Hoch, Chiara Pandolfi
Daniel T. Ruan, Michael D. Jones, Marlon A. Guerrero, Gerlof D. Valk, Inne H.M. Borel Rinkes, Menno R. Vriens

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Abstract

Purpose: Traditionally, total thyroidectomy has been advocated for patients with tumors larger than 1 cm. However, according to the ATA and NCCN guidelines (2015, USA), patients with tumors up to 4 cm are now eligible for lobectomy. A rationale for adhering to total thyroidectomy might be the presence of contralateral carcinomas. The purpose of this study was to describe the characteristics of contralateral carcinomas in patients with differentiated thyroid cancer (DTC) larger than 1 cm.

Methods: A retrospective study was performed including patients from 17 centers in 5 countries. Adults diagnosed with DTC stage T1b-T3 N0-1a M0 who all underwent a total thyroidectomy were included. The primary endpoint was the presence of a contralateral carcinoma.

Results: A total of 1313 patients were included, of whom 426 (32 %) had a contralateral carcinoma. The contralateral carcinomas consisted of 288 (67 %) papillary thyroid carcinomas (PTC), 124 (30 %) follicular variant of a papillary thyroid carcinoma (FvPTC), 5 (1 %) follicular thyroid carcinomas (FTC) and 3 (1 %) Hürthle cell carcinomas (HTC). Ipsilateral multifocality was strongly associated with the presence of contralateral carcinomas (OR 2.62).
Of all contralateral carcinomas, 82% were ≤10 mm and of those 99% were PTC or FvPTC.
Even If the primary tumor was a FTC or HTC, the contralateral carcinoma was (Fv)PTC in 92% of cases.

Conclusions: This international multicenter study performed on patients with DTC larger than 1 cm shows that contralateral carcinomas occur in one third of patients and, independently of primary tumor subtype, predominantly consist of microPTC.

Introduction

Differentiated thyroid cancer (DTC) is the most common endocrine malignancy and its incidence is rising. The prognosis is excellent with 10-year survival rates over 90% irrespective of the stage of disease.[1] Until recently, in western countries, treatment of DTC was similar for all stages of macroDTC (DTC larger than 1 cm): total thyroidectomy followed by radioactive iodine ablation (RAI) therapy.[2, 3] However, in the last decade single center studies performed in large volume centers showed no significant differences in recurrence and survival rates in patients diagnosed with macroDTC, who were either treated with lobectomy or total thyroidectomy.[4-7] This has evoked a new discussion about the optimal extent of surgery, whereby according to the ATA and NCCN guidelines (2015, USA), patients with tumors up to 4 cm are now eligible for lobectomy.[8, 9]

Traditional arguments for adhering to total thyroidectomy are the presence of contralateral carcinomas, the ability to perform RAI and the use of thyroglobulin as a follow-up marker. There is, however, increasing support for more selective use of RAI.[9-11] Contralateral carcinomas are reported in up to 44% of patients with DTC.12 Supporters of total thyroidectomy argue that contralateral carcinomas could affect disease recurrence and survival.[12-15] Interestingly, these data are mainly based on patients with microDTC (DTC smaller than 1 cm) and data on the incidence of contralateral carcinomas in macroDTC is currently scarce.[16-21]

We, therefore, aimed to describe the incidence and the characteristics of contralateral carcinomas, and subsequently assess determinants correlating with the presence of contralateral carcinomas in patients with macroDTC.

Patients and Methods

Patients

We conducted a descriptive, retrospective, cross-sectional, multicenter study in a total of 17 centers in 5 countries. Patients who underwent a total thyroidectomy for DTC, either in one or two stages, who were operated between January 2000 and December 2012 and aged ≥18 years were included. Indication for a completion thyroidectomy was confirmation of DTC larger than 1 cm in the histologic examination of the lobectomy specimen.

We specifically selected the patients for whom the discussion about the extent of surgery is most relevant. The TNM-stages that were included for the different histological subtypes were based on the currently recruiting study of Mallick et al..[10] This study investigates whether in a subgroup of low-risk patients ablation can be omitted, without compromising recurrence or survival rates. This concerns patients with a papillary thyroid carcinoma (PTC) including, follicular variant of papillary thyroid carcinoma (FvPTC) with stage pT1b-T2-T3, N0-N1a-Nx

and patients with a follicular thyroid carcinoma (FTC) or a Hürthle cell carcinoma (HTC) stages pT1b-T2, N0-N1a-Nx. The TNM classification from the 7th edition of the AJCC cancer staging manual was used.[22]

In Dutch University Medical Centers, all consecutive patients who were operated between 2000 and 2012 were included since these were only limited numbers. In the high-volume international centers, 150 patients were randomly generated from a list that included all patients that fulfilled inclusion criteria who were operated between 2000 and 2012. Cases were selected by creating a list of numbers generated by randomization software. Pathologic staging was performed according to the AJCC cancer staging manual. In the seven participating Dutch University Medical Centers data entry was performed by the same researcher (WPK). Outside the Netherlands, data were collected by a local investigator, using a well-defined data entry manual to ensure homogeneous input. The study was approved by the institutional review board of the University Medical Center Utrecht (the Netherlands) and in other centers if required.

Characteristics of contralateral carcinomas

The following characteristics of the contralateral carcinomas were collected: size, histological subtype and contralateral multifocality.

Determinants associated with contralateral disease

After performing a pilot study in 30 patients from the UMC Utrecht and by reviewing the recent literature, 13 determinants were selected.[17, 20, 23-25] Determinants included sex, age at diagnosis, size on ultrasound of primary tumor, Bethesda classification of the primary tumor, postoperative N-stage, size of the contralateral lobe on pathology (PA), size of the primary tumor, histological subtype of the primary tumor, multifocality in the lobe of the primary tumor (ipsilateral multifocality), angioinvasion, capsular invasion (cells invading the capsule of the tumor), extrathyroidal growth and surgical resection margins of the primary tumor (defined by evaluating resection margins at pathology). Data were collected from chart reviews, cytology reports of fine needle aspiration (FNA), reports of preoperative ultrasound and the histology reports.

Statistical analysis

All continuous variables were tested for linear association with the outcome and in the case of non-linearity the variable was categorized in clinically relevant groups.[26] The possible determinants were assessed for patients with- and without contralateral carcinoma, and univariate regression analysis and multivariate regression analysis were performed. Variables with a p-value <0.1 in the univariate regression analysis were selected for multivariate analysis. A p-value <0.05 was considered statistically significant. All statistical analyses were performed using SPSS version 22 (SPSS Inc., Chicago, IL).

Table 7.1: Distribution of number of included patients per center.

Country	Hospital	Number of patients
The Netherlands	UMC Utrecht	40
	UMC Groningen	50
	Leiden UMC	50
	Radboud UMC	31
	Maastricht UMC+	15
	Erasmus UMC	49
	VU Medical Center	26
	Amsterdam Medical Center	26
	Antonie van Leeuwenhoek Hospital	23
United States of America	University of California San Francisco	106
	Weill Cornell Medical College	106
	University of Chicago	128
	Brigham and Women's Hospital	145
	The University of Arizona Medical Center	77
Canada	McGill University Health Center	99
France	Centre Hospitalier Universitaire de Nancy	137
Australia	Royal North Shore Hospital	205

Results

Patients

In total, we included 1313 patients in 17 centers (Table 7.1). The mean age at time of surgery was 47.4 years (SD 14.5), and 967 (74%) patients were female. Total thyroidectomy as primary surgical intervention was performed in 961 (73%) patients, whereas 352 (27%) patients initially had a lobectomy followed by completion thyroidectomy. Central lymph node dissection was

Table 7.2: Descriptive statistics for the study population.

Determinants	N	Contralateral carcinoma +	OR (95% CI) Univariate analyses	P-value	OR (95% CI) Multivariate analyses	P-value
N	1313	426 (32%)				
Sex						
Female	967 (74%)	320 (75%)	0.89 (0.69-1.14)	0.40		
Male	346 (26%)	106 (25%)				
Age			1.01 (1.00-1.02)	0.07	1.02 (1.01-1.04)	0.01
≤45	579 (44%)	182 (43%)				
≥45	734 (56%)	244 (57%)				
Size primary tumor US (mm)			1.00 (1.00-1.01)	0.41		
<11	32 (3%)	13 (4%)				
11-20	384 (37%)	132 (39%)				
21-30	325 (31%)	87 (25%)				
31-40	148 (14%)	49 (14%)				
>40	162 (15%)	61 (18%)				
Missing	262					
FNA (Bethesda)						
1	57 (5%)	15 (4%)				
2	91 (8%)	34 (9%)	0.61 (0.33-1.13)	0.11	0.58 (0.21-1.60)	0.30
3	85 (8%)	25 (7%)	1.01 (0.64-1.61)	0.96	0.83 (0.35-2.00)	0.68
4	213 (19%)	55 (15%)	0.71 (0.43-1.17)	0.18	0.89 (0.37-2.15)	0.79
5	205 (19%)	63 (18%)	0.59 (0.41-0.85)	0.00	0.68 (0.37-1.25)	0.22
6	456 (41%)	169 (47%)	0.75 (0.53-1.07)	0.12	0.93 (0.50-1.72)	0.81
Missing	206					
Nodal status						
N0	979 (78%)	397 (73%)	1.56 (1.18-2.07)	0.00	1.89 (1.07-3.34)	0.03
N1a	269 (22%)	109 (27%)				
Missing	65					
Size primary tumor PA (mm)			1.01 (1.00-1.02)	0.07	1.02 (1.00-1.05)	0.03
11-20	618 (47%)	197 (46%)				
21-30	370 (28%)	115 (27%)				
31-40	163 (12%)	49 (12%)				
>40	162 (12%)	65 (15%)				
Angio-invasion						
No	901 (78%)	298 (79%)	0.93 (0.69-1.26)	0.66		
Yes	247 (22%)	78 (21%)				
Missing	165					
Capsular invasion	594 (59%)	187 (60%)	0.93 (0.71-1.22)	0.59		
No	415 (41%)	124 (40%)				
Yes	304					
Missing						

Determinants	N	Contralateral carcinoma +	OR (95% CI) Univariate analyses	P-value	OR (95% CI) Multivariate analyses	P-value
Extra-thyroidal growth						
No	1006 (81%)	307 (77%)	1.56 (1.16-2.10)	0.00	1.02 (0.53-1.93)	0.96
Yes	231 (19%)	94 (23%)				
Missing	76					
Negative margins						
No	374 (29%)	121 (30%)	0.99 (0.77-1.28)	0.95		
Yes	898 (71%)	289 (70%)				
Missing	41					
Multifocality						
No	1033 (79%)	285 (67%)	2.64 (2.01-3.47)	0.00	2.62 (1.60-4.29)	0.00
Yes	277 (21%)	139 (33%)				
Missing	3					
Subtype Carcinoma						
PTC	794 (61%)	280 (67%)				
FvPTC	354 (27%)	113 (27%)	0.86 (0.66-1.12)	0.27	0.58 (0.35-0.97)	0.04
FTC	116 (9%)	20 (5%)	0.38 (0.23-0.63)	0.00	0.54 (0.22-1.33)	0.18
HTC	38 (3%)	6 (1%)	0.34 (0.14-0.83)	0.02	0.16 (0.03-0.77)	0.02
Missing	11					
Size Contralateral Lobe (mm)			1.00 (1.00-1.00)	0.59		
<10	261 (24%)	68 (20%)				
>10-<15	254 (24%)	85 (25%)				
>15-<25	262 (24%)	81 (23%)				
>25	299 (28%)	113 (33%)				
Missing	237					

Number (N) of patients with a contralateral carcinoma is shown for each determinant. Odds-Ratios (OR) and p-values are shown for the uni- and multivariate analyses. Abbreviations: US; ultrasonography, FNA; Fine Needle Aspiration, PA; pathology

not standard of care but was performed dependent of the presence of suspicious lymph nodes on preoperative ultrasound, preference of the surgeon and the clinic. The histological subtype of the primary tumor was PTC in 794 patients (61%), FvPTC in 354 (27%), FTC in 116 (9%), and HTC in 38 (3%). Unilateral tumor multifocality was seen in 277 (21%) patients and 269 (22%) had central lymph node metastases. Capsular invasion, angioinvasion and extra-thyroidal growth were found in 415 (41%), 247 (22%), and 231 (19%) cases, respectively.

Table 7.3: Histological subtype of the primary tumors versus the histological subtype of the contralateral tumor. Missing: n = 14 (3 %).

	Subtype of primary tumor					
Subtype of contralateral tumor		PTC	FvPTC	FTC	HTC	Total
	PTC	244 (88%)	25 (23%)	13 (65%)	2 (33%)	284 (69%)
	FvPTC	30 (11%)	84 (76%)	6 (30%)	3 (50%)	123 (30%)
	FTC	1 (0%)	2 (2%)	1 (5%)	0 (0%)	4 (1%)
	HTC	2 (1%)	0 (0%)	0 (0%)	1 (17%)	3 (1%)
	Total	277 (100%)	111 (100%)	20 (100%)	6 (100%)	414 (100%)

Table 7.4: Size of the contralateral tumor versus the histological subtype of the contralateral tumors. Missing: n = 34 (8 %)

	Size of contralateral tumor				
Subtype of contralateral tumor		≤5 mm	6-10 mm	>10 mm	Total
	PTC	169 (71%)	54 (64%)	45 (63%)	268 (68%)
	FvPTC	68 (29%)	29 (35%)	21 (29%)	118 (30%)
	FTC	0 (0%)	1 (1%)	4 (6%)	5 (1%)
	HTC	1 (0%)	0 (0%)	2 (3%)	3 (1%)
	Total	238 (100%)	84 (100%)	72 (100%)	394 (100%)

Characteristics of contralateral carcinomas

The overall rate of contralateral carcinomas was 32% (table 7.2). The majority of contralateral carcinomas were PTC (N=284; 69%) or FvPTC (N=123; 30%), while only a few were FTC (N=4; 1%) and HTC (N=3; 1%) (Table 7.3). The median size of the contralateral carcinomas was 4 mm (IQR 2-9 mm). If the primary tumor was non-(Fv)PTC, so FTC or HTC, the contralateral

carcinoma was (Fv)PTC in 92% of cases. Sixty percent of the contralateral tumors were 5 mm or smaller, 21% were between 6 and 10 mm and 18% were larger than 10 mm. Of the 82% of tumors sized 10 mm or smaller, 99% were PTC or FvPTC. Six out of the total of eight contralateral FTCs or HTCs were 10 mm or larger (Table 7.4).
Ipsilateral multifocality was most frequent when the primary tumor was PTC (23%), followed by FvPTC (20%), HTC (18%), and FTC (9%). The histological subtype of the ipsilateral tumors was PTC or FvPTC in 99% of patients (data not shown).

Determinants associated with contralateral disease

Based on the presence of contralateral carcinomas, univariate analysis of possible determinants was performed (Table 7.2). Contralateral carcinoma was significantly more frequent in patients with N1a nodal metastasis (OR 1.56 95% CI 1.18-2.07), in tumors with extra-thyroidal growth (OR 1.56 95% CI 1.16-2.10), and when ipsilateral multifocality was found (OR 2.64 95% CI 2.01-3.47). When the histologic subtype of the primary tumor was a FTC or a HTC the likelihood that a contralateral carcinoma was present decreased significantly (FTC: OR 0.38 95% CI 0.23-0.63; HTC: OR 0.34 95% CI 0.14-0.83). In multivariate analysis, ipsilateral multifocality (OR 2.62 95% CI 1.60-4.29) and lymph node metastasis (OR 1.89 95% CI 1.07-3.34) were strongly correlated with the occurrence of contralateral carcinoma(s). Furthermore, when the primary carcinomas were FvPTC, FTC or HTC, there was a reversed correlation with the occurrence of contralateral carcinomas (FvPTC: OR 0.58 95% CI 0.35-0.97; FTC: OR 0.54 95% CI 0.22-1.33; HTC: OR 0.16 95% CI 0.03-0.77). The other investigated determinants, sex, age, size primary tumor (US or PA), angioinvasion, capsular invasion, negative resection margins and size of the contralateral lobe, did not correlate with the presence of contralateral carcinomas.

Discussion

In this international multicenter study, the incidence and characteristics of contralateral carcinomas were investigated in a large cohort of patients with primary macroDTC. The rate of contralateral malignancies was 32% and the dominant histological subtype of the contralateral carcinomas was PTC or fvPTC (94%). Median size was 4 mm and 82% of carcinomas was <1 cm. No correlation between histological subtype of the primary tumor and the subtype of the contralateral tumor was found. Multifocality in the lobe of the primary tumor had the strongest association with contralateral carcinoma in multivariate analysis with an OR of 2.62.
The rate of contralateral carcinomas is in agreement with current literature that reports rates between 17 and 43%. Most of these studies focused on contralateral carcinomas in primary papillary thyroid microcarcinomas (microPTC),[16, 17, 19-21] or had limited patient numbers, failed to report clear in- and exclusion criteria, or excluded patients with FTC.[25, 27-29] In contrast, our study

investigated contralateral carcinoma in a large, well-described and clinically relevant cohort, in which primary tumors were macroDTC. In our study, the rate of contralateral carcinomas was higher in PTC and FvPTC compared to FTC and HTC, 34 versus 17%. This is in line with a study by Machens et al., who found significantly more tumor multifocality in patients with PTC versus FTC.[30]

In our study, 82% of all contralateral carcinomas were microPTC. Based on several other studies, the clinical relevance of microPTCs can be questioned. In an observational trial performed in Japan, including 1235 patients with primary microPTC, tumor progression of more than 3 mm was noticed in only 8.0% of patients, novel nodal metastasis developed in 3.8%, and only 6.8% developed into clinical disease after 10 years of follow-up. Eventually, only 15% of patients underwent surgery.[11, 31] These low progression rates show that these primary microPTCs rarely develop into clinically significant thyroid carcinomas. Our study described contralateral microcarcinomas, while this study addressed primary microPTC, and currently, it is unknown whether the natural course of primary microPTC differs from those of contralateral microPTC. However, this is indirectly investigated by analyzing recurrence rates in the contralateral lobe in studies where DTC is treated by lobectomy. In a study with up to 20 years of follow-up comparing patients with microPTC treated with lobectomy versus treatment with total thyroidectomy, no difference in overall survival or in recurrence rates were found.[5] One might assume that in the remaining thyroid lobe, similar rates of contralateral carcinomas were present as in our population. Furthermore, from autopsy reports, it is known that when thyroid glands are thoroughly examined, malignancy rates of up to 36% are found which is similar to our contralateral carcinoma rate.[1, 32] Altogether, the clinical relevance of 82% of the contralateral carcinomas found in our study is questionable.

Currently, there is no consensus whether multifocal carcinomas arise as a result of true multicentricity or intrathyroidal spread of a primary tumor, as it is underlined by the report of the European Society of Endocrine Surgeons 2013.[33] In our study, no correlation was found between the histological subtype of the primary tumor and that of the contralateral carcinoma. Moreover, even when the primary tumor was of follicular or Hürthle cell origin, 99% of the contralateral carcinomas were PTC. This suggests that true multicentricity is more likely than intrathyroidal spread.

Patients with DTC have an excellent 10-year overall survival, but they do suffer from a relatively low quality of life (QoL) in comparison with other cancers, such as breast or colorectal cancer.[34, 35] This decreased QoL is expressed in several adverse physical, psychological, social, and spiritual challenges.[34] After RAI, patients had significantly more complaints of hypo- or hyperthyroidism, resulting in a decreased QoL.[35] Therefore, one of the factors to improve QoL of macroDTC patients is to pursue normal thyroid hormone homeostasis by performing parenchyma-sparing operations. After thyroid lobectomy, hormone replacement might be necessary in 10 to 50 % of patients; this is highly correlated to the TSH-level and presence of

microsomal antibodies. In patients with a low TSH level (<2.5 mIU/L) and without microsomal antibodies, the risk to become hormone replacement dependent is only 7%. Furthermore, after lobectomy, patients needed a lower dose of levothyroxine and less adjustments steps to become euthyroid.[36-38] Therefore, QoL might improve by performing a lobectomy instead of total thyroidectomy.

As the discussion continues, we believe treatment of DTC will become more and more a patient-tailored matter, in which the pros and cons of lobectomy or total thyroidectomy must be weighed one by one, based on existing evidence and discussed with the patient. The three main arguments in favor of total thyroidectomy are the ability to perform RAI, the use of thyroglobulin as a follow-up marker, and the high rate of contralateral carcinomas. Arguments in favor of lobectomy are reduced complication risk, especially recurrent laryngeal nerve injury and persisting hypocalcaemia, reduced risk of hypothyroidism, and no risk of complications from RAI. Taken the above-mentioned arguments into account, we question whether, in case the ultrasound of the contralateral lobe does not show suspicious lesions, the possible presence of microPTC should be an argument in favor of total thyroidectomy.

Conclusion

This international multicenter study is the largest study performed on patients with macroDTC and confirms that, in patients with macroDTC, the rate of contralateral carcinomas is 32%. This study shows that these contralateral carcinomas predominantly consist of microPTC.

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Chapter 8

Expression of HIF-1α in medullary thyroid cancer identifies a subgroup with poor prognosis

Lutske Lodewijk, Paul J. van Diest, Petra van der Groep, Natalie D. ter Hoeve, Abbey Schepers, Hans Morreau, J.J. Bonenkamp, A.C.H. van Engen – van Grunsven, Schelto Kruijff, Bettien M. van Hemel, Thera P. Links, Els J. Nieveen van Dijkum, Susanne van Eeden, Gerlof D. Valk, Inne H.M. Borel Rinkes, Menno R. Vriens

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Abstract

Background: Medullary thyroid cancer (MTC) comprises only 4% of all thyroid cancers and originates from the parafollicular C-cells. HIF-1α expression has been implied as an indicator of worse prognosis in various solid tumors. However, whether expression of HIF-1α is a prognosticator in MTC remained unclear. Our aim was to evaluate the prognostic value of HIF-1α in patients with MTC.

Methods: All patients with MTC who were operated on between 1988 and 2014 in five tertiary referral centers in The Netherlands were included. A tissue microarray was constructed in which 111 primary tumors could be analyzed for expression of HIF-1α, CAIX, Glut-1, VEGF and CD31 and correlated with clinicopathologic variables and survival.

Results: The mean age of patients was 46.3 years (SD 15.6), 59 (53.2%) were male. Of the 111 primary tumors, 49 (44.1%) were HIF-1α negative and 62 (55.9%) were HIF-1α positive. Positive HIF-1α expression was an independent negative indicator for progression free survival (PFS) in multivariate cox regression analysis (HR 3.1; 95% CI 1.3 – 7.3). Five-years survival decreased from 94.0% to 65.9% for the HIF-1α positive group (p=0.007). Even within the group of patients with TNM-stage IV disease, HIF-1α positivity was associated with a worse prognosis, shown by a decrease in 5-years survival of 88.0% to 49.3% (p=0.020).

Conclusion: Expression of HIF-1α is strongly correlated with adverse prognosis of MTC. This could open up new ways for targeted systemic therapy of MTC.

Introduction

Medullary thyroid cancer (MTC) accounts for 4% of all thyroid cancers and, in contrast to other forms of thyroid cancer, it arises from the parafollicular C-cells. It occurs either as a sporadic disease or in a hereditary context as a manifestation of the endocrine tumor syndrome Multiple Endocrine Neoplasia type 2 (MEN2) (20-25%).[1] Hereditary tumors are characterized by activating mutations in the rearranged-during-transfection (RET) proto-oncogene. Sporadic MTCs harbor, in 50% of cases, an acquired mutation in the RET proto-oncogene. About 45% of all patients present with advanced disease (stage III-IV), which has a 10-year survival rate of 71% and 21%, respectively.[2]

Currently, clinical variables such as age, extent of the primary tumor, lymph node metastases and distant metastases have been identified as prognostic factors.[2] No further stratification for stage III-IV patients is available, while prognosis within these groups can vary widely.[3]

To set the indication for follow-up or adjuvant treatment, patients with poor prognosis need to be identified, especially within the subgroup of stage III-IV patients.

Hypoxia inducible factor-1 (HIF-1) is the key regulator of the hypoxia response.

HIF-1 is a protein complex composed of two subunits; constitutive HIF-1α and oxygen-sensitive HIF-1α. HIF-1α can either be upregulated due to oncogenic signaling or as a response to tumor hypoxia. Under normal conditions the HIF-1α subunit is degraded by the ubiquitin-proteasome pathway. Under hypoxia or as a result of oncogenic signaling, degradation is inhibited resulting in its accumulation, subsequent binding to HIF-1α, translocation to the nucleus and activation of downstream signaling pathways by binding to hypoxia responsive elements in the promoters of target genes.[4-9] Increased expression of HIF-1α in tumor cells, whether induced by hypoxia or by aberrant oncogenic signaling, actively drives tumor growth and progression by regulating the expression of crucial target genes such as vascular endothelial growth factor (VEGF), carbonic anhydrase IX (CAIX) and glucose transporter 1 (Glut-1).[8, 10]

In this regard, the importance of HIF-1α and its downstream targets has been investigated in various solid tumors, and the correlation of high HIF-1α expression with poor prognosis has been well established.[10] In sporadic MTC, expression of HIF-1α has been reported in 89% of tumors, and associated with clinical features, as lymph node positivity, higher T-stage and extrathyroidal extension, which are known to adversely affect prognosis.[11] However, due to the lack of survival data, prognostic value of HIF-1α in MTC could not be determined. Our aim was therefore to investigate the prognostic value of HIF-1α expression in MTC.

Materials and methods

Patients

Patients who underwent surgery between 1988 and 2014 for MTC were identified from the pathology databases of Leiden University Medical Center (LUMC), Amsterdam Medical Center (AMC), Radboud University Medical Center (RadboudUMC), University Medical Center Groningen (UMCG) and University Medical Center Utrecht (UMCU), The Netherlands (all tertiary referral centers). Formalin fixed paraffin embedded (FFPE) tissues were collected from the pathology archives. In total 111 patients were identified from who primary tumor tissue was available for inclusion in the tissue microarray (TMA).

Whole slides were scored for necrosis, angioinvasion and desmoplasia. Necrosis and angioinvasion were scored as absent or present and desmoplasia as negative, some, moderate or severe. These scorings were performed on the same FFPE blocks that were used for the construction of the TMA.

Clinical and pathological patient information was retrieved from patient files in all five centers. All MEN2 diagnoses were confirmed by germline mutation analysis, sporadic patients were either patients with negative germline mutation analysis or with a negative family history. Microscopic positive resection margins were considered as part of the T-stage and not included as a separate variable. Disease status was based on postoperative calcitonin and CEA measurements; this was scored as a dichotomous variable. Since we included patients from five centers over almost three decades different assays were used for CEA and calcitonin measurements, therefore making it impossible to compare exact values. An elevation in CEA or calcitonin was interpreted as persistent disease, an CEA or calcitonin within normal range was interpreted as cured. Only postoperative CEA and calcitonin measurements were taken into account. Due to the fact that CEA and calcitonin measurements were performed in five centers over almost three decades and different assays were used, doubling times could not reliably be assessed. This study was performed according to national guidelines with respect to the use of leftover tissue and approval for this study was obtained from the Institutional Review Board of the UMCU.[12]

Construction of tissue microarray

The TMA was developed on the TMA machine (TMA grand master, 3D Histec, Budapest, Hungary). Three cores of 0.6 mm were punched from FFPE blocks of the primary tumor. To assure that cores were punched from tumor areas, cell rich areas were marked on H&E slides by a pathologist (PJvD), scanned, and marks were manually circled with the TMA software (3D Histech). In this manner cell rich punches were automatically inserted into the recipient block.

Immunohistochemistry

After deparaffinization and rehydration, endogenous peroxidase was blocked in a buffer solution containing 0.3%hydrogen peroxidase for 15 minutes. For HIF-1α antigen retrieval was performed using EDTA buffer, pH = 9.0, at boiling temperature for 20 minutes. A cooling period of 30 minutes preceded the incubation of the slides with protein block (Novolink Max Polymer detection system, ready to use, Novocastra Laboratories Ltd, Newcastle Upon Tyne, UK) for 5 minutes at room temperature. Incubation of the slides with the HIF-1α mouse monoclonal (BD Biosciences, Pharmingen, Lexington, MA, USA), was done at a dilution of 1:50 overnight at 4°C. For detection, a polymer (Novolink Max Polymer detection system, ready to use, post primary for 30 minutes and Novolink Polymer for 30 minutes) was used and developed with diaminobenzidine (5 minutes, Novolink Polymer detection system). For Glut-1, CAIX and VEGF-A, downstream targets of HIF-1α, antigen retrieval was performed in citrate buffer, pH = 6.0, for 20 minutes at boiling temperature. For Glut-1 and CAIX, a cooling period of 30 minutes preceded the incubation (60 minutes at room temperature) with the primary antibodies. Polyclonal primary antibodies used were Glut-1 (1:200, DAKO, Santa Clara, USA) and CAIX (1:1000, Abcam, Cambridge Science Park, Cambridge, UK). For VEGF-A, a cooling period of 30 minutes preceded the incubation of the slides with the VEGF-A rabbit polyclonal antibody (0.2 αg/mL, RB-9031, ThermoFisher, Fremont, USA).[13] For detection of the primary antibodies a poly HRP anti- Mouse/Rabbit/Rat IgG (Brightvision ready to use, 30 minutes. ImmunoLogic, Duiven, The Netherlands) was used. All slides were developed with diaminobenzidine (10 minutes) followed by hematoxylin counterstaining. Before the slides were mounted, all sections were dehydrated in alcohol and xylene. Positive and negative controls were used throughout.

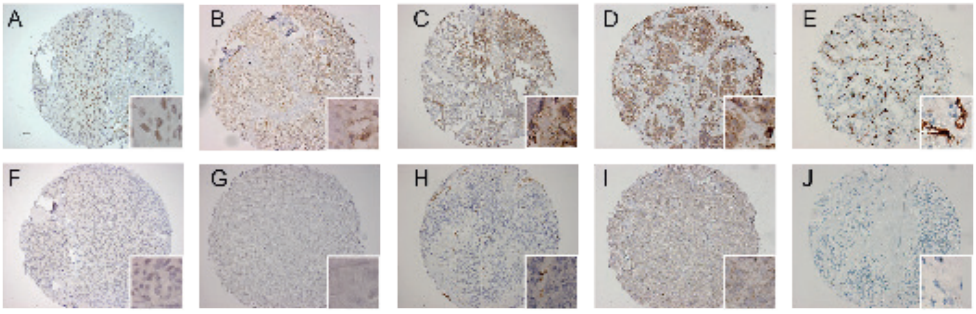

Figure 8.1: *Representative examples of immunohistochemical staining pattern for HIF-1α, CAIX, Glut-1, VEGF and MVD. A. HIF-1α nuclear staining pattern in 50% of cells, B. focal membranous CAIX immunoreactivity, C. focal membranous Glut-1 immunoreactivity, D. strong cytoplasmic VEGF immunoreactivity, E. high MVD shown by CD31 immunoreactivity, F. absent HIF-1α staining, G. absent CAIX staining, H. absent Glut-1 in tumor cells, with control positivity in red blood cells, I. weak VEGF immunoreactivity, J. MVD is 0 shown by absence of CD31 immunoreactivity.*

Table 8.1: Clinicopathological characteristics of all patients stratified by HIF-1α status.

	HIF-1α neg (N=49)	HIF-1α pos (N=62)	p-value
Mean age in years (SD)	45.0 (14.1)	47.2 (16.7)	0.46
Sex			
Male (%)	25 (51.0)	34 (54.8)	0.71
Heritability			
Sporadic (%)	33 (67.3)	32 (54.2)	0.33
MEN2a/b (%)	16 (28.6)	27 (42.4)	
Stage			
I – III (%)	28 (10.2)	30 (16.1)	0.23
IV (%)	21 (42.9)	32 (51.6)	
Size mean in mm (SD)	26.1 (15.2)	25.1 (15.5)	0.75
< 20mm (%)	20 (40.8)	22 (38.6)	0.84
≥ 20mm (%)	29 (59.2)	35 (61.4)	
Lymph node metastasis	28 (57.1)	42 (67.7)	0.32
Overall Survival	46 (93.9)	49 (79.0)	0.03
Progression Free Survival	42 (85.7)	38 (61.3)	0.01
Disease status			
Normal CEA/calcitonin (%)	17 (37.8)	22 (36.7)	1.00
Elevated CEA/calcitonin (%)	28 (62.2)	38 (63.3)	
Presence of necrosis	3 (6.2)	6 (10.0)	0.73
Presence of angioinvasion	4 (8.3)	6 (10.0)	1.00
Presence of desmoplasia			
None – some	32 (66.7)	20 (33.3)	0.00
Moderate – severe	16 (33.3)	40 (66.7)	
CAIX			
Negative (%)	29 (59.2)	30 (49.2)	0.34
Positive (%)	20 (40.8)	31 (50.8)	
Glut-1			
Negative (%)	48 (98.0)	57 (91.9)	0.23
Positive (%)	1 (2.0)	5 (8.1)	
MVD mean vessels/core	15.9 (9.7)	13.2 (5.8)	0.10
< 14 vessels/core	22 (48.9)	35 (58.3)	0.43
≥ 14 vessels/core	23 (51.1)	25 (41.7)	
VEGF			
Negative (%)	18 (41.9)	20 (33.3)	0.41
Positive (%)	25 (58.1)	40 (66.7)	

Abbreviations: SD = Standard Deviation; MEN2a/b = Multiple Endocrine Neoplasia type 2a/b; CAIX = carbonic anhydrase IX; Glut-1 = glucose transporter 1; MVD = microvessel density; VEGF = Vascular Endothelial Growth Factor.

To calculate microvessel density (MVD) CD31 immunohistochemistry was performed on the automatic system (BenchMark ULTRA, Ventana Medical System, Tucson, Arizona). The CD31 mouse monoclonal antibody, clone JC70A (1:100, DAKO, Santa Clara, USA).

Scoring of immunohistochemistry

All TMA slides were scored by an experienced pathologist (PJvD) and an experienced researcher (LL), when there was inconsistency between both a 2nd experienced pathologist was consulted. For HIF-1α the percentage of positive nuclei per core was scored as an absolute number. For statistical analysis this was transformed in a dichotomous variable, which was positive when in either one of the three cores ≥1 percent of nuclei were positive as previously used.[8, 11, 14] Glut-1 and CAIX were scored as absent, cytoplasmic or membranous for each core separately. For statistical analysis, only membranous staining was taken into account, when this was present for either one of the cores the tumor was considered positive.[8] VEGF-A was scored as absent (0), weak (1), moderate (2) or strong (3) for each core separately. For statistical analysis the average score over three cores was calculated and when this was ≥2 it was considered positive. MVD was calculated by the average number of CD31 positive vessels per core. For the dichotomous variable of CD31, a cut-off of 14 (mean) was chosen. Representative scores of all immunostainings are shown in figure 8.1.

Statistical Analysis:

Categorical data were summarized with frequencies and percentages, and continuous data were summarized with medians and ranges. Progression was defined as development of distant metastases or dead. This excluded development of lymph node metastases or elevation in CEA/calcitonin. We chose this definition since MTC is an incurable disease when distant metastases occur. Progression-free survival (PFS) was therefore defined as the time to development of distant metastases or dead. To increase the power of the statistical analysis categorical data were recoded into dichotomous variables. Stage I, II, III and IV was recoded into stage I - III and stage IV; hereditability was recoded in either sporadic or hereditable; grade of desmoplasia was recoded in none - some and moderate - severe. The chi-square test was used to assess associations between the dichotomous variables, the Student's t-test was used to test for differences between continuous variables. Kaplan-Meier survival curves were plotted, and univariate survival analysis was performed and the log-rank test was used to calculate significance. Multivariate Cox-regression analysis was performed and Hazard Ratios (HR) of clinicopathologic characteristics on PFS were calculated. Violations of the proportional hazards assumption were tested by the log minus log plot and by adding a time dependent covariate. All reported p-values were two sided. Analysis was performed using SPSS version 22.0 software (SPSS, Inc., Chicago, IL, USA).

Table 8.2: Univariate Kaplan-Meier survival analysis on PFS and OS.

	Progression Free Survival				Overall Survival			
	N	PF (N)	PF (%)	p- value	N	OS (N)	OS (%)	p- value
Stage				0.00				0.14
I-III	56	53	94.6		53	48	90.6	
IV	52	24	46.2		49	38	77.6	
Gender				0.09				0.62
Male	56	36	64.2		53	44	83.0	
Female	52	11	78.8		49	42	85.7	
Tumor size				0.54				0.71
< 20mm	40	33	82.5		39	34	87.2	
≥ 20mm	63	42	68.3		59	50	84.7	
Lymph node metastasis				0.00				0.09
Yes	39	37	94.9		37	34	91.9	
No	69	40	68.4		65	52	80.0	
Heritability				0.00				0.01
Sporadic	62	40	64.5		57	45	78.9	
MEN2a/b	43	36	83.7		42	39	92.9	
HIF-1α				0.01				0.04
Negative	47	40	85.1		44	41	93.2	
Positive	61	37	60.7		58	45	77.6	
Disease status				0.00				0.05
Normal CEA/calcitonin	39	37	94.9		39	37	94.9	
Elevated CEA/calcitonin	66	37	56.1		62	48	77.4	
Necrosis				0.01				0.01
Negative	96	25	74.0		92	79	85.9	
Positive	9	6	33.3		7	4	57.1	
Angioinvasion				0.18				0.67
Negative	95	26	72.6		90	75	83.3	
Positive	10	5	50.0		9	8	88.9	
Desmoplasia				0.16				0.30
None – Some	51	10	80.4		49	44	89.8	
Moderate – Severe	54	21	61.1		50	39	78.0	
CAIX				0.69				0.49
Negative	59	45	76.3		58	51	87.9	
Positive	48	32	66.7		43	34	79.1	
Glut-1				0.02				0.51
Negative	102	74	72.5		97	81	83.5	
Positive	6	3	50.0		5	5	100.0	

	Progression Free Survival				Overall Survival			
	N	PF (N)	PF (%)	p- value	N	OS (N)	OS (%)	p- value
VEGF				0.37				0.01
Negative	37	26	70.3		34	32	94.1	
Positive	64	48	75.0		61	49	80.3	

Abbreviations: N = number; PF = progression free; OS = overall survival; MEN2a/b = Multiple Endocrine Neoplasia type 2a/b; CAIX = carbonic anhydrase IX; Glut-1 = glucose transporter 1.

Results

Clinicopathologic variables

The mean age of the 111 patients was 46.3 years (SD 15.6), 59 (53.2%) were male. Sixty-five patients (60.2%) had a sporadic medullary thyroid carcinoma, 39 patients (36.1%) had MEN2a and 4 patients (3.7%) had MEN2b, from 3 patients the RET-mutation status was unknown. Fifteen patients presented with stage 1 (13.5%), 26 with stage II (23.4%), 17 (15.3%) with stage III and 53 (47.7%) with stage IV disease. Median tumor size was 25.6 mm (IQR 25; minimum 2 mm; maximum 80 mm) and 79 (63.1%) patients presented with lymph node metastases.
Mean follow-up was 79.2 months (SD 60.6).

Table 8.3: *Multivariate cox-regression analysis on PFS.*

	Events/patients	Adjusted Hazard Ratio (95% CI)	p-value
HIF-1α			0.01
Negative	7/49	1	
Positive	24/62	3.1 (1.3 – 7.3)	
Stage			0.00
I-III	3/56	1	
IV	28/52	6.8 (2.0 – 22.9)	
Heritability			0.04
Sporadic	22/62	1	
MEN2a/b	7/43	0.4 (0.16 – 0.95)	

Abbreviations: HIF-1α = Hypoxia Inducible Factor 1α; MEN2a/b = Multiple Endocrine Neoplasia type 2a/b.

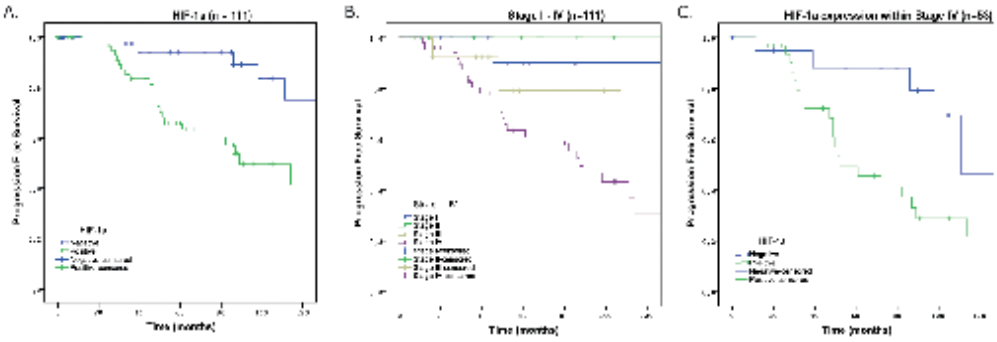

Figure 8.2: 10-years PFS in Kaplan-Meier survival curve in patients with HIF-1α positive MTC compared to patients with HIF-1α negative MTC. A. Analyses over total group of 111 patients comparing HIF-1α positive versus HIF-1α negative MTC. B. Analyses over total group comparing TNM-stage I-IV. C. Analyses over subpopulation of TNM-stage IV patients comparing HIF-1α positive versus HIF-1α negative MTC.

Association between HIF-1α expression and clinicopathologic variables

Forty-nine (44.1%) patients were HIF-1α negative and 62 (55.9%) HIF-1α positive. In univariate analysis, age, gender, heritability, stage, tumor size, lymph node metastases, disease status, microvessel density (MVD) and presence of necrosis or angioinvasion did not differ significantly between HIF-1α negative and positive groups (Table 8.1). For overall survival (OS) and PFS a significant association with HIF-1α status was found. Forty-six (93.9%) patients in the HIF-1α negative versus 49 (79.0%) patients in the HIF-1α positive group survived (p = 0.03). Forty-two (85.7%) patients in the HIF-1α negative versus 38 (61.3%) patients in the HIF-1α positive group did not show progression, i.e. they did not develop distant metastasis (p = 0.01).
Grade of desmoplasia correlated significantly with HIF-1α expression, in the moderate to severe group 40 patients (66.7%) showed HIF-1α positivity (p = 0.00). CAIX, Glut-1 and VEGF showed a positive correlation with HIF-1α, with an OR of 1.5, 4.2 and 1.4 respectively, however, this correlation was not significant (Table 8.1).

Prognostic value

To verify which variables were associated with PFS and OS a univariate survival analysis was performed (Table 8.2). For PFS a significant association was found for TNM-stage, presence of lymph node metastasis, heritability, HIF-1α, necrosis and Glut-1. For OS an association was found for heritability, disease status, necrosis, HIF-1α and VEGF. Our total number of events, patients that developed metastases and/or died, (n = 33) restricted us in the number of variables for multivariate analysis; therefore, we studied in a Cox regression analysis the association of HIF-1α, TNM-stage and heritability on PFS. The variable lymph node metastasis

was left out of the Cox regression analysis since this is also taken into account by the TNM-stage; disease status was left out since this is predominantly base on postoperative CEA/calcitonin measurements which are of value to evaluate over time to show tumor progression, but not as a single postoperative measurement, Glut-1 was left out as a down-stream effector of HIF-1α; necrosis was left out since only 9 patients showed necrosis, and with a prevalence of only 8.1% it is not suitable as a prognostic factor. In this multivariate analysis, we found that HIF-1α positivity, TNM-stage IV and sporadic MTC were all significantly and independently correlated to PFS (Table 8.3). HIF-1α positivity increased the risk of developing distant metastases with a HR of 3.1 (95% CI 1.3 – 7.3) per year. Hazard-ratios were 6.8 (95% CI 2.0 – 22.9) and 0.4 (95% CI 0.16 – 0.95) for Stage IV disease and MEN2a/MEN2b syndromes respectively. No violations of the proportional hazards assumption were found. In figure 8.2A and figure 8.2B Kaplan-Meier curves visualize the prognostic effect of HIF-1α positivity and of Stage IV disease. In figure 8.2C we show that also within TNM-stage IV disease, HIF-1α is able to identify patients with a less favorable outcome. Five-year survival rates were 55% for the HIF-1α positive group versus 95% for the HIF-1α negative group. Within the TNM-stage IV group survival decreased to 35% for the HIF-1α positive group versus 90% for the HIF-1α negative group.

Discussion

This study shows that HIF-1α is associated with the disease course of MTC and could, therefore, be a valuable prognostic marker. Survival decreases significantly when HIF-1α was expressed, with five-year survival rates of 95% for HIF-1α positive MTC versus 55% for HIF-1α negative MTC. As almost half of patients with MTC present with stage III-IV disease, indicating that the tumor already spread to either lymph nodes in the neck or to distant organs such as lung, liver or bones, a marker that can distinguish patients with a good survival within this group is warranted. Until now, prognostic markers in MTC that were identified, seemed to be of little clinical relevance, especially within the group with distant metastasis.[15-19] In contrast, HIF-1α did discriminate between less and more favorable prognosis within the group with TNM-stage IV in the present study.

Besides TNM-stage, CEA and calcitonin are well known valuable prognostic factors in MTC. In this study, only direct postoperative measurements were included. CEA and calcitonin have the greatest prognostic value in the course of disease, i.e. faster doubling times give a worse prognosis.[20, 21] This means CEA and calcitonin are able to monitor tumor progression over time. However, we were more interested in pre- or postoperatively identifying patients who have a worse prognosis, thus identifying those patients who are likely to have short doubling times of CEA and calcitonin later on, to be able to prevent extensive tumor progression.

Koperek et. al. investigated HIF-1α expression in tumor tissue from 100 patients with sporadic MTC. They found expression of HIF-1α in 51% of cases, using the same threshold for HIF-1α positivity as we did. In line with their findings, our percentage of HIF-1α positivity was 56%. They did not show survival data, however correlations were found with variables that are known to correlate to poor prognosis i.e. grade of desmoplasia, T-stage and lymph node metastasis.[11] Our data confirmed their findings, since a significant correlation was found between HIF-1α expression and TNM-stage, lymph node metastasis and grade of desmoplasia.

Our study demonstrates that high HIF-1α expression in the primary MTC specimen is associated with poor outcome, especially in patients with advanced stage. Patients who are in need of a more intense follow-up can be identified, and this might have consequences for adjuvant therapeutic regimens. The prognostic value of HIF-1α in MTC is in line with findings in numerous studies performed in other cancer-types like; bladder -, breast -, cervical -, colorectal -, gastric -, head and neck -, non-small cell lung -, ovarian -, pancreatic -, prostate cancer and glioblastoma, glioma, melanoma, gastrointestinal stromal tumor and renal cell carcinoma. This has led to the generally accepted idea that increased expression of HIF-1α actively drives tumor growth and progression by regulating the expression of important target genes.[10]

For CAIX expression only a weak-positive correlation was found with HIF-1α positivity. The relationship between CAIX and HIF-1α is complicated, since HIF-1α can also be upregulated by oxygen independent factors. This may affect the extent of their co-localization. Furthermore, the tissues examined are a 'snap-shot' in time, due to the very swift and dynamic kinetics of HIF-1α and the slower transcription of CAIX co-localization can be missed.[22] For Glut-1 a strong correlation (OR 4.2) was found, the lack of significance might be best explained by the relative low number of Glut-1 positive tumors (5.4%). Cytoplasmic staining of VEGF was seen in all MTCs however in a varying degree, we interpreted the VEGF staining as positive when there was a moderate to strong reactivity.[23, 24] No significant correlation was found with HIF-1α, however VEGF is known to be upregulated by RET mutations and sporadic RET mutations are not included in our data.[23] However, we did find that patients with high VEGF expression had a significant shorter overall survival.[25]

Our results also raise interest in targeting HIF-1α in MTC. Targeting hypoxia is challenging. There are in short two main approaches: bioreductive prodrugs and inhibition of molecular targets in hypoxic cells. The main advantage of the bioreductive prodrugs is the ability to, selectively, attack hypoxic cells, resulting in a low toxicity profile. Therefore, they have a greater opportunity for a combination with current standards of therapy; Vandetanib and Cabozantinib.[26] However, the present study is merely a prognostic one, and further in vitro - and in vivo studies should be developed to investigate the role of bioreductive prodrugs in combination with tyrosine kinase inhibitors in MTC.

One of the strengths of this study is the fact that we combine immunohistochemical data with clinical endpoints such as survival or the occurrence of distant metastases in a relatively large

sample size. Furthermore, since MTC is in most cases a relatively low-proliferating tumor, event-rates are low and a long follow-up is needed to detect them.

Our follow-up is long (mean 70.2 months; SD 60.6) and we used PFS to increase the total number of events. One of the limitations is that immunohistochemistry is inherently a more qualitative than quantitative method. Furthermore, one might argue that due to heterogeneity of the HIF-1α, CAIX and Glut-1 staining pattern the use of tissue microarrays is suboptimal. However, studies investigating concordance between whole slide analysis and TMA results found good concordance in general.[27] Moreover, tissue microarrays are described as the standard for the validation of prognostic biomarker,[28, 29] and have been used in studies investigating the same proteins.[14, 30-32] Further limitations are merely due to its retrospective character and the low incidence of MTC. A total of 5 tertiary referral centers have participated and patients over almost 3 decades have been included. To overcome this we limited our analyses to variables least subject to treatment changes overtime or interinstitutional differences.

In summary, HIF-1α overexpression is a prognostic biomarker in MTC indicating a worse prognosis, particularly, in the subpopulation with TNM-stage IV. Thus, HIF-1α may be clinically useful to identify patients in need of more intense follow-up or adjuvant therapy, and may provide an interesting therapeutic target in MTC.

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Chapter 9

The theranostic target Prostate-Specific Membrane Antigen is expressed in medullary thyroid cancer

Lutske Lodewijk, Stefan M. Willems, Koen M.A. Dreijerink, Bart de Keizer4, Paul J. van Diest, Abbey Schepers, Hans Morreau, Han J. Bonenkamp, Ilse A.C.H. Van Engen – van Grunsven, Schelto Kruijff, Bettien M. van Hemel, Thera P. Links, Els Nieveen van Dijkum, Susanne van Eeden, Gerlof D. Valk, Inne H.M. Borel Rinkes, Menno R. Vriens.

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Abstract:

Background: Medullary thyroid cancer (MTC) accounts for 4% of all thyroid cancers and originates from the parafollicular C-cells. Prostate-Specific Membrane Antigen (PSMA) is known for its expression in the epithelium of prostate cancer and has been demonstrated to be useful both for therapeutic and diagnostic purposes as a so-called theranostic target. As PSMA is also expressed in the neovasculature of other solid tumor types, our aim was to assess PSMA expression and its prognostic role in MTC.

Materials and Methods: Tissues from patients that underwent surgery for MTC between 1988 and 2014 in five tertiary referral centers in The Netherlands were included in a tissue microarray. Using immunohistochemistry, total numbers of PSMA and CD31 positive microvessels were evaluated.

Results: 92% of MTC expressed PSMA in the neovasculature, whereas the tumor cells were consistently negative. The average number of PSMA positive microvessels did not differ significantly between the primary tumor and initial lymph node metastases (p=0.09), nor between initial and recurrent lymph node metastases (p=1.00). The PSMA score was found to be correlated with progression free survival and overall survival. In a multivariate analysis, a higher number of PSMA positive microvessesls was associated with a favorable prognosis (OR 3.6; 95%CI 1.0 – 12.8; p=0.05).

Conclusions: Over 90% of MTC appears to express PSMA in the neovasculature. A higher number of PSMA positive microvessels is prognostically favorable. PSMA is an interesting novel target for imaging and potentially also peptide radioligand therapy in MTC.

Introduction:

Medullary thyroid cancer (MTC) accounts for 4% of all thyroid cancers and, in contrast to other types of thyroid cancer, it arises from the parafollicular C-cells. MTC occurs either as a sporadic disease or in a hereditary context as a manifestation of the endocrine tumor syndrome Multiple Endocrine Neoplasia type 2 (MEN2) (20-25%).[1] Hereditary tumors are characterized by activating mutations in the rearranged-during-transfection (RET) proto-oncogene. Sporadic MTCs harbor, in 50% of cases, an acquired mutation in the RET proto-oncogene. About 45% of all patients present with advanced disease (stage III-IV), which is associated with a 10-year survival rate of 71% and 21%, respectively. Clinical variables such as age, extent of the primary tumor, lymph node metastases and distant metastases have so far been identified as prognostic factors.[2] Patients with advanced disease might either have a long-term survival, due to an indolent course of the disease, or develop rapidly progressing disease leading to death from distant metastases. At this moment, it cannot be predicted what will happen within individual cases.[3]

Several clinical issues need to be addressed in recurrent or persistent MTC. First, to guide treatment and follow up, patients with poor prognosis need to be identified, especially within the subgroup of stage III-IV patients.[4] Second, current imaging modalities lack specificity and sensitivity, and often no anatomical substrate can be found in patients with elevated calcitonin and/or CEA levels.[2] Third, current therapies for patients who show progression of distant metastases seem to be able to prolong progression free survival (PFS) but no benefits for overall survival (OS) have been shown, while high adverse event rates are seen.[2]

Prostate-specific membrane antigen (PSMA) is a zinc-dependent exopeptidase. Sites of expression in normal physiology are prostate, kidney, nervous system and small intestine. It is known for its expression in prostate adenocarcinoma.[5] However, more recently, PSMA has been implicated in the neovasculature of several solid tumors including urothelial and renal cell carcinomas, gynecologic cancers, breast cancer, colorectal tumors and thyroid tumors.[6-10]

PSMA is a transmembrane protein, which makes it an interesting target for nuclear imaging and for targeted therapy. To date, several small compounds for labelling PSMA have been developed and are currently being investigated as imaging probes for (68)Gallium-labelled PET scans.[11] For therapeutic purposes several compounds have been investigated, of which (177) Lutetium-labelled PSMA inhibitors and (225)Actinium-labelled PSMA inhibitors are promising.[12, 13]

Our aim was threefold: 1) to find out whether PSMA is expressed in the neovasculature of MTC, 2) whether expression correlated with clinicopathologic variables and 3) whether expression levels remained stable in primary tumors, initial and recurrent lymph node metastases.

Materials & Methods

Patients

Patients who underwent surgery between 1988 and 2014 for MTC were identified from the pathology databases of Leiden University Medical Center (LUMC), Amsterdam Medical Center (AMC), Radboud University Medical Center (RadboudUMC), University Medical Center Groningen (UMCG) and University Medical Center Utrecht (UMCU), The Netherlands (all tertiary referral centers). Formalin fixed paraffin embedded (FFPE) tissues were collected from the pathology archives. In total 111 patients were identified. From all of the 111 patients primary tumor tissue was available for inclusion in the tissue microarray (TMA), from 35 patients lymph node metastases at initial surgery were included and respectively from 10 and 2 patients recurrent lymph node metastases could be included which were removed during a second or third operation.

Whole slides were scored for necrosis, angioinvasion and desmoplasia. Necrosis and angioinvasion were scored as absent or present and desmoplasia as negative, some, moderate or severe. These scorings were performed on the same FFPE blocks that were used for the construction of the TMA.

Both clinical and pathological patient information was retrieved from patient records in all five centers. All MEN2 diagnoses were confirmed by germline mutation analysis, sporadic patients were either patients with negative germline mutation analysis or with a negative family history. Microscopic positive resection margins were considered as part of the T-stage and not included as a separate variable. Disease status was based on postoperative calcitonin and CEA measurements; this was scored as a dichotomous variable. Since we included patients from five centers over almost three decades different assays were used for CEA and calcitonin measurements, therefore making it impossible to compare exact values. An elevation in CEA or calcitonin was interpreted as persistent disease, an CEA or calcitonin within normal range was interpreted as cured. Only postoperative CEA and calcitonin measurements were taken into account. Due to the variety of CEA and calcitonin measurements over the years, doubling times could not reliably be assessed. This study was performed according to national guidelines with respect to the use of 'leftover tissue' 7 and approval for this study was obtained from the Institutional Review Board of the UMCU.

Construction of tissue microarray

The TMA was developed on the TMA machine (TMA grand master, 3D Histec, Budapest, Hungary). Three cores of 0.6 mm were punched from FFPE blocks of the primary tumor. To assure that cores were punched from tumor areas, cell rich areas were marked on H&E slides by a pathologist (PJvD), scanned, and marks were manually circled with the TMA software (3D Histech). In this manner cell rich punches were automatically inserted into the recipient block.

Immunohistochemistry

From the TMA tissue blocks 4 µm slides were mounted on glass slides, deparaffinized and rehydrated. Immunostaining of PSMA and CD31 was performed on the automatic system (BenchMark ULTRA, Ventana Medical System, Tucson, Arizona, USA). For the CD31 immunostaining the mouse monoclonal anti-CD31 antibody, clone JC70A (1:100, DAKO, Santa Clara, USA) was used. For the PSMA immunostaining the mouse monoclonal anti-PSMA antibody, clone 3E6 (1:20, DAKO, Santa Clara, USA) was used. HIF-1α, Glut-1, CAIX and VEGF-A immunohistochemistry were performed manually as described before. Incubation of the slides with the HIF-1α mouse monoclonal antibody (BD Biosciences, Pharmingen, Lexington, MA, USA), was done at a dilution of 1:50 overnight at 4°C. For Glut-1 and CAIX polyclonal primary antibodies were used; Glut-1 (1:200, DAKO, Santa Clara, USA) and CAIX (1:1000, Abcam, Cambridge Science Park, Cambridge, UK). For VEGF-A a rabbit polyclonal antibody was used (0.2 αg/mL, RB-9031, ThermoFisher, Fremont, USA).

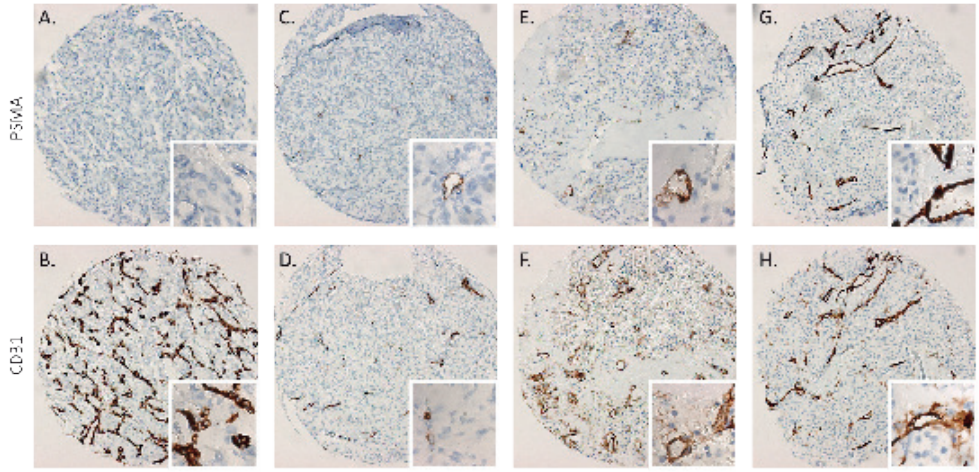

Figure 9.1: *Representative cores of PSMA and CD31 immunohistochemistry in tissue microarrays of medullary thyroid cancer. A and B: tumor core with no PSMA positivity and high CD31 expression; C and D: PSMA – and CD31 positivity in cell-rich tumor; E and F: PSMA – and CD31 positivity in tumor with desmoplasia; G and H: strong PSMA – and strong CD31 positivity.*

Scoring of immunohistochemistry

All TMA slides were scored by a dedicated pathologist (SMW or PJvD) and an experienced researcher (LL). PSMA was scored as the number of PSMA positive vessels in the core, irrespective of staining intensity, and an average number per core was calculated for each patient. MVD was calculated by the average number of CD31 positive vessels per core.

Table 9.1: Clinicopathological characteristics of all patients stratified by average PSMA value.

	PSMA			MVD			PSMA/MVD-ratio		
	N	Value	p-value	N	Value	p-value	N	Value	p-value
Sex			0.38			0.29			0.14
Male	55	5.6		54	15.7		55	0.40	
Female	49	5.9		51	12.9		49	0.50	
Heritability			0.46			0.52			0.17
Sporadic	60	5.7		60	14.8		60	0.42	
MEN2a/b	41	5.9		42	13.5		41	0.50	
Stage			0.07			0.24			0.16
I – III	54	6.3		54	14.6		54	0.49	
IV	50	5.1		51	14.1		50	0.41	
Size			0.57			0.00			0.27
< 20 mm	39	6.1		39	17.6		39	0.39	
≥ 20 mm	60	5.5		61	12.2		60	0.49	
Lymph node metastasis			0.08			0.41			0.11
No	38	7.0		37	14.0		38	0.53	
Yes	66	5.0		68	14.0		66	0.40	
Overall Survival			0.03			0.27			0.04
Alive	82	6.0		83	14.5		82	0.46	
Death	14	3.5		14	11.9		14	0.28	
Progression Free Survival			0.01			0.07			0.08
No progression	73	6.3		74	14.6		73	0.49	
Progression	28	4.1		28	12.2		28	0.35	
Disease status Normal			0.23			0.04			0.58
CEA/calcitonin Elevated	37	6.6		37	15.0		37	0.46	
CEA/calcitonin	62	5.4		63	14.0		62	0.44	
Presence of necrosis			0.56			0.13			0.81
Negative	92	5.8		93	14.8		92	0.45	
Positive	9	4.9		9	10.8		9	0.47	
Presence of angio-invasion			0.13			0.10			0.33
Negative	91	5.9		92	14.8		91	0.46	
Positive	10	3.9		10	10.7		10	0.36	
Presence of desmoplasia			0.46			0.15			0.84
None – some	50	6.4		51	14.7		50	0.46	
Moderate – severe	51	5.1		51	14.2		51	0.44	
CAIX			0.95			0.29			0.73
Negative	54	6.1		54	14.5		54	0.44	
Positive	50	5.3		50	13.8		50	0.45	

	PSMA			MVD			PSMA/MVD-ratio		
	N	Value	p-value	N	Value	p-value	N	Value	p-value
MVD			0.02						0.09
< 14 vessels/core	56	4.7					56	0.50	
≥ 14 vessels/core	47	7.1					47	0.39	
VEGF			0.26			0.42			0.64
Negative	38	5.0		38	13.8		38	0.43	
Positive	64	6.3		64	14.7		63	0.46	
HIF-1α			0.73			0.20			0.26
Negative	45	5.3		45	15.9		45	0.41	
Positive	59	6.1		60	13.2		59	0.47	

Abbreviations: MEN2a/b = Multiple Endocrine Neoplasia type 2a/b; CAIX = carbonic anhydrase IX; Glut-1 = glucose transporter 1; MVD = microvessel density; VEGF = Vascular Endothelial Growth Factor; HIF-1a = Hypoxia Induced Factor-1 alpha.

Representative cores are shown in figure 9.1.

For HIF-1α the percentage of positive nuclei per core was scored as an absolute number. For statistical analysis, this was transformed in a dichotomous variable, which was positive when in either one of the three cores ≥1 percent of nuclei were positive as previously used. Glut-1 and CAIX were scored as absent, cytoplasmic or membranous for each core separately. For statistical analysis, only membranous staining was taken into account, when this was present for either one of the cores the tumor was considered positive. VEGF-A was scored as absent (0), weak (1), moderate (2) or strong (3) for each core separately. For statistical analysis, the average score over three cores was calculated and when this was ≥2 it was considered positive.

Statistical analysis

Categorical data were summarized with frequencies and percentages, and continuous data were summarized with means and standard deviations. Progression was defined as development of distant metastases or death. This excluded lymph node metastases or elevation in CEA/calcitonin without anatomical substrate. This definition was chosen since MTC is an incurable disease when distant metastases occur. Progression-free survival (PFS) time was therefore defined as the time from diagnosis to development of distant metastases, and overall survival as the time to death.

To increase the power of the statistical analysis categorical data were recoded into dichotomous variables. Stage I, II, III and IV was recoded into stage I – III and stage IV; hereditability was categorized in either sporadic or hereditable, grade of desmoplasia was

Table 9.2: Univariate Kaplan-Meier survival analysis on PFS and OS.

	Progression Free Survival				Overall Survival			
	N	PF (N)	PF (%)	p- value	N	OS (N)	OS (%)	p- value
Sex				0.09				0.62
Male	56	36	64.2		53	44	83.0	
Female	52	11	78.8		49	42	85.7	
Heritability				0.00				0.01
Sporadic	62	40	64.5		57	45	78.9	
MEN2a/b	43	36	83.7		42	39	92.9	
Stage				0.00				0.14
I-III	56	53	94.6		53	48	90.6	
IV	52	24	46.2		49	38	77.6	
Tumor size				0.54				0.71
< 20mm	40	33	82.5		39	34	87.2	
≥ 20mm	63	42	68.3		59	50	84.7	
Lymph node metastasis				0.00				0.09
Yes	39	37	94.9		37	34	91.9	
No	69	40	68.4		65	52	80.0	
Disease status				0.00				0.05
Normal CEA/calcitonin	39	37	94.9		39	37	94.9	
Elevated CEA/calcitonin	66	37	56.1		62	48	77.4	
Necrosis				0.01				0.01
Negative	96	25	74.0		92	79	85.9	
Positive	9	6	33.3		7	4	57.1	
Angio-invasion				0.18				0.67
Negative	95	26	72.6		90	75	83.3	
Positive	10	5	50.0		9	8	88.9	
Desmoplasia				0.16				0.30
None – Some	51	10	80.4		49	44	89.8	
Moderate – Severe	54	21	61.1		50	39	78.0	
PSMA				0.04				0.14
< 5 vessels/core	50	30	60.0		49	39	79.6	
≥ 5 vessels/core	51	43	84.3		47	43	91.5	
MVD				0.53				0.74
< 14 vessels/core	58	39	67.2		54	45	83.3	
≥ 14 vessels/core	45	36	80.0		43	38	88.4	
VEGF				0.37				0.01
Negative	37	26	70.3		34	32	94.1	
Positive	64	48	75.0		61	49	80.3	

	Progression Free Survival				Overall Survival			
	N	PF (N)	PF (%)	p- value	N	OS (N)	OS (%)	p- value
CAIX				0.69				0.49
Negative	59	45	76.3		58	51	87.9	
Positive	48	32	66.7		43	34	79.1	
Glut-1				0.02				0.51
Negative	102	74	72.5		97	81	83.5	
Positive	6	3	50.0		5	5	100.0	

Abbreviations: N = number; PF = progression free; OS = overall survival; MEN2a/b = Multiple Endocrine Neoplasia type 2a/b; CAIX = carbonic anhydrase IX; Glut-1 = glucose transporter 1.

recoded in none – some and moderate – severe, PSMA was recoded in < 5 microvessels/core and ≥ 5 microvessels/core, MVD was recoded in < 14 microvessels/core and ≥ 14 microvessels/core. PSMA/MVD-ratio was calculated by dividing the average number of PSMA positive vessels by the average MVD.

The chi-square test was used to assess associations between the dichotomous variables, the Student's t-test was used to test for differences between continuous variables. Wilcoxon Signed Ranks test was used to perform paired analysis, between primary tumors and subsequent lymph node metastases. Variables with a p-value <0.05 in the univariate regression analysis were eligible for selection in the multivariate analysis. Kaplan-Meier survival curves were plotted, and univariate survival analysis was performed and the log-rank test was used to calculate significance. All reported p-values were two sided. Analysis was performed using SPSS version 22.0 software (SPSS, Inc., Chicago, IL, USA).

Results

Baseline Characteristics

The total group consisted of 111 patients, the mean age was 46.3 years (SD 15.6), 59 (53.2%) were male. Sixty-five patients (60.2%) had sporadic MTC, 39 patients (36.1%) were diagnosed with MEN2a and 4 patients (3.7%) were diagnosed with MEN2b, and from 3 patients the RET-mutation status was unknown. Fifteen patients presented with TNM stage 1 (13.5%), 26 with TNM stage II (23.4%), 17 (15.3%) with TNM stage III and 53 (47.7%) with TNM stage IV disease. Median tumor size was 25.6 mm (IQR 25; minimum 2 mm; maximum 80 mm) and 79 (63.1%) patients presented with lymph node metastases. Mean follow-up was 79.2 months (SD 60.6).

Assessment of PSMA expression in primary tumors and lymph node metastases

Ninety-six out of 104 patients (92%) of MTC expressed PSMA in the neovasculature,

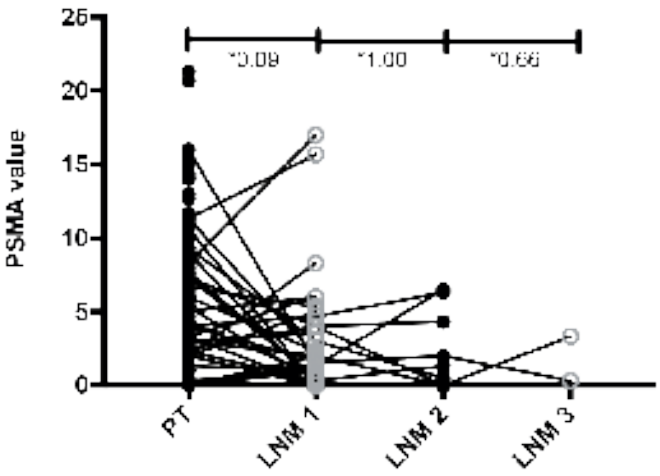

Figure 9.2: *Average PSMA values in primary tumors and their subsequent lymph node metastases. The vertical axis shows the average number of PSMA positive microvessels/ core. PT; primary tumor. LNM; lymph node metastasis.*
** = p-value between average PSMA values calculated by Wilcoxon Signed Ranks Test.*

but never in the tumor cells. The average PSMA score over all primary tumors (n = 104) was 5.74; SD 4.68. As to stability of PSMA expression during progression (Figure 9.2), the average number of PSMA positive microvessels was 4.56; SD 4.03 in the primary tumor (n = 35) compared to 2.92; SD 3.97 in the initial lymph node metastases included from the same patient (p = 0.09). Subsequently, the average PSMA positive microvessels in the recurrent lymph node metastases were 2.08; SD 1.75 and 2.10; SD 2.65 for the later recurrence of lymph node metastases (n = 10; p = 1.00) (Table 9.1).

Table 9.3: *Multivariate logistic regression analysis on PFS.*

	Events/patients	Odds Ratio (95% CI)	p-value
PSMA			0.05
< 5 vessels/core	20/50	3.6 (1.0 – 12.8)	
≥ 5 vessels/core	8/51	1	
TNM-stage			0.00
I-III	3/56	1	
IV	28/52	23.6 (4.8 – 115.5)	
Heritability			0.11
Sporadic	22/62	2.9 (0.8 – 11.0)	
MEN2a/b	7/43	1	
HIF-1α			0.02
Negative	7/47	1	
Positive	24/61	4.9 (1.3 – 18.1)	

Abbreviations: PSMA = Prosate-Specific Membrane Antigen; HIF-1α = Hypoxia Inducible Factor 1α; MEN2a/b = Multiple Endocrine Neoplasia type 2a/b.

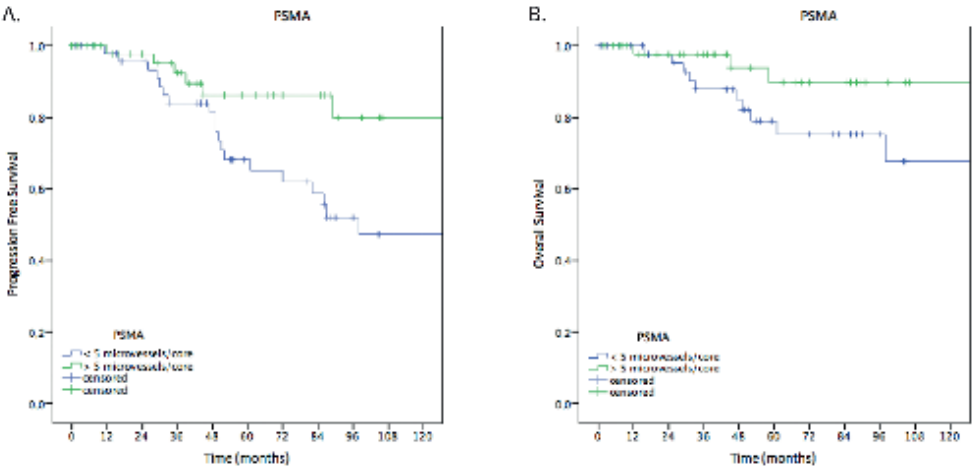

Figure 9.3: *10-years PFS and OS in Kaplan-Meier survival curve in patients with < 5 PSMA positive microvessels/core to ³ 5 PSMA positive microvessels/core. A. PFS in total group of 111 patients comparing < 5 PSMA positive microvessels/core to ³ 5 PSMA positive microvessels/core; log-rank 0.04. B. OS in total group comparing < 5 PSMA positive microvessels/core to ³ 5 PSMA positive microvessels/core; log-rank 0.14.*

Association and prognostic value of PSMA with clinicopathologic variables

As shown in table 9.2, a low number of PSMA positive microvessels was significantly associated with survival status. For patients surviving, average PSMA scores were 6.0 versus 3.5 in patients who died (p = 0.03). Average PSMA scores were 6.3 in patients who were progression-free versus 4.1 in patients who did show tumor progression, respectively (p = 0.01). Average scores for patients without lymph node metastasis at initial surgery were 7.0 versus 5.0 for patients with lymph node metastasis at initial presentation (p = 0.08). No significant association was found with one of the aggressive histologic features investigated as; necrosis, angio-invasion and desmoplasia, nor was any significant association found with the immunohistochemical variables; HIF-1α, CAIX, Glut-1, VEGF. There was, however, a significant association with MVD, which showed an average PSMA score of 4.7 vs 7.1 in the group with < 14 vessels/core and ≥ 14 vessels/core, respectively.

The association of the clinicopathological variables with MVD and PSMA/MVD-ratio was also investigated. In short, for MVD a correlation was found with average tumor size showing a higher MVD (17.6) in tumors < 20 mm compared to 12.2 for tumors ≥ 20 mm (p = 0.00). The PSMA/MVD-ratio was significantly higher for patients that survived versus patients who deceased during follow-up; 0.46 versus 0.28 respectively (p = 0.04). All other clinicopathologic variables did not show any relevant significant differences (Table 9.1).

To verify which variables were associated with PFS and OS, univariate survival analysis was

performed (Table 9.3). For PFS, significant associations were found for heritability, TNM-stage, presence of lymph node metastasis, postoperative CEA/calcitonin levels, presence of necrosis, PSMA, HIF-1α and Glut-1. For OS, significant associations were found for heritability, postoperative CEA/calcitonin levels, necrosis, VEGF, and HIF-1α. Figure 9.3 shows the PFS and OS survival curves for MTC patients stratified according to the number of PSMA positive microvessels.

The number of variables that was tested in multivariate analysis was limited to four because of the low number of patients that showed progression (n = 33): mean PSMA-levels, TNM-stage, heritability and HIF-1α. The variable lymph node metastasis was left out of the multivariate regression analysis since this is also taken into account by the TNM-stage; disease status was left out since this is predominantly base on postoperative CEA/calcitonin measurements which are of value to evaluate over time to show tumor progression, but not as a single postoperative measurement, Glut-1 was left out as a down-stream effector of HIF-1α; necrosis was left out since only 9 patients showed necrosis, and with a prevalence of only 8.1% it is not suitable as a prognostic factor. A low mean of PSMA positive microvessels, TNM-stage IV and HIF-1α positivity were significantly associated with PFS with ORs of 3.6 (95% CI 1.0 – 12.8; p = 0.05), 23.6 (95% CI 4.8 – 115.5; p = 0.00) and 4.9 (95% CI 1.3 – 18.1; p = 0.02), respectively (Table 9.3).

Discussion

This is the first study to examine expression of PSMA in neovasculature of MTC. Our results show that over 92% of MTC have PSMA positive microvessels. Expression of PSMA in neovasculature was relatively stable over time from primary tumors to lymph node metastases. Furthermore, the average number of PSMA positive microvessels was, in univariate analysis associated with OS, PFS and MVD. In multivariate analysis, a higher number of PSMA positive microvessels was significantly associated with a favorable prognosis. Since >90% of tumors contain PSMA positive microvessels, PSMA might be an interesting target for imaging studies and targeted therapies in MTC.

The transmembrane protein PSMA gained attention for its abundant expression in prostate adenocarcinoma and its strong prognostic value with regard to impaired survival.[14-16] Immunohistochemical expression of PSMA in prostate adenocarcinoma is in the epithelium and predominantly cytoplasmic and membranous. In most other solid tumors expression is not in the epithelium, but in the endothelium of the neovasculature.[17] The role of PSMA expression in endothelial neovasculature is not well understood. PSMA has folate hydrolase activity and may increase folic acid levels at the site of neovaculature formation. Folic acid is necessary to assure nitric oxide formation by nitric oxide synthase (eNOS). Nitric oxide is responsible for endothelial progenitor mobilization and vasculogenesis. In the absence of folic acid, eNOS generates

superoxide radicals, which impair endothelial proliferation.[17] The role of superoxide radicals, which is family of the reactive oxygen species, in tumorigenesis is complicated, increased ROS levels are able to promote tumor growth and malignant progression.[18]

Data on the prognostic value of PSMA expression in neoasculature of non-prostate solid tumors is scarce. We found a favorable prognosis for patients with a high number of PSMA positive vessels. Haffner et al. evaluated PSMA expression in gastric – and colorectal cancer and found endothelial expression in 66% of gastric and 85% of cases of colorectal cancer. No correlations with disease free – or overall survival were found. Similar to our data they found stable expression between primary tumors and their corresponding metastases.[8] In oral squamous cell carcinoma, endothelial PSMA was strongly correlated with impaired survival.[19] In accordance with our data, Wang et al. reported a higher number of PSMA positive microvessels in patients with TNM stage I – II lung cancer compared to patients with TNM stage III – IV.[20] Currently, there is no consensus how to interpret immunohistochemical data, some studies score by intensity of expression, some by a percentage of positive cells and some by absolute number of PSMA positive microvessels. We scored any endothelial expression as positive, irrespective of staining intensity. These varying methods might influence the prognostic results of the different studies. In summary, while data on this topic is scarce, the prognostic role of endothelial PSMA expression seems to be tumor-specific.

As to the predictive value of PSMA expression for PSMA imaging, Milowsky et al. verified in 10 patients whether there was a correlation between expression on immunohistochemistry and PSMA imaging signals of patients with varying cancer types. All four patients with strong expression on immunohistochemistry, of which 2 patients with renal cancer, 1 with colon cancer and 1 with bladder cancer, had a positive (111)In-J591 PSMA scan. Of the six patients, 3 patients with renal cancer, 2 with bladder cancer, 1 with pancreas cancer, that had no or showed weak expression of PSMA still half had a positive PSMA scan.[21] In adrenocortical carcinoma, high expression of PSMA was found in 3 out of 3 tested metastatic sites. One of these patients was imaged with Zr89-J591, and the metastases were found to demonstrate strong uptake in vivo.[22] This shows that many patients have positive scans, irrespective of PSMA expression on the tissue level. Too few PSMA negative patients have yet been scanned to allow conclusions on the predictive value of tissue PSMA expression for the PSMA scan result.

Anyway, PSMA is an interesting target for nuclear imaging, since it has a large extracellular domain5 and is thereby well accessible for tracers. In prostate cancer, results have been promising so far with a large decrease in PSA values in the majority of patients treated with anti-PSMA.[12, 23-25] PSMA-targeted imaging and therapy is continuously changing and improving, antibodies to the extracellular domain of PSMA have been developed and more recently the PSMA peptide inhibitor PSMA-617 was developed and tested. (117)Lu-PSMA-617 showed favorable pharmacokinetics and favorable safety and efficacy in a multicenter study.[26] Currently, data on PSMA-617 is solely available for prostate cancer and a biochemical response

was seen in 45 out of 99 (45%) of patients. However, effect on overall survival rates still need to be studied in phase II/III trials. Data on the efficacy of (177)Lu-PSMA-617 in tumors with expression of PSMA in neovasculature, like MTC, is not yet available. In contrast, some experience has been gained with (111)In-J591, a monoclonal antibody targeting the extracellular domain of PSMA, in advanced solid tumors.[21] Uptake has been identified in a fairly high number of metastatic sites in different types of solid tumors, but long-term survival data is not yet available.[21, 27, 28] Common adverse events were disease-related fatigue, pain, anemia and mild liver function test abnormalities.[21, 24, 28] To overcome these adverse events an α-radionuclide–labeled PSMA ligand, (225)Ac-PSMA-617 was evaluated and showed promising results, both patients had no measurable PSA levels after three cycles without any hematologic toxicity identified.[13] Even though we show a favorable prognosis in patients with a high number of PSMA positive microvessels, our results may form the basis for the evaluation of PSMA imaging and peptide radioligand therapy for patients with metastasized MTC. If the metastasis show uptake on nuclear imaging, it is still an interesting target for therapeutic interventions.

One of the strengths of this study is the combination of immunohistochemical data with clinical endpoints such as survival or the occurrence of distant metastasis in a relatively large sample size. Furthermore, since MTC is in most cases a relatively low-proliferating tumor, event-rates are low and a long follow-up is needed to detect them. Our follow-up is long (mean 70.2 months; SD 60.6) and we used PFS to increase the total number of events. One of the limitations is that immunohistochemistry is inherently a more qualitative than quantitative method. Further limitations are merely due to its retrospective character and the low incidence of MTC. A total of 5 tertiary referral centers have participated and patients over almost 3 decades have been included. To overcome this, we limited our analyses to variables least subject to treatment changes overtime or interinstitutional differences. Further, it would have been interesting to study distant metastases, but such tissue was unfortunately not available.

In conclusion, this is the first time that PSMA expression in microvessels of MTC has been investigated. Over 90% of patients showed expression of PSMA, and patients with a higher number of PSMA positive microvessels showed favorable prognosis. Since a relative stable expression was seen from primary tumors to lymph node metastases, PSMA is altogether an interesting target for imaging and peptide radioligand therapy.

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Chapter 10

General discussion

The major subjects discussed in this thesis relate to the management of diagnosing thyroid nodules, genetic and molecular alterations in thyroid nodules and the ability to minimize the number of diagnostic lobectomies by testing for these alterations, histologic aspects of contralateral carcinomas in patients with DTC, and prognostic factors in patients with MTC. This chapter provides a general discussion and summary of all presented studies.

Thyroid Nodules

Currently, the work-up for a patient with a thyroid nodule may evolve over a protracted period of time, often months, causing uncertainty and anxiety in the patient's life. In **chapter 2** we describe how, in our tertiary referral center this issue was addressed via a same-day diagnosis outpatient clinic for thyroid nodules. Where the patient was seen by an endocrinologist, a radiologist and, if necessary, a surgeon, in order to get a diagnosis and treatment plan in 1 day. In this setting, we investigated feasibility and patient anxiety throughout the diagnostic process. The 'standard' diagnostic algorithm differs between regions of the world as well as at the local level between hospitals, or is sometimes based on geographical differences, therefore it might be hard to compare time to diagnosis between different countries.[1] Patel et al. describe a one-stop thyroid clinic in Australia where ultrasound guided FNAC is performed by the treating endocrine surgeon, and find this to be a safe and fast alternative to diagnose thyroid nodules.[2] This would clearly benefit the logistics for patients with a thyroid nodule compared to our current system. Nonetheless, we would advocate that, within the system of the country or hospital, the treating physicians should aim for a diagnosis as soon as possible. In **chapter 2** we, furthermore, showed that patients with a thyroid nodule experience just as much anxiety related to the potential diagnosis of cancer as patients with a lump in the breast.[3] Therefore, the aim of each rapid assessment and care clinic should be to minimize time to diagnosis, and if applicable time to surgery. Indications for acceptable time from presentation to diagnosis and subsequently to surgery, should be part of future national guidelines. As of 2004, the National Institute for Clinical Excellence of the United Kingdom has proposed head and neck 'one-stop clinics', and same day diagnostics for thyroid nodules is already mentioned in other national guidelines.[4-6] In the Netherlands, such recommendations are lacking in our yearly published standardisation report (SONCOS), which defines the conditions that good cancer care should fulfil. Regretfully, this report does not suggest about time between presentation and diagnosis, neither about time between diagnosis and surgery. As shown in **chapter 2** such recommendations would improve thyroid cancer care and therefore we would encourage to encompass this in future reports. By doing so quality of thyroid cancer care can be more improved and unified for all treating centers in the Netherlands.

Multiple Endocrine Neoplasia type 1 (MEN1) is caused by an inactivating germline mutation in the MEN1 gene, which encodes for the tumor suppressor protein menin.[7]

It is characterized by the occurrence of pituitary tumors, primary hyperparathyroidism, pancreatic and duodenal neuroendocrine tumors, adrenal adenomas and neuroendocrine tumors of stomach, lung and thymus.[8] Screening for disease in MEN1 families starts at the age of 7, and encompasses of annual biochemical tests and abdominal imaging which should be repeated at 1 to 3 year intervals. It has been shown that throughout this period of time, patients with this disease entity experience substantial uncertainty and anxiety.[9] In this regard, it is important to give MEN1 patients certainty while keeping the number of doctor visits and diagnostic procedures as low as possible. In the diagnostic process of hyperparathyroidism an ultrasound of the neck is essential; when present, thyroid nodules are unavoidably detected. In **chapter 3**, we describe that the prevalence of thyroid incidentalomas on a neck ultrasound in patients with MEN1 is equal to a matched reference group with non-MEN1 patients. These epidemiologic results are supported by the presence of a positive nuclear menin stain in a representative subset of thyroid tumors from MEN1 patients, supporting the hypothesis that the tumorigenesis of these thyroid incidentalomas is not MEN1 related, because loss of menin is a key step in the tumorigenesis of MEN1 related tumors. In general, worry about the development of cancer is a significant problem in MEN1 patients. In 116 female MEN1 patients worry about cancer development was tested using the validated Cancer Worry Scale (CWS) the mean score was 14.7.[10] A CWS > 14 represents severe levels of fear of cancer occurrence, underlining the importance to be informed about thyroid cancer risk for this particular patient population. With the findings presented in **chapter 3** we hope to inform physicians that thyroid incidentalomas found in MEN1 patients can be treated according to guidelines for thyroid incidentalomas in the general population.[11] This conservative management may contribute to reduce unnecessary uncertainty and anxiety as well as overtreatment of benign thyroid lesions in this vulnerable population.

Cytopathology is currently the most important factor in the evaluation of thyroid nodules. Unfortunately, there is still a substantial number of patients who have inconclusive cytopathology and this can either be a nondiagnostic or unsatisfactory (Bethesda I) result or an indeterminate FNAC (Bethesda III, IV or V) result.[12] For a nodule with a nondiagnostic cytology result, FNA should be repeated and, if available, on-site cytologic evaluation should be performed to ensure adequate cellular yield. De Koster et al, at our institution, showed that on-site cytologic evaluation reduced the number of nondiagnostic FNA results.[13] Patients with an indeterminate cytology result should undergo an initial lobectomy, as surgical histology is the gold standard for diagnosis of these tumors.[12] When the nodule turns out to be malignant and larger than 1 cm or smaller than 1 cm with aggressive pathologic features a completion lobectomy is performed. In the current guidelines lobectomy is offered as a safe alternative for low-risk patients with tumors 1–4 cm.[12] However, for the majority of patients this initial thyroid lobectomy may turn out to be futile when histology shows a benign thyroid nodule,

thus patients might be exposed to potentially harmful complications. Even though complication rates are low; transient or permanent hypocalcemia, transient or permanent recurrent laryngeal nerve injury may occur postoperatively. Further, some patients need (temporary) levothyroxine replacement after a diagnostic lobectomy.[14] Most importantly, in clinical practice patients are often relieved when the nodule turns out benign and as shown in **chapter 2** uncertainty about a thyroid nodule causes high anxiety rates. Furthermore, patients with a benign thyroid nodule might also consider a lobectomy due to esthetics or functional complaints, these factors should be considered when discussing the diagnostic lobectomy with the patient at the outpatient clinic.

In the past years, there has been substantial effort to lower the number of indeterminate results, mainly by focusing on the tumor cell expression of molecular markers. Currently, commercial classifiers have been developed including Afirma, ThyGenX, ThyraMIR and ThyroSeq v2.[15-17] None of these classifiers are used routinely in Dutch clinical practice although the most recent national guidelines state that molecular tests can be beneficial, especially for patients with FNA results showing Bethesda 3.[18] Further, the guideline of the American Thyroid Association states that the risk of malignancy in each of the six diagnostic categories should be independently defined at each institution to guide clinicians on risk estimates and help choose appropriate molecular testing for patients with indeterminate cytology.[12] In this regard we commented on the well-designed study of Kleiman et al. where the hypothesis was addressed whether preoperative BRAF(V600E) testing would alter the initial surgical management of patients with indeterminate thyroid nodules.[19] They concluded that in the authors' institution using BRAF testing for indeterminate cytopathology results was not helpful. It is important to note that in this institution, patients with Bethesda 5 results undergo total thyroidectomy whereas in our institution the majority of patients initially receive a diagnostic lobectomy. In **chapter 6** we show that by re-analyzing the data presented by Kleiman et al. and using the approach of our institution it actually would alter initial surgical management even in a cost-effective manner. Underlining the statement in the ATA guidelines that inter-institutional differences in the risk of malignancy in each of the six diagnostic categories can lead to different outcomes.

Other than genetic alterations, microRNA expression levels are frequently investigated to distinguish benign thyroid nodules from malignant thyroid nodules.[20] In **chapter 5** we summarized the current literature on the value of altered microRNA expression levels within thyroid nodules and we identified overlapping results between the different studies. Best results, in terms of sensitivity, specificity, negative – and positive predictive value, to distinguish benign from malignant nodules are found when a combination of microRNAs is used, in 3 out of 6 studies these involved miR146b, miR-221 and miR-222.[21-26] We believe that

when such a combination of microRNAs is used; the aim should be to identify benign nodules and thereby to reduce the number of unnecessary diagnostic lobectomies. Even though Afirma does not include microRNAs but uses a set of genetic alterations instead, they do focus on identifying the benign nodules and results in literature show their test is able to reduce the number of diagnostic lobectomies by 50%.[27, 28] This test is most helpful as a rule-out test and should - in our opinion- be used as such in the clinical context for optimal cost-effectiveness. Due to the logistic hick-ups and lack of cost-effectiveness studies in the Netherlands, use of this test is not yet implemented in our practice. Ruling out is especially beneficial when the expected number of malignant cases is low, underlining the statement in our national guidelines that molecular tests are especially beneficial in Bethesda category 3.

Differentiated Thyroid Cancer

Over the past decade, research aims in the treatment of DTC are slowly changing. Twenty-year survival rates now approach 98%, moving research goals towards a focus on quality of life of patients surviving DTC instead of improving survival rates.[29] These excellent survival perspectives for patients with DTC are traditionally achieved by a total thyroidectomy and high dose of RAI ablation therapy, however, this resulted in a relatively low survivorship quality of life in comparison to other cancers such as melanoma or colorectal cancer.[30] The current trend in the treatment of DTC is to de-intensify the initial treatment strategy, by either; a lower dose of radioiodine ablation therapy, changing surgical treatment strategy from a total thyroidectomy to a lobectomy, or by shortening of the time between surgery and radioiodine ablation.[12, 31-34] All these initiatives aim to increase quality of life postoperatively while maintaining high survival rates. In the initial treatment strategy, the treatment plan is similar for all patients with DTC tumors > 1 cm, while within the new developments a more patient tailored approach is pursued.[35]

In light of these recent developments we wanted to gain more insight in the characteristics of contralateral carcinomas, when the initial carcinoma was larger than 1cm. From literature, it is known that these are present in about 30% of patients. Bilateral disease is generally an indication to perform a total thyroidectomy with adjuvant radioiodine ablation therapy.[12] Currently there is no consensus whether multifocal carcinomas arise as a result of true multicentricity or intrathyroidal spread of a primary tumor, nor is there consensus whether papillary microcarcinomas (tumors < 1 cm) require any therapy.[36-38] Ito et al. observed 1235 microcarcinomas and found that only 3.5% progressed to clinical disease, young age was an independent predictor for this disease progression.[37] This might indicate that papillary microcarcinomas have no clinical relevance.

In **chapter** 7 histologic characteristics such as; size and tumor subtype were analyzed from a large multicenter cohort of 1313 patients. We found that the majority of the identified contralateral carcinomas are papillary microcarcinomas and that no correlation was found between histologic subtype of the primary tumor and the contralateral carcinomas. It can be concluded that, unless a thyroid cancer is found in the contralateral lobe which would independently mandate surgical management, multifocality should not be an argument to perform bilateral surgery. The clinical relevance of these incidentally found contralateral microcarcinomas is negligible and observation can be a safe alternative.[37, 38] The major advantage of a lobectomy in these cases would be that most patients will not need levothyroxine substitution therapy which will most likely increase quality of life.[14, 39-42]

Longitudinal research on DTC is impeded by its relatively low incidence in combination with high survival rates. To evaluate whether new treatment strategies are safe and effective, studies with high statistical power should be designed. In medicine the Randomized Controlled Trial (RCT) is still considered to be the most reliable form of scientific evidence in the hierarchy of evidence.[43, 44] However, the validity of a RCT is strongly based on sample size, i.e. studies with high survival rates are easily underpowered.[45] To overcome such shortcomings large-scale collaborative consortia should be formed. With the introduction of centralization, statistical power in studies on outcomes of thyroid cancer in the Netherlands has already been mitigated.[18] Such centralization will strengthen future research, because this will secure a more unified diagnostic process and treatment for patients with DTC. Other models of clinical care should be applied to thyroid cancer e.g. in the form of national collaborative consortia, wherein endocrinologists and endocrine surgeons of a country work closely together. The Prospective Dutch ColoRectal Cancer cohort is an alternative to the more traditional RCT, which is based on a strong collaborative effort. This 'cohort multiple randomized controlled trial' (cmRCT) is characterized by: (1) patient-centered informed consent; (2) framework to systematically collect long-term clinical follow-up as well as patient-reported outcomes; and (3) efficient recruitment for trials by asking patients to give 'broad consent for randomization' in future trials.
Cohort multiple randomized controlled trials also allow multiple randomized trials to be conducted simultaneously and patients to participate in multiple non-conflicting trials at the same time.[46] When such an approach would be introduced on the base of strong national collaborative consortia it would secure efficient recruitment for trials and thereby benefit to improve studies with high statistical power in patients with DTC.

Medullary Thyroid Cancer

Medullary Thyroid Cancer (MTC) is a rare form of thyroid cancer and its etiology, diagnosis, prognosis and treatment are completely different from DTC, it develops from the c cells that are found in the center of the thyroid lobe.[47] The fact that c cells are the only cells in the human

body to produce calcitonin, makes this a very specific marker to diagnose the first presentation of MTC but also to identify disease recurrences and to monitor growth speed of these recurrences.[48] However, there are still some pitfalls in the imaging, prognosis and treatment of MTC, of which some are studied in this thesis.

Currently, prognosis is mainly based on TNM-stage and on postoperative calcitonin levels.[49-51] It is well known that in various other solid tumors Hypoxia Inducible Factor-1 alpha (HIF-1α) is strongly associated with worse prognosis.[52] For MTC such a correlation was already reported, however, this was only based on clincopathological parameters, while lacking survival data.[53] We established a large cohort of patients with MTC with long follow-up data, and, we show in **chapter 8** for the first time the correlation of HIF-1α and impaired survival in patients with MTC. In hypoxic conditions HIF-1α is strongly upregulated, but this upregulation might also be due to altered oncogenic signaling pathways. Increased expression of HIF-1α actively drives tumor growth and progression, mainly due to its downstream effectors as; vascular endothelial growth factor (VEGF), carbonic anhydrase IX (CAIX) and glucose transporter 1 (Glut-1).[52, 54] In our study we used progression free survival (PFS) as a composite endpoint where death or the development of distant metastases were combined. We found a strong correlation between HIF-1α expression and PFS, which resulted in a significant hazard ratio of 3.1. Furthermore, within the group of patients with TNM stage IV we could distinguish between a worse (5-years PFS 35%) and a relatively good (5-years PFS 90%) prognosis. Upregulation of HIF-1α can be by oxygen dependent factors, but also by oncogenic signaling pathways, in the latter an interplay between HIF-1α and REarranged during Transfection (RET) was proposed.[55] In our database, all patients with inherited MTC are identified, however, it is unknown which of the sporadic tumors harbor RET mutations resulting in constitutively activated RET signaling pathway. Therefore, we could not study this relationship. The fact that the HIF-1α pathway seems to be involved in tumor development or progression raise interest to use HIF-1α as a targetable agent. Currently two types of HIF-1α inhibitors are available. Indirect HIF regulators reduce the level of HIF-1α protein through regulation of HIF-1α translation or stabilization. Direct HIF regulators inhibit the interaction between HIF-1α and p300 without affecting levels of HIF-1α protein. Some HIF inhibitors are under pre- and early clinical development as anticancer agents, these inhibitors lack specificity and, therefore, exhibit significant side effects.[56, 57]

Currently, the clinical management of patients with persistent or recurrent MTC is still under debate, because these patients either have a long-term survival, due to an indolent course of the disease, or develop rapidly progressing disease leading to death from distant metastases.[58] Expression of HIF-1α distinguished within patients with TNM stage 4 between a relatively good and worse prognosis. Therefore, HIF-1α can be used in future as a marker to guide follow-up and therapy. Nevertheless, more research should be performed to find such a relationship. A

prospectively validation of our findings is necessary to verify whether it will influence treatment strategies. But also, to validate the reproducibility of measuring increased HIF-1α expression and to set a reliable cut-off point, as we now performed the staining on tissue microarrays. The management guideline for thyroid nodules states that, with regard to imaging, no single procedure provides optimal whole body imaging.[47] Positron Emission Tomography – Computed Tomography (PET/CT) is able to generate functional whole body imaging data. Its role in MTC is currently limited, however, the field of functional imaging is fast evolving and different radiotracers are of interest. FDG, F-DOPA and somatostatin analogues are radiotracers tested for utility in MTC. FDG and F-DOPA have an average sensitivity of 68% and 67% respectively.[59-61] For somatostatin analogues, data on sensitivity is not yet available but it seems to be lower than FDG, however, the major advantage is that it can be used as a therapeutic agent.[62, 63] Prostate Specific Membrane Antigen (PSMA) is a radiotracer that initially gained interest in the diagnosis and treatment of prostate cancer where it is expressed in the epithelial cells of prostate adenocarcinoma.[64] More recently, the expression of PSMA in neovasculature of other malignancies was identified.[65-68] The advantage of PSMA is that is easy targetable since it has a large extracellular domain, furthermore it is multifunctional, as it can be used to diagnose tumor sites and as a therapeutical modality with radioligand therapy to decrease tumor burden.[69-71] To investigate the utility of PSMA in MTC, we studied in **chapter 9**, for the first time, the expression of PSMA in MTC. We found that 93% of MTC expressed PSMA in the microvessels and we found that expression was relatively stable between the primary tumors and subsequent lymph node metastases. Confirmation of these findings would render PSMA an interesting target for further investigation in imaging and therapeutic studies. The high percentage of tumors expressing PSMA is encouraging, however, metastatic MTC frequently involves multiple sites and at each site, metastases are often multiple and small, which can compromise the utility of PET/CT in general.[47, 72] The difficulty in analyzing literature regarding imaging and targeting the extracellular domain of PSMA is that there is a wide variety of antibodies and peptides available that target PSMA and these are combined with various nuclear imaging and therapeutic modalities. The recently developed PSMA peptide inhibitor PSMA-617 seems to have favorable pharmacokinetics and favorable safety and efficacy in a multicenter study on patients with prostate cancer.[73] However, effect on overall survival rates still need to be studied in phase II/III trials, efficacy was measured by PSA levels. When PSA levels declined more than 50% it was considered as a biochemical response. A biochemical response was seen in 45 out of 99 (45%) patients. In concordance with prostate cancer, where PSA is very specific marker to measure disease recurrences, calcitonin can easily be used to identify biochemical response in MTC.

Summary

- Physicians should aim for a short time to diagnosis for patients with a thyroid nodule since these patients fear for cancer.
- Thyroid incidentalomas in patients with MEN1 show different tumorigenesis than MEN1 related tumors and therefore should be treated as thyroid incidentalomas in the non-MEN1 population.
- When FNAC shows a Bethesda V result, BRAF(V600E) testing is a useful and cost-effective method to reduce the number of diagnostic lobectomies.
- Thirty-two percent of patients with DTC have a contralateral carcinoma, and this contralateral carcinoma is PTC smaller than 1 cm in 82% percent of patients.
- MTC tumors expressing HIF-1α have a HR of 3.1 for developing distant metastases or death compared to HIF-1α negative tumors.
- PSMA is expressed in 92% of neovasculature of MTC tumors, therefore it is an interesting target to further evaluate as an imaging and therapeutic target.

Future perspectives

For the future, three keyword should be central in the discussion about the management of thyroid nodules, DTC and MTC and those are centralization, personalization and collaboration. To achieve the aimed level of personalized cancer care, centralization of care and (inter)national collaborations are essential.

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Chapter 11

Summary in Dutch – nederlandse samenvatting

Dit proefschrift beschrijft verschillende facetten van de diagnostiek, genetica en prognose van schildkliertumoren. De schildklier is een vlindervormig orgaan dat net voor de luchtpijp en onder de adamsappel ligt. Het is belangrijk voor de regulatie van de schildklierhormonen T3 en T4, deze hormonen zorgen voor de regulatie van het energiemetabolisme in je lichaam. In de schildklier kunnen goed- en kwaadaardige tumoren ontstaan, welke soms als een knobbel te voelen zijn. Deze palpabele knobbels (nodi) in de schildklier komen bij 3 – 8% van de volwassenen voor. Een klein deel van deze nodi is kwaadaardig. De diagnostiek van patiënten met een schildkliernodus bestaat uit de anamnese (gesprek met de dokter), lichamelijk onderzoek, laboratoriumonderzoek (schildklierhormoon (T4) en schildklierstimulerend hormoon (TSH)) en een echo gecombineerd met een punctie. Bij deze punctie, ook wel dunne naald aspiraat, wordt in de tumor geprikt om daar cellen vandaan te zuigen die door de patholoog onder de microscoop beoordeeld worden. Ondanks dat de punctie de gouden standaard is, is de uitkomst niet zwart of wit. Er zijn grofweg vier uitkomsten, goedaardig, kwaadaardig, niet te beoordelen (celarm) en inconclusief. Bij de inconclusieve groep kan de patholoog niet met zekerheid zeggen of het goed- of kwaadaardig is, terwijl er wel voldoende cellen zijn.

Tot op heden neemt deze diagnostiek een aantal weken in beslag, waardoor de patiënt langdurig in onzekerheid verkeert. Voor de evaluatie van veel andere tumoren, zoals bij verdenking op borst-, long of darmkanker, wordt de diagnostiek in de meeste ziekenhuizen al in 1 dag uitgevoerd. In **hoofdstuk 2** evalueren we de mogelijkheid om het diagnostisch traject in 1 dag te laten plaatsvinden zodat patiënten direct een behandelplan krijgen of gerustgesteld kunnen worden. We laten zien dat dit op een veilige en accurate manier uitgevoerd kan worden. Tevens tonen we in **hoofdstuk 2** aan dat patiënten, over het algemeen, voorafgaand aan dit onderzoek een hoge mate van angst, c.q. stress, hebben en dat deze angst snel afneemt als de uitslag goedaardig is. Opvallend is dat de, door middel van vragenlijsten, gemeten angst bij patiënten met een schildkliernodus vergelijkbaar is als bij patiënten met een knobbel in de borst. Wij concluderen dat sneldiagnostiek voor de evaluatie van een schildkliernodus veilig is en adviseren implementatie in de kliniek.

In **hoofdstuk 3** richten we ons op patiënten die een syndroom genaamd Multipele Endocriene Neoplasie Type 1 (MEN type 1) hebben. Dit is, met ongeveer 400 patiënten in Nederland, een erg zeldzame aandoening. Patiënten met dit syndroom ontwikkelen gedurende hun leven verschillende endocriene tumoren doordat ze een aangeboren genetische afwijking hebben. Deze tumoren bevinden zich met name in “hormoonproducerende organen” zoals de bijschildklieren, alvleesklier, hypofyse en/of de twaalfvingerige darm. In het UMC Utrecht, een expertisecentrum, worden relatief veel patiënten met dit syndroom behandeld en wordt er veel onderzoek naar gedaan. Het viel de behandelende artsen op dat er vaak onrust ontstaat bij deze patiënten als er een afwijking in de schildklier gezien wordt op de echo. Het

bijschildklieradenoom (goedaardige zwelling van de bijschildklier) is vaak een van de eerste verschijnselen. Voor de diagnostiek van een bijschildklieradenoom wordt er altijd een echo van de hals gemaakt, waarbij de schildklier ook in beeld wordt gebracht. Aangezien de incidentie van niet-palpabele schildkliernodi (schildklier incidentalomen) in de algemene bevolking hoog is, worden deze ook vaak gevonden op de echo van de hals bij patiënten met het MEN type 1 syndroom. Het was voor de behandelend artsen onduidelijk of de afwijkingen in de schildklier bij patiënten met MEN type 1 een uiting is van hun syndroom of dat dit een toevalsbevinding is. Om deze vraag op te lossen onderzoeken we dit in **hoofdstuk 3**. We tonen aan dat deze schildklier incidentalomen bij deze patiëntenpopulatie net zo vaak voorkomen als bij de populatie 'gezonde' mensen. Dit ondersteunen we door met behulp van immunohistochemie te onderzoeken of het eiwit menine tot expressie wordt gebracht. Menine is verantwoordelijk voor de tumorgenese van MEN type 1 tumoren. De resultaten hiervan tonen aan dat het ontstaan van de schildklier incidentalomen op een andere manier verloopt. We concluderen dat schildklier incidentalomen bij MEN type 1-patienten niet gerelateerd zijn aan het syndromen daarom adviseren we de behandelaars van deze patiënten de schildklier incidentalomen op dezelfde manier te behandelen als bij de 'gezonde' patiënt. Tevens voorkomt dit overdiagnostiek voor een patiëntenpopulatie die sowieso al veel diagnostiek moet ondergaan en daardoor veel onzekerheid kent.

Het hierop volgende hoofdstuk somt de verschillende genetische en epigenetische veranderingen op die bij het ontstaan van een schildkliernodus en schildklierkanker een rol spelen. **Hoofdstuk 4** belicht de rol van deze genetische en epigenetische alteraties en beschrijft hun functie. Een aantal van deze alteraties wordt al gebruikt om de onderscheidende waarde van de inconclusieve punctie in de schildklier te verbeteren. Met de onderscheidende waarde wordt de mate waarin de uitslag kan differentiëren tussen goedaardig en kwaadaardig bedoeld. Bij een inconclusieve uitslag wordt in de huidige praktijk de schildklierhelft waar de tumor in zit operatief verwijderd (hemithyreoïdectomie), daarna onderzoekt de patholoog de tumor in z'n geheel en concludeert of het om een goed- of kwaadaardige tumor gaat. Indien het een kwaadaardige tumor betreft, moet de patiënt vaak nog een keer geopereerd worden om de andere schildklierhelft ook te verwijderen (totaliserende thyreoïdectomie). Als de genetische en epigenetische alteraties gebruikt kunnen worden als zijnde een soort van voorspeller, dan zou daarmee het aantal onnodige operaties verminderd kunnen worden.

Hoofdstuk 5 is een reviewartikel waarin de beschikbare literatuur over expressie van microRNAs in schildkliertumoren wordt samengevat en de waarde van verschillende microRNA alteraties is geëvalueerd. MicroRNAs vallen onder de epigenetica. Het zijn korte stukjes enkelstrengs DNA die de expressie van genen in het DNA reguleren en daarmee een rol spelen in het ontstaan van kanker. De mate van expressie van bepaalde microRNAs is gemakkelijk

te bepalen in het materiaal van een schildklierpunctie. In de schildkliercellen is veelvuldig onderzoek gedaan om de aanvullende waarde van bepaalde microRNA expressiepatronen te bepalen met als doel de groep inconclusieve puncties te verkleinen.

Binnen de genetica van schildkliertumoren is er één genmutatie, BRAF, die extra aandacht behoeft en wel omdat de aanwezigheid hiervan bewijst dat een nodus kwaadaardig is. Bij ongeveer de helft van alle kwaadaardige tumoren kan deze mutatie worden aangetoond. Daartegenover staat dat hij altijd afwezig is in goedaardige tumoren, dit maakt deze mutatie heel waardevol in de diagnostiek. We hebben in **hoofdstuk 6** onderzocht of het in de Nederlandse situatie kosteneffectief is om deze genmutatie te analyseren op alle puncties die verdacht zijn voor een kwaadaardige tumor, maar waarbij dit niet met zekerheid te zeggen is. Ondanks dat deze uitslag niet vaak voorkomt, is een BRAF-analyse van deze puncties kosteneffectief.

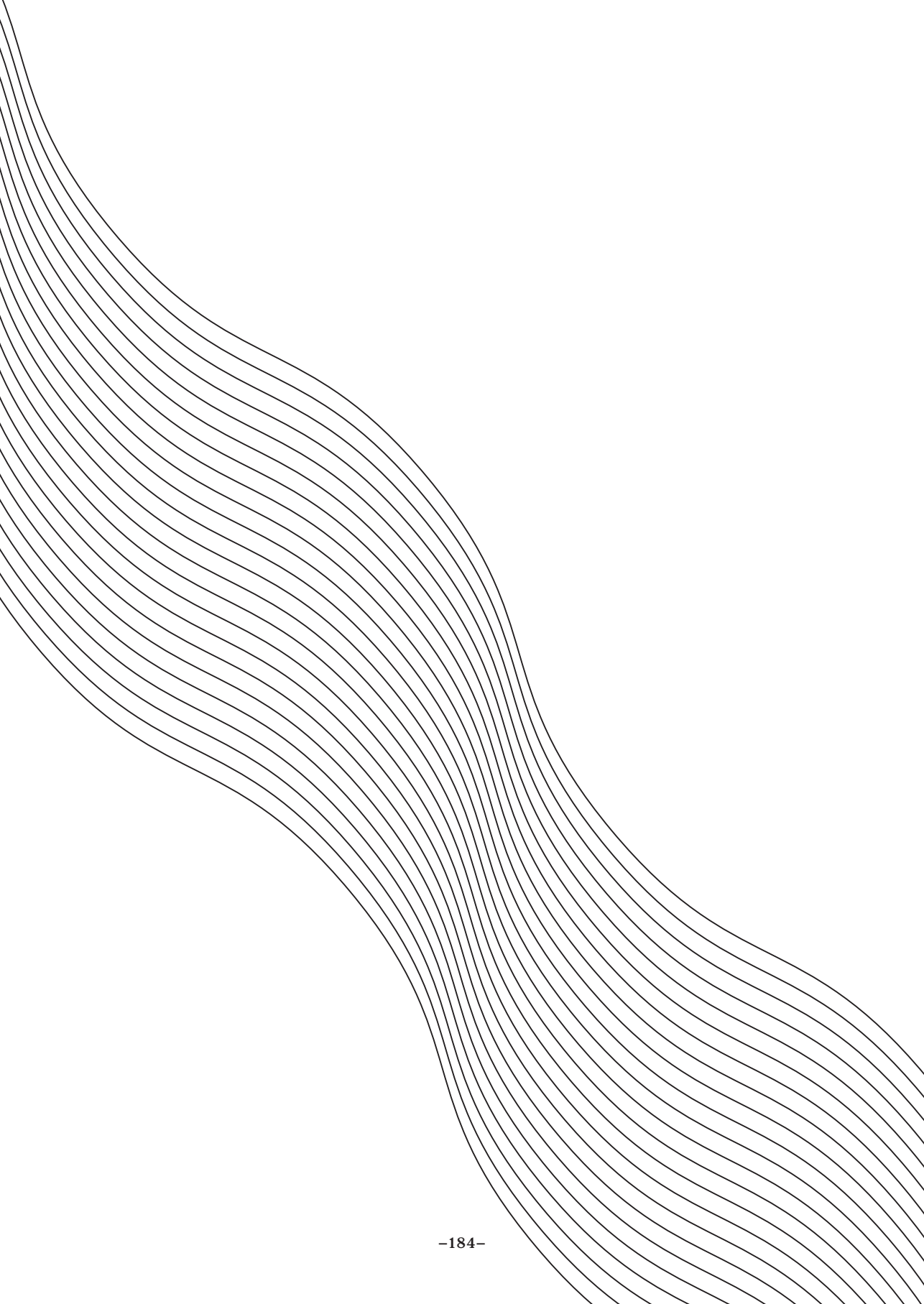
De hierop volgende hoofdstukken gaan over schildklierkanker. Er zijn vier verschillende typen schildklierkanker; het papillair en folliculair schildkliercarcinoom (gedifferentieerd schildkliercarcinoom), het medullair schildkliercarcinoom en het anaplastisch schildkliercarcinoom. In 80 – 85% van de gevallen gaat het om het gedifferentieerd schildkliercarcinoom, dit type schildklierkanker heeft een goede prognose, met een 10-jaars overleving van meer dan 90%. De behandeling van het schildkliercarcinoom bestaat uit een operatie, in de meeste gevallen het verwijderen van de hele schildklier (totale thyreoïdectomie) aangevuld met radioactief jodium (I131). Na de totale thyreoïdectomie zijn patiënten afhankelijk van medicamenteuze suppletie van schildklierhormoon. De behandeling is de laatste decennia steeds minder intensief geworden en wordt meer patiënt-specifiek afgestemd. Zo is de dosis van het radioactief jodium gehalveerd vergeleken met 5 jaar geleden en wordt bij steeds meer patiënten volstaan met een hemithyreoïdectomie. In **hoofdstuk 7** hebben we in een groot internationaal cohort bij 1313 patiënten, gediagnosticeerd met laag-risico gedifferentieerd schildkliercarcinoom, onderzocht in hoeveel procent van de verwijderde schildklieren er ook nog een kwaadaardige tumor in de andere schildklierkwab zou zitten. Deze tumoren zouden dus achterblijven als patiënten met een hemithyreoïdectomie behandeld zouden worden. Het bleek dat dit wel frequent voorkomt, in zo'n 30% van de gevallen, maar dat het in 80% van de gevallen gaat om een papillair schildkliercarcinoom welke kleiner dan 1 cm is. Uit de literatuur blijkt dat deze kleine papillaire schildkliercarcinomen zich in de loop van de jaren zeer zelden ontwikkelen tot klinische ziekte. Wat betekent dat een hemithyreoïdectomie een veilig alternatief kan zijn voor patiënten met laag-risico gedifferentieerd schildkliercarcinoom. Een bijkomstig voordeel van een hemithyreoïdectomie is dat patiënten niet meer afhankelijk zijn van medicamenteuze suppletie van schildkliercarcinoom.

In **hoofdstuk 8** bestuderen we patiënten met het medullair schildkliercarcinoom, dit is een zeer zeldzame variant van schildklierkanker met jaarlijks ongeveer 30 nieuwe gevallen in Nederland. De overleving hiervan is een stuk lager met een 10-jaars overleving van ongeveer 50%. Naar dit type schildklierkanker wordt weinig onderzoek gedaan. We hebben daarom alle beschikbare tumoren van dit type in vijf academische ziekenhuizen in Nederland verzameld. Op deze tumoren hebben we met behulp van immunohistochemie analyses gedaan naar expressie van bepaalde eiwitten en deze gecorreleerd aan overleving. 'Hypoxia-inducible factor 1 alpha' ofwel HIF-1α is zo'n eiwit waarvan we weten dat in andere soorten solide tumoren het samen met zijn signaleringscascade een belangrijke factor is voor agressiviteit van tumoren. De rol van HIF-1α was bij het medullair schildkliercarcinoom nog niet onderzocht. De resultaten laten zien dat ongeveer 60% van de tumoren dit eiwit tot expressie brengt en dat de 5-jaarsoverleving, onafhankelijk van andere factoren, daalt van 94% naar 66% als het medullair schildkliercarcinoom HIF-1α positief is. Dit is om verschillende redenen een belangrijke bevinding; ten eerste bewijst het dat HIF-1α en zijn signaleringscascade een belangrijke rol speelt in het ontstaan en de progressie van het medullair schildkliercarcinoom. Wat mogelijk een interessant aanknopingspunt zou kunnen zijn voor het ontwikkelen van nieuwe therapieën. Verder geeft het de mogelijkheid om patiënten een beter inzicht te geven in hun prognose.

In dezelfde groep patiënten als in hoofdstuk 8 hebben we in **hoofdstuk 9** een eiwit genaamd 'prostate-specific membrane antigen' (PSMA) onderzocht. PSMA is bekend als invloedrijk eiwit bij patiënten met een prostaatcarcinoom, maar recent is ontdekt dat nieuwe bloedvaatjes in verschillende typen solide tumoren dit ook tot expressie brengen. Het is nog eens een extra interessant eiwit omdat het gelokaliseerd is op de celmembraan. Deze lokalisatie maakt dat PSMA als een soort vlag kan dienen, die gemakkelijk herkent kan worden wanneer toegediende antilichamen eraan binden. Dit biedt geruime mogelijkheden binnen de nucleaire beeldvorming, bijvoorbeeld als tracer voor een PET/CT-scan en voor nucleaire therapie als een PSMA-antilichaam gekoppeld wordt aan een radioactieve stof. We hebben met behulp van immunohistochemie gevonden dat 93% van de medullair schildkliercarcinomen PSMA in meer of mindere mate tot expressie brengt en de mate van expressie stabiel blijft tussen de primaire tumor en uitzaaiingen in de lymfklieren. De verwachting is dat als we met behulp van de PET/CT-scan de uitzaaiingen kunnen signaleren, er geen variatie is tussen de verschillende uitzaaiingen. De eerstvolgende stap is nu om te onderzoeken of met de PSMA gelabelde PET/CT-scan het medullair schildkliercarcinoom en eventuele uitzaaiingen opgespoord kunnen worden, **hoofdstuk 9** vormt hier een goede basis voor. Daarnaast kwam naar voren, dat onafhankelijk van andere factoren, PSMA-expressie een relatief gunstige prognose geeft voor patiënten met een medullair schildkliercarcinoom.

Appendices

Review Committee
List of Publications
Acknowledgements
About the Author



Review Committee

Prof. dr. P.J. van Diest
Department of Pathology
University Medical Center Utrecht
The Netherlands

Prof. dr. H. Morreau
Department of Pathology
Leiden University Medical Center
The Netherlands

Prof. dr. O. Kranenburg
Cancer Center
University Medical Center Utrecht
The Netherlands

Prof. dr. R. Goldschmeding
Department of Pathology
University Medical Center Utrecht
The Netherlands

Prof. dr. G.J. de Borst
Department of Vascular Surgery
University Medical Center Utrecht
The Netherlands

List of Publications

1. Lodewijk L, van Diest P, van der Groep P, Ter Hoeve N, Schepers A, Morreau J, Bonenkamp J, van Engen-van Grunsven A, Kruijff S, van Hemel B, Links T, Nieveen van Dijkum E, van Eeden S, Valk G, Rinkes IB, Vriens M. Expression of HIF-1alpha in medullary thyroid cancer identifies a subgroup with poor prognosis. Oncotarget. 2017.

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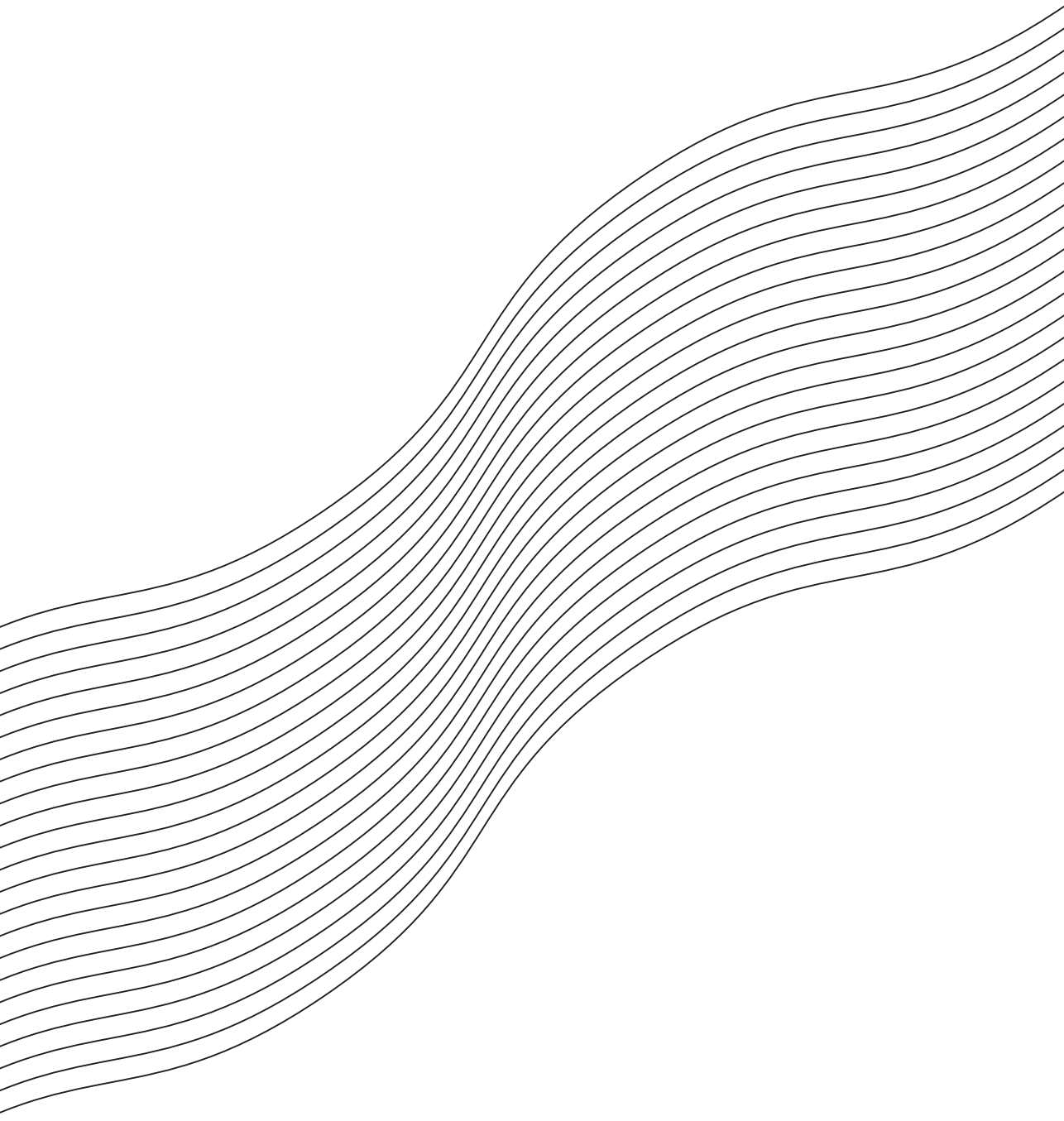

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Aan het begin van m'n promotietraject dacht ik dat het ontwikkelen wetenschappelijke en statistische vaardigheden de uitdaging zou zijn, maar de grootste uitdaging was misschien wel om op de goede momenten met de beste mensen samen te werken. En dat is gelukt!

Wat is een promovenda zonder haar promotoren? En wat mag je van geluk spreken als je promotoren zulke ontzettend inspirerende, grappige, intelligente en energieke mensen zijn!

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Dirk-jan van Beek | Selena Brouwer | Farahnaz Waissi | Anne Prins | Siegrid de Meer
Lisanne de Koster | Linde van Veenendaal | Benjamin Emmink | Ernst Steller | Klaas Govaert
Jamila Laoukili | Andre Verheem | Danielle Raats | Suzanne van Schelven | Inge Ubink
Thomas Vellinga | Frederik Hoogwater | Maarten Nijkamp | Charlotte van Kessel
Szabolcs Fatrai | Tijana Borovski | Nicola Frenkel | Domenico Castigliego | Petra van der Groep
Natalie ter Hoeve | Biobank medewerkers | Thijs van Dalen | Egbert-Jan Verleisdonk
Collega's Diakonessenhuis | Pieter van der Sluis | Laurien Waaijer | Anne den Hartog
Stefanie Peeters Weem | Tesse Leunissen | Pieter Leliefeldt | Okan Bastian | Marjolein Heeres
Kevin Parry | Steven van Haelst | Anne Huibers | Roy Verhage | Claire Pennekamp
Marieke Straver | Margriet Fokkema | Martin Teraa | Bryan van der Wal | Jasmijn Smits
Willemijne Schrijver | Dominique Buck | Maarten Burbach | Frank Voskens | Djurre de Waard
Marieke Walma | Vanessa Pourier | Janine Simons | Annique Pieters | Yoen van der Linden
Quirine Manson | Sieske Hoendervangers | Jacqueline van Laarhoven | Cas van 't Hullenaar
Marijn Houwert | Mark van Heijl | Vrienden en Vriendinnen | Floor Bijkersma
Douwe &let Bauke & Brenda Gerlof & Hanna
Teun

About the Author

Lutske Lodewijk was born on the 6th of September 1984 in Leeuwarden. She is the daughter of Douwe Lodewijk and Iet Lodewijk – Anema, and has two brothers Bauke and Gerlof. Until the age of six she lived in St. Annaparochie, and spent the rest of her childhood in Marknesse. After graduating from secondary school at the Zuyderzee College in Emmeloord, she moved to Groningen to study medicine at the University of Groningen. She joined a team of medical students that assisted with liver transplantations in the University Medical Center Groningen and this fortified her interest in surgery. During her medical training, she enrolled in a research project during her internship at the Groote Schuur Hospital in Cape Town under supervision of prof. dr. D. Kahn. She studied the effect of liver regeneration on the pharmacokinetics of immunosuppressive drugs.

After graduating in January 2009, Lutske started to work in the Sint Lucas Andreas Hospital as a surgical resident (not in training). In 2010 she worked 6 months in Nanatha in Mozambique for the public health project Anan Clinica, a charity she chaired for the following years.
Back in the Netherlands Lutske met prof. dr. M.R. Vriens and prof. dr. I.H.M. Borel Rinkes in her search for a PhD position. They sparked her interest for endocrine surgery and gave her the opportunity to start as PhD candidate. The results of her research are presented in this thesis.
Lutske commenced her surgical residency in January 2015 at the University Medical Center Utrecht under the supervision of prof. dr. M.R. Vriens, and continued her surgical training in january 2016 in the Diakonessenhuis in Utrecht under the supervision of dr. T. van Dalen.
Besides her work Lutske enjoys spending time with family and friends, travelling and sports. She lives with Teun Bimbergen in ‘s Graveland.