Differentiated Thyroid Carcinoma

Diagnostic and Therapeutic Studies

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Differentiated Thyroid Carcinoma: *Diagnostic and Therapeutic Studies*
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Dedicated to people who love me
&
to my motherland

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Chapter 1

General Introduction

1. Introduction

Differentiated thyroid carcinoma (DTC) is a fascinating tumor for multiple aspects. First, from a biological point of view, DTC has many intriguing aspects. Recent insights into the pathogenesis of DTC have revealed a clear picture of the relation between genetic alterations and the different subtypes of DTC that all arise from the thyroid epithelium. These insights not only have added to the understanding of the pathogenesis of DTC, but have also provided new candidate targets for therapy. In addition, the pathogenesis of DTC has also revealed important knowledge about normal thyroid physiology, in particular the pathophysiology of molecules involved in iodide metabolism, like the sodium iodide symporter (NIS) and thyroid peroxidase (TPO). The defects in iodide metabolism in DTC that are present in advanced tumors, offer a model to study the contribution and significance of the components involved in iodine metabolism and may also offer targets for redifferentiation approaches. The accomplishments of basic research in these areas may ultimately provide valuable directions for clinical management of DTC.

Second, from a clinical point of view, DTC is fascinating because the approach to the patient differs essentially from many non-endocrine tumors. The central role of therapy with radioactive iodine is unique for DTC. Another special aspect is the fact that despite the good prognosis, a substantial proportion of patients develop metastases, that are not life threatening but may impair quality of life considerably, a situation that is not often encountered in general oncology. The unique features of DTC offer opportunities for basic and clinical research and indeed insights from the pathophysiology of DTC have often lead to a broader understanding of biological mechanisms involved in cancer.

Despite these fascinating aspects of DTC, the diagnosis and therapy remain a challenge to the physician. DTC has a low incidence and in general an excellent prognosis. Therefore, intervention studies are difficult to perform because to reach relevant endpoints high numbers of patients with long follow-up periods are required. As a result, most protocols are based on retrospective studies. The low incidence logically would require centralized treatment and registration of DTC patients in order to develop optimal follow-up protocols. The problem, however, is that treatment of DTC is often decentralized, resulting in many different follow-up protocols. A typical example of the decentralized follow-up is the fact that many different staging systems have been developed, which complicates the comparison of treatment results between centers. Fortunately, in recent years, several national and multinational guidelines have become available (published by the British, American and European Thyroid Associations)(1;2). Although these guidelines agree in many aspects, they are still based on moderate evidence levels, leaving many questions to be answered. Prominent questions are the diagnostic criteria of DTC, the optimal follow up strategies in DTC patients and strategies to improve radioiodide (RaI) therapy.

The present thesis is focused on several clinical questions involved in treatment and follow-up of patients with DTC. In this chapter a general overview of DTC will be provided and the questions addressed in this thesis will be introduced.

2. Characterization of thyroid carcinomas

DTC has a low incidence, varying from 2-10/100.000 (3-6) with a female to male preponderance of 2:1. In general, 80% of newly diagnosed thyroid carcinomas are differentiated tumors with a median age at diagnosis of 45 to 50 years (7). DTC has a relatively favourable prognosis with a 10-yr survival of 90-95% (Table 1).

Table 1. Clinico-pathological features of thyroid cancer (adapted from ref.2)

Tumour type	Prevalence	Sex ratio (female:male)	Age (years)	Lymph-node metastasis	Distant metastasis	Survival rate (5 year)
Papillary thyroid carcinoma	85–90%	2:1–4: 1	20–50	<50%	5–7%	>90%
Follicular thyroid carcinoma	<10%	2:1–3: 1	40–60	<5%	20%	>90%
Poorly differentiated thyroid carcinoma	rare–7%	0.4:1–2.1: 1	50–60	30–80%	30–80%	50%
Undifferentiated thyroid carcinoma	2%	1.5: 1	60–80	40%	20–50%	1–17%
Medullary thyroid carcinoma	3%	1:1–1.2: 1	30–60	50%	15%	80%
Mixed medullary and follicular-cell carcinoma	rare					

This high survival rate is the result of the biological behaviour of most of these tumors and the efficacy of primary therapy, consisting of surgery and RaI therapy. However, when distant metastases occur, the prognosis is worse because the results of RaI therapy, which is virtually the only curative treatment option, are moderate. Although these metastases are rarely life threatening, they may affect quality of life for years depending on the localization and size.

The tumor-node-metastases (TNM) classification system is based primarily on pathologic findings and separates patients into four stages, with progressively poorer survival with increasing stage (Table 2) (8). Recently, the 6th edition of the TNM system has become available (9). The most important difference with he 5th edition is the fact hat the dimension of T1 has been extended to 2 cm, which has implications for the prognosis of DTC (10). Therefore, some experts propagate to continue the use of the 5th edition. In this thesis the 6th edition of he TNM staging system is used (11).

Table 2. Postoperative TNM stage of Differentiated Thyroid Carcinomas (6th Edition, Ref. 9)

T0	No primary tumor
T1	Tumor diameter < 2 cm
T2	Tumor diameter 2- 4 cm
T3	Tumor diameter > 4 cm, limited to the thyroid or with minimal extrathyroid extension
T4a	Tumor of any size extending beyond the thyroid capsule to invade subcutaneous soft tissues, larynx, trachea, esophagus or recurrent laryngeal nerve
T4b	Tumor invades prevertebral fascia or encases carotid artery or mediastinal vessels
N0	No metastatic nodes
N1a	Metastases to level VI (pretracheal, paratracheal and prelaryngeal lymph nodes)
N1b	Metastases to unilateral, bilateral, contralateral cervical or superior mediastinal nodes
M0	No distant metastases
M1	Distant metastases

3. Pathogenesis of DTC

3.1 Molecular Pathogenesis

Human thyroid tumors originate from epithelial follicular cells or from parafollicular C-cells. Follicular cell-derived tumors represent a wide spectrum of lesions, ranging from benign adenomas to differentiated (follicular and papillary) and undifferentiated (anaplastic) carcinomas, thus providing a good model for finding a correlation between specific genetic lesions and histological phenotype.

Recent developments have provided a detailed map of the role of the genetic alterations involved in the pathogenesis of thyroid neoplasms and DTC. The dissection of the genetic alterations has important implications not only for the diagnosis, but also for the understanding of the molecular (patho)physiology of thyroid disorders (12-14). Follicular adenomas and carcinomas frequently have mutations in one of the three RAS genes . Mutations of $G_s\alpha$ protein (GSP) and thyroid-stimulating hormone (TSH) receptor genes are associated with benign hyperfunctioning thyroid nodules and adenomas. The understanding of the molecular pathogenesis of papillary carcinoma (PTC) has improved considerably by the recent identification of mutations in B-RAF, which are present in 40-60% of the carcinomas. B-RAF is a component of the RET, RAS, RAF cascade that activate MAP kinase. Indeed, mutations and rearrangements of B-RAF, RAS, RAF and TRK (neurotrophic tyrosine kinase receptor) account for almost all cases of PTC. Translocations of RET observed in DTC result in a chimeric protein consisting of an activated RET tyrosine kinase domain. (13;15-30). MET (receptor-tyrosine kinase) overexpression in DTC is thought to be regulated

by transcriptional or post-transcriptional mechanisms as a secondary effect (31). The situation in follicular thyroid carcinoma (FTC) is less clear (32), but a very interesting observation has been the presence of a rearrangement of the PAX-8 and PPARγ genes (33), a unique combination of genes that traditionally is associated with thyroid development (the transcription factor PAX-8) and cell differentiation and metabolism (PPARγ). The chimeric protein acts as a dominant negative competitor for PPARγ. Indeed, in experimental models of DTC, downregulation of the PPARγ signaling route has been observed (34). Anaplastic carcinomas are frequently associated with mutations in the p53 tumor suppressor gene (35). This is in contrast with many other tumors in which p-53 mutations play a role early in the process of tumorigenesis.

In the pathogenesis of thyroid carcinoma, it is believed that the genetic alterations lead to both proliferations via multiple pathways, and the loss of thyroid specific protein expression. The disappearance of the functional expression of thyroid specific proteins is a complex chain of events, in which the mechanism is incompletely understood. From many observations, it is believed that there is a sequential disappearance of specific proteins. The disappearance of thyroid peroxidase (TPO) is believed to be an early event, followed by NIS. TSH receptor (TSHR) expression and thyroglobulin (Tg) expression are usually still present in advanced stages (36;37;37;38). The mechanisms involved in the decreased expression of thyroid specific proteins may be genetic, involving the absence of thyroid transcription factors, epigenetic changes (observed for NIS and TSHR), mutations (not frequently observed) or by post-translational regulation (NIS) (39).

3.2 Sodium iodide symporter and iodide metabolism

The main treatment option for recurrent or metastatic thyroid carcinoma is therapy with radioiodide (RaI). The discovery and molecular cloning of the rat and later NIS have contributed greatly to the understanding of the physiology and pathophysiology of iodide uptake by the thyroid gland (40;41). NIS resides at the basolateral membrane of thyroid epithelial cells and is responsible for the uptake of iodide from capillaries into the thyroid epithelial cell. The relation between decreased RaI uptake in thyroid carcinoma and decreased NIS activity has been well established. However, controversy exists on the mechanism. There is evidence that genetic alterations in DTC lead to decreased NIS mRNA and protein expression (42). Indeed, the causal chromosomal rearrangements in PTC have been demonstrated to inactivate NIS expression by decreased expression of the transcription factors TTF-1 and PAX-8 (43). TTF-1 and PAX-8 are involved in the gene expression of important thyroid proteins, including NIS. In contrast, experimental gene transfer with PAX-8 lead to re-expression of NIS in a dedifferentiated thyroid cell-line (44;45). In other studies however, a defect in targeting of NIS to the cell membrane is reported, which is accompanied by an intra-cytoplasmatic overexpression of NIS in about 80% of thyroid tumors (39). These differences have important consequences for

interventions aimed at increasing NIS expression.

The ultimate dose of radioactivity in thyroid tumors (expressed in Gray (Gy)) is not only determined by the amount (activity) of RaI administered to the tumor (specific activity), the rate of uptake but also by the tumor volume and the effective half life of RaI, which on its turn is determined by the physical half life and the biological half life (46). The exact mechanism of iodide efflux remains elusive. Although candidate molecules for apical iodide efflux (pendrin) (47) have been discovered, their exact role in apical iodide transport has not been determined yet. The putative apical iodide symporter (48) has been proven not to transport iodide (49). In addition to iodide efflux, organification of iodide by TPO together with the three dimensional architecture of the thyroid is also likely to contribute to the dose of RaI achieved.

4. Initial diagnosis of DTC

Despite the increasing standards of imaging techniques like ultrasound, fine needle aspiration (FNA) is the procedure of choice in patients presenting with thyroid enlargement. The sensitivity of FNA for DTC in most series is 90-95%. The specificity of FNA is lower, 60-80% when all patients with a non-benign FNA are referred for surgery (50). As a consequence, the frequency of FTC in hemi-thyroidectomies performed after suspicious results from FNA is only 20-30%. The problem is that the distinction of benign and malignant follicular neoplasms is difficult to make by FNA, as the crucial criterion for FTC vs. adenoma (FA) is capsular invasion, which cannot be determined by cytology. In addition, the distinction between FA and Follicular variant of PTC (FVPTC) is also difficult, because the crucial criterion here is the aspect of the nuclei. The implication is that 70-80% of the patients with suspicious results from FNA, who undergo thyroid surgery have a benign tumor (51). Therefore, approaches to improve the accuracy of FNA are warranted (51). Candidate molecular markers for diagnosis and prognosis can be distinguished in 3 groups: 1) gene mutation or chromosomal rearrangements; 2) lack of thyroid specific protein expression and 3) markers associated with malignant transformation:

1) Genomic instability in DTC

As chromosomal alterations have only recently been identified, their place in the diagnosis of DTC is still limited. B-RAF mutations can be identified in FNA and would facilitate the diagnosis of PTC, which is however, usually not difficult. Unfortunately, B-RAF mutations are uncommon in FVPTC (52). The detection of a PPARγ/PAX8 rearrangement in 75% of follicular carcinoma's and not in papillary carcinoma and follicular adenomas (33), suggested that this rearrangement could be a used as a diagnostic tool. However, in a recent study (53) the rearrangement was observed in 13% of follicular adenomas, which weakens the diagnostic value of this marker.

2) Thyroid specific proteins that lose expression during thyroid dedifferentiation

Absence of TPO has been reported to be a specific marker both in cytology and histology of thyroid malignancies. In the process of dedifferentiation, TPO appears to be the first protein with diminished expression. Clinically, this leads to decreased organification of iodine, which may have consequences for RaI therapy. In studies originally initiated by De Micco et al, a cut-off value of 80% of thyroid epithelial cells staining positive with the anti-TPO antibody MoAb47 has been found to have superior sensitivity (100%) and specificity (up to 99%) for follicular carcinoma in surgical specimens (54). Later studies, mostly from the same center, confirmed the diagnostic value of TPO immunostaining in FNA. In the distinction between follicular adenoma and carcinoma, sensitivity is reported around 100%, specificity varying from 61-99% (55;56). However, most of these observations have come from one group, suggesting that the technique (both antibody and staining procedures) may be relatively complicated.

3) Markers associated with malignant transformation in general

From the non-thyroid specific markers, galectin-3 has been a promising marker. The galectins are carbohydrate binding proteins involved in cell adhesion, cell growth and cell death. Galectin-3 (Gal-3) has been considered a marker with a high diagnostic potential to identify FTC (55-65), but in recent publications Gal-3 staining was also reported in benign lesions (66;67). Other immunohistochemical markers that have been reported of various use are HBME-1 (Hector Battifora mesothelial) (68-72) and Cytokeratin-19 (73-78). Other molecular markers that have been investigated include telomerase activity. Telomerase is an enzyme that adds nucleotides to telomeres, DNA sequences at the ends of chromosomes that enhance chromosomal stability. Assessment of telomerase in thyroid FNA reveals telomerase in 14-38% of follicular adenoma and in 75% of follicular carcinoma (79;80), indicating that these assays alone have insufficient diagnostic properties as compared with TPO and Gal-3.

The introduction of high-throughput techniques in molecular biology has opened new potential perspectives for the identification of novel diagnostic molecular markers for thyroid carcinoma (81-85). Recent studies based on cDNA expression arrays have identified immunohistochemical markers for the differentiation between thyroid neoplasms. Markers emerging from these studies are Gal-3, Fibronectin-1 (FN-1) and CITED-1 (*C*BP/p300-*I*nteracting *T*ransactivators with glutamic acid *[E]* and aspartic acid *[D]*-rich C-terminal domain) were found to be overexpressed in PTC (85). In most of these studies fixed cut-off levels for positive staining are used and with the exception of Casey et al (75), de Matos et al (76) and Prasad et al no panels of antibodies are studied.

The introduction of tissue micro-array (TMA) facilitates a comprehensive expression analysis of thousands of genes at the protein level in a given tissue. Maximal standardization of the procedure is a major strongpoint of the TMA methods as

all tissues analyzed are located on one glass section and treated under absolutely identical conditions. Most TMAs have been used in cancer research to investigate the prevalence of molecular changes and their associations with tumor progression and/or prognosis (86;87).

We decided to evaluate the diagnostic value of Gal-3, HBME-1, CK-19, CITED-1, FN-1, NIS and PPARγ in a TMA containing a large panel of thyroid neoplasms, using receiver operator curve (ROC) analyses to calculate cut-off levels and evaluating the diagnostic accuracy of panels identified by hierarchical cluster analysis **Chapter 2**.

5. Initial therapy of DTC

The guidelines for the initial therapy of TC have been extensively reviewed in the guideline papers mentioned above. In all patients with DTC except unifocal T1 (5^{th} edition TNM (11)) PTC, initial therapy consists of near-total thyroidectomy followed by RaI ablative therapy of thyroid remnants. Although there is still some controversy about the extent of thyroid surgery, there are strong arguments in favor of total or near-total thyroidectomy (leaving only as limited thyroid tissue as is necessary to keep vital structures intact) in all patients (88). Total or near-total thyroidectomy results in a lower recurrence rate than more limited thyroidectomy, because many papillary carcinomas are multifocal and bilateral. Furthermore, total thyroidectomy facilitates total ablation with RaI and reveals a higher specificity of Tg as a tumor marker (89-93).

Although controversy exists about the routine application of RaI ablation of thyroid remnants, many clinics follow this procedure. Postoperatively RaI therapy is given for three reasons. First, it destroys any remaining normal thyroid tissue, thereby increasing the specificity of detectable serum Tg and positive whole-body scintigraphy as markers for persistent or recurrent tumour (7;89;94). Second, RaI therapy may destroy occult microscopic carcinomas, thereby decreasing the long-term risk of recurrent disease (89;95-97). Third, the use of a large amount of RaI for therapy permits post ablative scanning, a test for detecting persistent carcinoma (98;99).

However, in a recent meta-analysis (100) the beneficial effect of RaI ablation to prevent recurrence or death was doubtful. A beneficial effect was only shown in patients with a high risk or irradical surgery (91;95;101;102). In addition, doubts have arisen about the safety of routine RaI ablation, a recent paper suggesting a relation between excess non-thyroidal malignancies and RaI (103). This has led to a more careful positioning of RaI ablation in recent papers (2;104). In conclusion, there is consensus about the efficacy of RaI ablation therapy in patients with: (i) tumor stages T2-4; (ii) evidence for remaining thyroid tumor remnants and (iii) metastases (105;105;106).

The efficacy of RaI therapy depends on the effective radiation dose delivered to the thyroid remnant or tumor (46). The radiation dose is negatively affected by

decreased uptake and the shorter effective half-life of RaI in tumor tissue compared with normal thyroid tissue (37;42;107).

Strategies to increase RaI uptake include the establishment of high TSH levels, either by thyroid hormone withdrawal or by therapy with recombinant human TSH (108;109). Another method to increase RaI uptake is to deplete the plasma inorganic iodine pool before RaI therapy. Low plasma iodine concentrations may increase the expression of the sodium iodine transporter (hNIS) leading to a higher specific activity of RaI which can be achieved by limiting iodide intake through a low-iodine diet (110).

6. Follow-up of patients with DTC

The purpose of follow-up protocols in DTC is to detect and prevent persistent or recurrent DTC. Recurrences are usually detected during the early years of follow-up but may be detected later, even after more than 15 years after initial treatment. Most patients during follow up have been cured definitely, and, as a consequence, have a low pre-test probability for recurrent disease. Therefore, the sensitivity of the diagnostic test must be adequate to detect the few patients with evident thyroid carcinoma, whereas specificity must also be high to avoid unnecessary treatments in patients without recurrent disease. In addition, the burden of diagnostic tests for the patient should be kept at a minimum. The most important tools in follow up protocols are serum measurements of Tg, diagnostic whole body RaI scintigraphies and neck-ultrasound.

Numerous studies have been performed on the diagnostic value of Tg measurements. The consensus is that the TSH stimulated Tg measurements have superior diagnostic value in DTC (111). The interpretation of many studies and consequently of the guidelines on Tg performed so far is difficult because the analytical aspects of Tg measurements are complicated.

6.1 Thyroglobulin

Tg is a glycoprotein that is produced only by normal or neoplastic thyroid follicular cells. It should not be detectable in patients, who have undergone total thyroid ablation. The presence of Tg in such patients reveals the presence of persistent and/or recurrent disease. The type of analysis (RIA or immunometric assay) affects the interpretation of serum Tg values (112). Currently, the clinical interpretation of serum Tg levels is hampered by analytical and statistical problems. Analytical problems are the lack of universal standardization, leading to significant inter-method variability (112;113), poor inter-assay precision across the relevant concentrations used for monitoring patients with DTC (6-12 months), "hook" effects which primarily affect immunometric assay methods and can lead to inappropriately low- or normal range Tg values in sera with very high Tg concentrations (113) and Tg auto-antibody (TgAb) interference, which can lead to under- or overestimation of the serum total

Tg concentration, regardless of the type of method used (112;114-117). Statistical problems are the use of fixed Tg cut-off levels without using receiver operator curve (ROC) analyses. The application of ROC data is essential, as a chosen cut-off level is a subjective choice based on the balance between desired percentages of missed recurrences versus unnecessary therapies. Therefore, in a recent European consensus paper, it was recommended to define institutional Tg cut-off levels (118). In addition to diagnostic purposes, Tg could also be used as a prognostic factor in DTC. The few studies that were published on the prognostic significance of Tg measurements used fixed cut-off levels, contained selected subgroups of patients and included either Tg measurements at one time point or at undefined time points (119-123).

In **Chapter 3**, we describe a study on the diagnostic and prognostic value of Tg in a homogeneous group of DTC patients with respect to initial therapy, using Tg measurements at 5 defined time-points after diagnosis, in combination with ROC analyses. In addition, we studied the diagnostic and prognostic value of Tg antibodies for tumor presence or death. We also looked into the potential diagnostic use of Tg auto-antibodies.

6.2 New serological markers

Because of the limitations of Tg, novel serological markers have been searched for. Of interest is the demonstration of Tg mRNA in peripheral blood, which indicates the presence of circulating Tg producing cells (e.g. thyroid cancer cells). In a number of studies, Tg mRNA alone did not have sufficient diagnostic power to discriminate between patients with active tumor and thyroid remnants (124) or thyroid carcinoma and healthy volunteers (125). However, the combination of Tg and Tg mRNA allowed the identification of all patients with active disease in another study (34).

Interestingly, RT-PCR can also be applied to detect cells that produce other thyroid specific proteins. In a study on TPO (126), RT-PCR correlated significantly with metastatic disease.

6.3 Iodine-131 total body scitigraphy

The result of iodine-131 whole body scitigraphy depends on the ability of thyroid-cancer tissue to accumulate RaI in the presence of high serum TSH concentrations. The sensitivity of diagnostic RaI scintigraphies is much lower than that of ultrasound and Tg measurements and consequently, the routine use of RaI scintigraphy in the diagnostic follow-up of DTC patients is no longer recommended (2;127).

6.4 Ultrasound

In recent publications, ultrasound combined with FNA had the highest sensitivity (even higher than Tg) for local recurrent DTC and lymphnode metastases (128-130). This has led to an important place for ultrasound in he follow up of DTC.

6.5 18-F Fluorodeoxyglucose-positron emission tomography (FDG-PET)

The diagnostic accuracy of FDG-PET in patients suspected of recurrent DTC is not well defined. Many studies are biased by selection of patients or have other methodological problems (131). The general idea is hat FDG-PET may be useful in patients with elevated serum Tg levels in whom no RaI is observed after diagnostic or post-therapeutic scintigraphy. The sensitivity of FPG-PET is better when serum Tg levels are higher (132). FDG-PET during TSH stimulation may be more sensitive than during suppressive therapy (133).

6.6 Somatostatin Receptor Scintigraphy (SRS)

The expression of somatostatin receptors (SSTR3 and SSTR5) by DTC is the basis for SRS imaging and therapy. Interestingly, in a considerable proportion of DTC, SRS imaging shows pathological lesions, which has diagnostic and therapeutic consequences (134;135).

6.7 Thyroxine withdrawal versus recombinant human TSH (rhTSH)

Serum Tg measurements, thyroid ablation, diagnostic scintigraphies with RaI during follow-up and RaI therapy for recurrent disease are based on the responsiveness of DTC to TSH (136).

High serum TSH levels can be realized by conventional thyroxin withdrawal or more recently by recombinant human TSH (rhTSH), which has advantages with respect to quality of life (137). The first indication for rhTSH has been diagnostic RaI scintigraphy and Tg measurements (108;109;128;130;138-143). rhTSH has also been used for RaI therapy in active DTC (144-148). Although these studies suggest a comparable efficacy with thyroxine withdrawal, no randomized studies have been performed. A good indication for rhTSH for RaI therapy is when contraindications are present for withdrawal. rhSH has also been registered for the ablation of thyroid remnants (149-151). Tg measurements during rhTSH have comparable accuracy with thyroxine withdrawal (111;139). Some authors, however, have observed a lower sensitivity of diagnostic RaI scintigraphies performed after rhTSH (152;153). The efficacy of RaI therapy after rhTSH may be comparable with withdrawal, but no randomized studies have been performed to allow a direct comparison (144;154). Efficacy of RaI ablation after rhTSH was comparable after thyroxin withdrawal in a recent randomized trial (151), although earlier studies with lower activities of RaI showed a lower efficacy (155). One of the possible explanations for the supposedly decreased RaI uptake during rhTSH may be that triiodothyronin (T3) directly influences iodine uptake in the thyroid. This may be relevant as patients with rhTSH will continue thyroxine therapy and have consequently higher T3 levels. We therefore studied the in vitro effects of T3 on iodide uptake in **Chapter 4**.

6.8 TSH-suppressive L-thyroxine therapy

Although the rationale for this therapy is evident, it is not clear if all patients benefit from suppressed TSH levels, to what extent TSH should be expressed and for how long. In a retrospective study, a lower relapse-free survival was found in patients with a consistently suppressed TSH than in patients with TSH > 1 mU/L (156). In a large study with a median follow-up of 5 years, the level of TSH suppression (undetectable vs. normal) was a significant prognostic factor in high risk papillary carcinoma only (157). In a meta-analysis the role of TSH suppression in the follow-up of DTC was also not clear (158). The routine use of TSH suppression is limited by the supposed disadvantages of TSH suppression, like osteoporosis (159) and cardiac side effects (160;161).

7. Therapy in metastatic disease

Distant metastases, usually in the lungs and bones, occur in 10 to 15 % of patients with DTC. Lung metastases are most frequent in young patients with papillary carcinomas. In general, bone metastases are more common in older patients and in those with FTC.

In case of residual disease or metastases, surgery can be attempted when the lesion is accessible. In other cases, RaI therapy will be given to patients with metastases that accumulate RaI. The remission rate in pulmonary metastases treated with iodine -131 is ~50%, varying from 90% in patients with microscopic metastases to only 10% in macronodular disease (106;162;163). The remission rates of bone metastases in the same studies are worse, varying between 7-20 %. A major problem in this category of patients is the diminished or lost ability of thyroid cancer cells to accumulate RaI, indicated by negative post-therapeutic whole body scintigraphy. In these cases the prognosis is poor, as alternative treatment options (external radiotherapy or chemotherapy) have limited success (164).

Strategies to improve therapeutic options can be distinguished in 1) Therapies to improve RaI therapy or 2) Identification of new targets for therapeutic intervention.

7.1 Strategies to improve Radioiodide Therapy

Approaches to improve RaI therapy are subdivided in 1) Tumors that still accumulate iodide and 2) Tumors that do not.

7.1.1 Tumors that accumulate iodide

In tumors that still accumulate iodide, improving RaI therapy is essentially aimed at increasing the dose of RaI, which is determined by the factors mentioned above. Attempts to improve RaI can be aimed at all these factors. The specific activity of RaI presented to the tumor can be improved by a low iodide diet (110). The uptake rate can be influenced by high TSH levels, either by withdrawal of thyroxine or by

rhTSH. The half-life of RaI in DTC is an important factor. The loss of follicular architecture, and probably decreased activity of TPO may contribute to a decreased effective half life and thus by a lower tumor dose (46;165). Several attempts have been reported to improve half-life. Transfection with TPO did not result in an increased iodide retention (166). Another possibility reported in the literature to increase the effective half-life of RaI in DTC is to use a pharmacological approach with lithium salts.

7.1.1.1 Lithium

Lithium salts have been associated with an increased trapping of iodide by the thyroid gland (167;168). This property of lithium led to the assumption that lithium may enhance the dose of RaI in benign and malignant thyroid disorders. Indeed, lithium therapy increased RaI retention in Graves hyperthyroidism (169;170), although this could not be confirmed in other studies (171;172). Reports in DTC have indicated a positive effect of lithium as well (167;173-176). The design of these studies, however, does not allow a definite conclusion, as they contain small numbers of patients, vary in the time course of lithium application and used a sequential design resulting in higher TSH levels during lithium therapy (171;176) (167;169;173). Although in the study of Koong et al (176) it was advised to add lithium to RaI therapy in patients with metastatic DTC, no studies have been published to our knowledge in which the effects of the addition of lithium to RaI on the clinical course of patients was investigated. We, therefore, studied the clinical effects of RaI without and with lithiumcarbonate in 12 patients with proven metastatic DTC in **Chapter 5**.

In addition, the mechanism of the supposed beneficial effect of lithium salts is unclear. In the literature, however, variable effects of lithium salts on iodide uptake in vitro or in animal studies are reported. (177-184,185). We therefore studied the in vitro effects of lithium salts on iodide metabolism in a background of normal thyroid physiology, in a non-thyroid background and in the background of thyroid carcinoma, which is also reported in **Chapter 5**.

7.1.2 DTC with absent iodide uptake

When iodide uptake is completely lost in DTC, attempts to improve RaI should be targeted at the re-induction of functional NIS expression. These attempts are indeed complicated by he current uncertainty about the mechanism of decreased functional NIS expression in DTC which may involve both genetic defects (42) and post-translational (trafficking)(39) defects.

Genetic studies to reinduce NIS expression have been reported. NIS gene transfer in DTC cell lines and animal studies has been proven to restore the susceptibility to RaI therapy (4;186). Interestingly, gene transfer with PAX-8 also leads to re-expression of NIS in a dedifferentiated thyroid cell-line (44;45). Although these approaches are fascinating from a conceptual viewpoint, a potential clinical application appears not

to be within reach.

Therefore, medical approaches aimed at redifferentiation, or re-induction of thyroid specific proteins have gained much interest. Compounds that have been reported to reinduce NIS expression are retinoids, demethylation inducing compounds and histone-deacetylase inhibitors.

7.1.2.1 Epigenetic therapies

One of the mechanisms by which cells can block the expression of certain genes is by enzymes that methylate these genes or de-acetylate the histones that envelope a particular gene. These mechanisms also play a role in the silencing of genes in cancer. Therefore, compounds that can reverse methylation or inhibit histone deacetylation may lead to the re-expression of genes that are silenced in cancer.

Demethylation therapy has been proven successful in leukemia. In an in-vitro study in thyroid carcinoma, the demethylating agent 5-azacytidine led to re-induction of NIS expression, accompanied by RaI uptake in thyroid cancer cell lines (187). In parallel, the histone deacetylase inhibitor depsipeptide has been reported to reinduce NIS mRNA expression and RaI uptake in DTC (188;189), although toxicity may be a serious problem (190).

7.1.2.2 Retinoids

Retinoids are derivatives of vitamin A (i.e. retinol). Beneficial effects of retinoids have been reported in promyelocytic leukaemia and several types of carcinoma (191-193). In vitro studies have reported that retinoids have beneficial effects in DTC (194-197) including increased NIS mRNA expression and iodide uptake in some thyroid cancer cell lines (194). Interestingly, the promoter of the NIS gene has a retinoic acid response element (198). A limited number of human studies have been performed on the effects of retinoids on I-131 uptake with mixed results (199-203), all using the RAR agonist 13-cis retinoic acid. However, recent studies indicated a differential expression of both RAR and the retinoid receptor RXR in thyroid carcinoma cell-lines and tissues (204;205), which corresponded to the responsiveness to ligands for these receptors. The importance of RXR expression with respect to responsiveness to retinoid treatment was demonstrated in the latter study (205). We, therefore, decided to perform a prospective controlled clinical trial to investigate the efficacy of the novel ligand Bexarotene (Targretin, Ligand Pharmaceuticals, San Diego), in 12 patients with metastases of DTC and decreased or absent I-131 uptake. Bexarotene is an RXR agonist, which also induces RAR by transcriptional activation. The antineoplastic potential has been demonstrated in cutaneous T-cell lymphoma, but also in other malignant tumors (206-208). This study is described in **Chapters 6 and 7**.

7.2 Strategies aimed at non-thyroid specific targets

Over the last decade, exciting developments have taken place in the identification and molecular dissection of novel pathways involved in cancer. The avalanche of new approaches has lead to a considerable number of promising compounds. One of the disadvantages of DTC is that this low prevalent tumor is usually not included in initial clinical trials with these therapies. However, successful strategies that have survived these initial trials may well become available for thyroid carcinoma.

7.2.1 Conventional Chemotherapy

Although differentiated thyroid carcinoma is a low prevalent malignancy, many chemotherapeutic protocols that have been developed over the last decades for more common malignancies have been tried in progressive thyroid carcinoma. Overall, these approaches have been disappointing. Of the classical chemotherapeutic agents, adriamycin, alone or combined with cisplatin and bleomycin may induce temporary remissions or stationary disease in about 30-50% of the patients (164;209;20;21). The same has been reported for paclitaxel (210). Most remissions however, last only a few months and at the cost of a considerable reduction in quality of life, thus leading to the recommendation that there is no place in principle for chemotherapy (2;127).

7.2.2 Neovascularization

Molecular pathways involved in neovascularization have been demonstrated in DTC (211). The cascade of approaches to target tumor-induced neovascularization has led to a number of promising compounds that are now being tested in clinical trials in prevalent tumors. Reports have been published on beneficial effects of anti-VEGF antibodies in thyroid carcinoma cell-lines (212) and endostatin in animal experiments (213). A recently published clinical trial, including DTC patients was also successful (214).

7.2.3 Tyrosine kinase inhibitors

Another intriguing development is the advent of tyrosine kinase inhibitors. The development of imatinib mesylate (Gleevec) is prototypical for the innovative design of modern drugs with the molecular pathogenic defect as a starting point. Following imatinib, other small molecules have been developed, aimed at other tyrosine kinase activated pathways such as the epithelial growth factor receptor (EGFR) activated pathway (13;215). Activation of tyrosine kinase pathways is relevant for thyroid carcinoma. Several studies have been published reporting successful treatment with the tyrosine kinase inhibitors aimed at RET, VEGF or the EGFR (216-218).

7.2.4 PPAR γ agonists

An interesting new class of drugs is agonists of PPARγ. These drugs have been introduced as anti-diabetic agents. Their proposed mechanism is the differentiation

of pre-adipocytes into adipocytes, thereby increasing the fatty-acid storing capacity of adipose tissue. The involvement of PPARγ in differentiation processes extents beyond the area of adipose tissue. Indeed, altered expression of PPARγ and in vitro beneficial effects of PPARγ agonists have been described in a number of malignancies. In DTC, these compounds influence differentiation (219) induced apoptosis in thyroid tumors and prevented their growth in nude mice (220). In a recently published clinical study, rosiglitazone induced RaI uptake in DTC (219).

7.2.5 Radionuclide therapy

The expression of somatostatin receptors by DTC makes these tumors candidates for SRS based therapy. Recent studies have reported moderate effects of indium labeled octreotide (221) and promising effects of lutetium octreotate (222).

8. Scope of the present thesis

In the present thesis, questions regarding the diagnosis, follow-up and therapy of DTC will be addressed. These questions arise from the imperfections of current practice in DTC in which the lack of a centralized approach together with the low incidence and good prognosis have until recently prevented the introduction of optimalized diagnostic, therapeutic and follow-up protocols.

In **Chapter 2** we describe a study aimed at the improvement of the microscopic distinction between follicular thyroid lesions using antibodies against Galectin-3 (Gal-3), HBME-1, cytokeratin (CK)-19, CITED-1, Fibronectin (FN)-1, PPARγ and cytoplasmic NIS (cNIS). We therefore studied 156 thyroid tissues, using Receiver Operator Curve (ROC) analysis and the use of hierarchical cluster analysis.

In **Chapter 3** we describe an investigation aimed at the optimalization of serum Tg measurements in the follow up of DTC by defining institutional cut-off levels in 366 consecutive patients with DTC, who had all been treated according to the same protocol for initial therapy and follow-up. In addition, the prognostic values of serum Tg for cure and death, measured at fixed time points after initial therapy were studied as well.

In **Chapter 4** we investigate the effects of triiodothyronin (T3) on iodine uptake and expression of the sodium iodide symporter (NIS) in the rat thyroid cell line FRTL-5. This study was conducted, because some reports suggest that RaI uptake after rhTSH is inferior to thyroid hormone withdrawal.

In **Chapter 5** we evaluate the additional value of lithium therapy on the clinical outcome of RaI in 12 patients with insufficient effects of RaI. In addition, we studied the potential mechanism of he supposed beneficial effect of lithium salts on the uptake of RaI in the benign rat thyroid cell line FRTL-5, in the polarized non-thyroid MDCK cell-line stably transfected with hNIS to study lithium in a non-thyroid background and the human follicular thyroid carcinoma cell line FTC133-hNIS to study lithium effects in a background of DTC.

In **Chapter 6** we describe the results of an open prospective intervention study involving 12 patients with metastases of DTC, to evaluate the effects of 6-weeks treatment with the RXR agonist Bexarotene on the uptake of RaI.

In **Chapter 7** we describe the results of subsequent high dose RaI therapy in the patients in whom the diagnostic study with Bexarotene revealed increased RaI uptake. Eight patients received 7400 MBq RaI. The results of RaI were evaluated 6 months later, using CT scans and Tg measurements as outcome parameters.

Finally, in **Chapter 8** the results of the present thesis are summarized and put into perspective, which is also translated into Dutch in **Chapter 9**.

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Chapter 2

Combined Immunostaining with Galectin-3, Fibronectin-1, CITED-1, HBME-1, Cytokeratin-19, PPAR-gamma and NIS Antibodies Increases the Diagnostic Accuracy in the Differential Diagnosis of Thyroid Neoplasms

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Abstract

Background: The microscopic distinction between benign and malignant thyroid lesions is often difficult because in particular follicular lesions share many histological features.

Aim: This study was performed to evaluate the diagnostic value of Galectin-3 (Gal-3), HBME-1, cytokeratin (CK)-19, CITED-1, Fibronectin (FN)-1, PPAR-gamma (PPARγ) and cytoplasmic NIS (cNIS) staining in a large panel of thyroid neoplasms. Our study differed from earlier ones with regard to the identification of optimal semi-quantitative cut-off levels using Receiver Operator Curve (ROC) analysis and the use of hierarchical cluster analysis.

Methods: We used tissue arrays consisting of normal thyroid tissue (64), Graves disease (10), multinodular goiter (MNG, 14), follicular adenoma (FA, 12), papillary thyroid carcinoma (PTC, 53), follicular thyroid carcinoma (FTC, 13) and follicular variant of PTC (FVPTC, 11). Antibody staining was scored semi-quantitatively and differential expression was analysed in 2x2 tables and with hierarchical cluster analysis.

Results: In general, we found overexpression of FN-1, CITED-1, Gal-3, CK-19, HBME-1 and cNIS in malignant thyroid lesions. Gal-3, FN-1and cNIS had the highest accuracy in the differential diagnosis of follicular lesions. A panel of Gal-3, FN-1 and cNIS, identified by hierarchical cluster analysis had a 98% accuracy to differentiate between FA and malignant thyroid lesions. HBME-1 was found to be useful in the differentiation between FA and FVPTC (accuracy 88%).

Conclusion: A combination of antibodies increases the diagnostic value in the differential diagnosis of thyroid neoplasms. The combination of FN-1, Gal-3 and cNIS had the best accuracy (98%) whereas HBME-1 may be useful in the differentiation of FVPTC from FA.

Introduction

Although thyroid nodules are common, few are malignant and require surgical treatment. In particular, the microscopic distinction between follicular adenoma (FA), follicular thyroid carcinoma (FTC) and follicular variant papillary thyroid carcinoma (FVPTC)) is difficult because these follicular lesions share overlapping histological features. This is underscored by substantial inter-observer variability in the pathological and cytological assessment of thyroid nodules (1;2). As a result, up to 85% of patients with suspicious cytology who subsequently undergo surgery have benign lesions (3). Therefore, the identification of markers to distinguish benign from malignant tumours is important to avoid unnecessary surgery. In recent years, several immunohistochemical markers have been studied to improve the differential diagnosis of thyroid lesions.

The galectins are carbohydrate binding proteins involved in cell adhesion, cell growth and cell death. Galectin-3 (Gal-3) has been considered a marker with a high diagnostic potential to identify FTC (4-8), but in recent publications Gal-3 staining was also reported in benign lesions (9;10). HBME-1 (Hector Battifora mesothelial), is a monoclonal antibody developed against an unknown epitope of the microvillous surface of mesothelial cells and has been reported to be useful in the diagnosis of malignant thyroid tumours (11-15). Cytokeratins are intermediate filament proteins that are specific for epithelial cells. CK-19 has been found to be strongly and diffusely expressed in PTC, whereas it is heterogeneously expressed in FTC and absent or focally expressed in FA. However, CK-19 expression has also been reported in normal thyroid epithelium, Hashimoto's thyroiditis, and benign thyroid tumors (16-21).

Recent studies based on cDNA expression arrays have identified immunohistochemical markers for the differentiation between thyroid neoplasms. One study confirmed the differential expression of Gal-3 and also identified the extracellular matrix component Fibronectin-1 (FN-1) as a specific marker for PTC (22). In another study, Gal-3, FN-1 and the nuclear protein CITED-1 (*C*BP/p300-*I*nteracting *T*ransactivators with glutamic acid *[E]* and aspartic acid *[D]*-rich C-terminal domain) were found to be overexpressed in PTC (23). A combined approach using a panel of HBME-1, Gal-3 and CK-19 was followed by Casey et al (18), de Matos et al (19) and Prasad et al (24). In the study of de Matos et al, this combination had limited value. In contrast, in the study of Prasad et al (24) this panel had a high sensitivity and specificity for carcinomas.

In most of the before mentioned studies, fixed cut-off levels for positive staining are used. Therefore, we decided to evaluate the diagnostic value of Gal-3, HBME-1, CK-19, CITED-1, FN-1, the sodium iodide symporter (NIS) and peroxisome proliferator activated receptor gamma (PPARγ) in a large panel of thyroid neoplasms. We calculated cut-off levels for positive staining for each antibody using

receiver operator curve (ROC) analyses. We not only analyzed the diagnostic value of each individual antibody, but also the diagnostic accuracy of panels identified by hierarchical cluster analysis. We decided to include NIS and PPARγ because intracellular overexpression of NIS has been reported in a considerable percentage of malignant thyroid tumors (25). Apart from the pathophysiological implications, this expression pattern, if confirmed, may be helpful in the distinction between benign and malignant lesions. In the pathogenesis of thyroid tumors, decreased expression of PPAR-γ has been reported (26-28). Apart from the pathogenetic significance, PPAR γ may therefore also be used as a diagnostic marker.

Material and methods

Patients

One hundred and seventy seven histological samples from surgically removed thyroid lesions, representing 7 different histological thyroid disorders and adjacent normal thyroid tissue were obtained from the pathological archive of the Leiden University Medical Center, the Netherlands. We selected normal thyroid tissue (64), Graves disease (10), MNG (14), FA (12), PTC (53), FTC (13, minimally invasive 5) and FVPTC (11).

Tissue microarrays

Ten percent formalin-fixed, paraffin-embedded blocks routinely prepared from surgical specimens of thyroid tumours were selected for this study. Representative areas containing tumor or adjacent normal tissues were identified by a pathologist (HM). Triplicate tissue cores with a diameter of 0.6 mm were taken from each specimen (Beecher Instruments, Silver Springs, MD, USA) and arrayed on a recipient paraffin block, using standard procedures (29).

Immunohistochemistry

Four μm consecutive tissue sections were cut from each arrayed paraffin block and prepared on pathological slides. The sections were deparaffinised in xylene followed by 0.3% hydrogen peroxide methanol at room temperature for 20 minutes for blocking endogenous peroxidase. After rehydration, except for Gal-3, antigen retrieval treatment was done for CK-19, HBME-1, FN-1, CITED-1, NIS and PPAR-γ immunostaining by microwave treatment in 0.01 M citrate buffer at pH 6.0. After 2 hours cooling down, endogenous avidin activity blocking was performed for NIS immunostaining by incubation with egg-white for 5 minutes followed by biotin for 15 minutes. The sections were incubated with primary antibodies (specified in Table 1) in PBS with 1% bovine serum albumin overnight at room temperature. The negative controls were stained with the primary antibody omitted. Next, sections

Table 1. Specification of Antibodies

Primary antibody	Dilution	Manufacturer	Type	Secondary Antibody	Antigen Retrieval	Extra procedure
NIS	1:200	Donated by Nancy Carrasco, NY, USA	Polyclonal	1	Na-Citrate heating	Endogenous Avidin activity block
PPAR γ	1:30	Santacruz, California, USA (sc-7273)	Monoclonal	2	Na-Citrate heating	No
Galectin-3	1:200	Novocastra, Newcastle, UK (NCL-GAL3	Monoclonal	2	No	No
HBME-1	1:50	DacoCytomation Glostrup, Denmark (M3505)	Monoclonal	2	Na-Citrate heating	No
Fibronectin	1:2000	DacoCytomation, Glostrup, Denmark (A0245)	Polyclonal	1	Na-Citrate heating	No
CITED-1	1:100	Abcam, Cambridge, UK (ab15096)	Polyclonal	1	Na-Citrate heating	No
CK-19	1:100	DacoCytomation, Glostrup, Denmark	Polyclonal	1	No	No
Secondary Antibodies						
(1) Swine-anti-Rabbit	1:400	DacoCytomation, Glostrup, Denmark				
(2) Rabbit-anti-Mouse	1:200	DacoCytomation, Glostrup, Denmark				

were incubated for 30 minutes with either the biotinylated rabbit-anti-mouse conjugate (Dako, Glostrup, Denmark, 1:200) or swine-anti-rabbit (1:400), followed by incubation for 30 minutes with the streptavidin-biotin-peroxidase conjugate (Dako, Glostrup, Denmark 1:100). This step was by a 10-minute incubation with 3,3'-diaminobenzidinetetrachloride substrate in a buffered 0.05 M Tris/HCl (pH 7.6) solution containing 0.002% hydrogen peroxide. The sections were counterstained with haematoxylin.

Scoring

A semi-quantitative assessment of immunohistochemical scoring was performed according to both the intensity of staining and the percentage of positive cells. The criteria are summarized in Table 2. Score results for triplicate samples were summarized in one total score. The resulting score ranged from 1 – 6.

Table 2. Immunohistochemistry staining score levels according to proportion of positive cells and staining intensity

Cells with positive staining (%)	0	10	30	50	100
Intensity			*Score*		
Faint	0	1	2	3	4
Moderate	0	2	3	4	5
Intense	0	3	4	5	6

Statistical analyses

Statistical analyses were performed using SPSS 12.0. Staining scores were summarized and expressed as median and ranges and proportion of samples with scores above the cut-off level. Analyses of significant differences in staining scores were analyzed on a 2x2 base using the Kruskall-Wallis test. Optimal cut-off values for each antibody were identified using Receiver Operator Curve (ROC) analysis for each individual marker. Diagnostic validity was expressed using Bayesian statistics as sensitivity, specificity and accuracy.

In addition to individual protein markers, analysis of the diagnostic accuracy of panels of antibodies was performed using hierarchal clustering analysis of tissue microarray data using Cluster and TreeView (Cluster and TreeView 2.11, Eisen Lab, University of California at Berkely, California). A p value of <0.05 was considered significant.

Results

Protein expression in thyroid lesions

Because a distinct intracellular distribution was observed for some antibodies, their staining scores were categorized according to these patterns: NIS staining was differentially categorized as membranous (mNIS) or cytoplasmic (cNIS). Accordingly, FN-1 was also categorized as mFN-1 and cFN-1. Gal-3 was categorized as cGal-3 or nuclear Gal-3 (nGal-3).

The median values, ranges of expression of the proteins and the proportion of samples with staining scores above the cut-off levels are given in Table 2. Statistically significant differences in protein expression between all categories of thyroid tissues were investigated in 2x2 tables, the results of which are given in Table 3. Examples of staining patterns are given in Figure 1 (see color image at page 150).

In general, malignant tumors showed overexpression of Gal-3 (predominantly PTC), cFN-1 (all carcinomas), CK-19 (mostly PTC), HBME-1 (mostly PTC and FTC) and cNIS (mostly PTC and FTC). In contrast, expression of PPAR-γ and membranous NIS (mNIS) were low or absent in thyroid carcinomas.

In Graves disease, expression of mNIS was abundant as expected. PPAR-γ was also higher in adjacent normal tissues and benign thyroid lesions.

In general the most prominent differences were observed in PTC in comparison with benign lesions and adjacent normal thyroid tissues: PTC showed high expression levels of cFN-1 (median level 5, 96% of tumors), cGal-3 (median level 5, 92% of tumors), cNIS (median level 4, 83% of tumors), HBME-1 (median level 3, 74% of tumors), CITED-1 (median level 5, 98% of tumors) and CK-19 (median level 3, 78% of tumors) and absence of PPARγ and mNIS.

CK-19, Gal-3 and HBME-1 were differentially expressed between PTC and FTC.

FN-1, CK-19, Gal-3, HBME-1 and cNIS were differentially expressed between PTC and FVPTC (Table 4).

FTC had high expression levels of cFN-1 (median level 5, 86% of tumors), CITED-1 (median level 5, 86% of tumors) and cNIS (median level 4, 67% of tumors). In the comparison between FTC and FA, proteins differentially expressed were cFN-1 and cNIS (Table 4). No significant differences were observed in staining patterns between minimally invasive FTC and widely invasive FTC. In the comparison between FTC and FVPTC, the only differentially expressed protein was HBME-1 (Table 4).

FVPTC had a high expression of FN-1 (median level 4, 89% of tumors), CITED-1 (median level 5, 100% of tumors) and HBME-1 (median level 5, 89% of tumors)

Table 3. Protein expression in thyroid lesions

Protein	**Fibronectin 1**		**CITED-1**	**CK-19**	**Gal-3**		**HBME-1**	**NIS**		**PPAR-γ**
Cut-off level	*Cytoplasmic (1.5)*	*Membranous (0.5)*	*(3.0)*	*(1.5)*	*Cytoplasmic (2.5)*	*Nuclear (2.5)*	*(0.5)*	*Membranous (0.5)*	*Cytoplasmic (1.0)*	*(1.0)*
Normal Thyroid (64)	0(0-5) 14%	0(0-2) 2%	3(0-4) 51%	0(0-2) 4%	0(0-4) 10%	0(0-3) 2%	0(0-2) 2%	1(0-4) 59%	0(0-3) 12%	2(0-5) 56%
Benign lesions										
Graves(10)	0(0-4) 22%	0(0-0) 0%	4(2-5) 89%	0(0-0) 0%	1(0-2) 0%	0(0-3) 10%	0(0-0) 0%	6(2-6) 100%	0(0-0) 0%	3(2-4) 100%
MNG (14)	0(0-4) 15%	0(0-1) 8%	3(0-4) 79%	0(0-1) 0%	0(0-2) 0%	0(0-2) 0%	0(0-0) 0%	0(0-5) 45%	0(0-0) 0%	2(0-4) 64%
Benign tumors										
FA (12)	0(0-5) 40%	0(0-0) 0%	4(2-6) 80%	0(0-0) 0%	0(0-0) 0%	0(0-2) 0%	0(0-4) 11%	0(0-0) 0%	0(0-4) 22%	1(0-3) 50%
Malignancy										
PTC (53)	5(0-6) 96%	0(0-6) 40%	5(1-6) 98%	3(0-4) 78%	5(0-6) 92%	4(0-6) 80%	3(0-6) 74%	0(0-5) 11%	4(0-6) 83%	0(0-3) 10%
FTC (13)	5(0-6) 86%	0(0-3) 15%	5(0-6) 86%	0(0-1) 0%	0(0-5) 33%	0(0-5) 29%	0(0-6) 17%	0(0-0) 0%	4(0-5) 67%	0(0-6) 15%
FVPTC (11)	4(0-5) 89%	0(0-0) 0%	5(4-5) 100%	0(0-2) 22%	0(0-6) 33%	0(0-4) 33%	5(0-6) 89%	0(0-2) 11%	0(0-4) 33%	0(0-0) 0%

Classification of disorder (n)
MNG: Multinodular Goiter; FA: Follicular Adenoma; PTC: Papillary Thyroid Carcinoma; FTC: Follicular Thyroid Carcinoma; FVPTC: Follicular Variant PTC
Expressed as Median (Range) and proportion of samples with scores above the cut-off level for positivity.

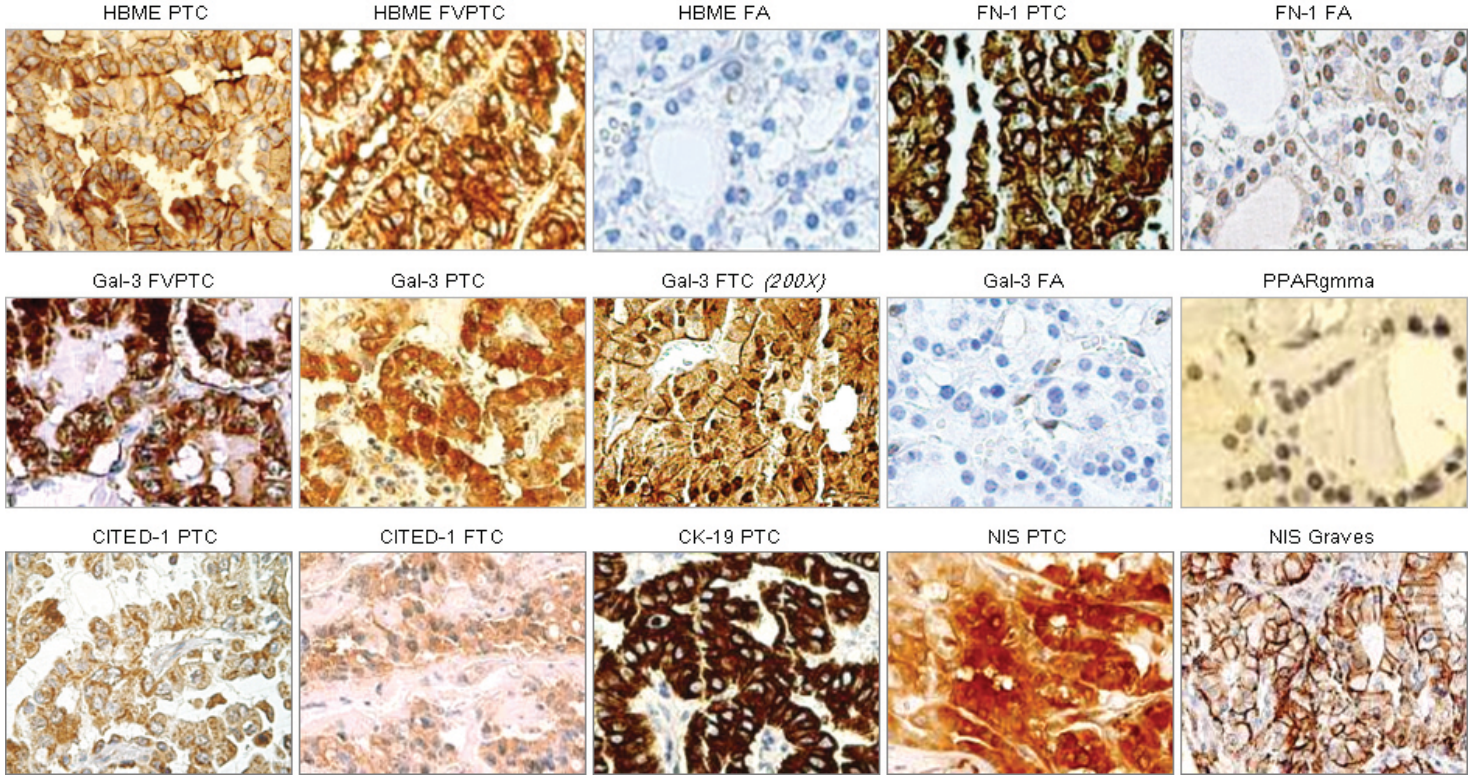

Figure 1. Immunostaining of thyroid tissues with HBME-1, Fibronectin-1 (FN-1), Galectin-3 (Gal-3), PPARγ, CITED-1, cytokeratin-19 (CK-19) and Sodium Iodide Symporter (NIS). Magnification was 200x. For immunohistochemical staining procedures, see Materials and Methods. HBME-1 gave membranous staining in Papillary Thyroid Carcinoma (PTC) and Follicular Variant PTC (FVPTC), and was absent in Follicular Adenoma (FA). FN-1 gave cytoplasmic staining in PTC. Gal-3 gave cytoplasmic or nuclear staining in thyroid carcinomas. PPARγ staining nuclear staining was observed in benign thyroid lesions. CITED-1 gave cytoplasmic staining in benign and malignant thyroid lesions. CK-19 was overexpressed in PTC. Cytoplasmic NIS was observed in FTC and PTC. Typical membranous staining was observed in Graves disease (see color image on page 150).

(Table 3). FVPTC differed from FA for cFN-1, PPAR-γ, HBME-1 and CK-19, whereas protein expression was different for FN-1, Gal-3, cNIS, HBME-1 and CK-19 in the comparison between FVPTC and PTC (Table 4).

Cytoplasmic NIS was mainly observed in PTC (median level 4, 83% of tumors) and FTC (median level 4, 67% of tumors) (Table 3). Remarkably, differences in CITED-1 were not prominent between benign thyroid tissues (median levels 3-4 in normal or benign lesions, with 51-89% of tissues positive) and malignant lesions (86-100% positive cases) in 2x2 comparisons.

Protein expression in follicular lesions

Because the clinical distinction between follicular lesions proves to be the most difficult, we focused our analyses on the diagnostic value of proteins found to be differentially expressed in follicular lesions (Tables 4 and 5). In 2x2 comparisons of the different follicular lesions, we first identified the optimal cut-off levels using ROC-analyses, aiming at the highest combination of sensitivity and specificity for each comparison. The cut-off values are given in Table 5. We subsequently calculated the percentages of correct diagnoses of both lesions in a 2x2 comparison as well as the accuracy, using these cut-off levels. The accuracy (total percentage of correct diagnoses) is the best indicator of the diagnostic or discriminating value of the antibody.

The highest accuracies were found in the discrimination between PTC and FVPTC, with the highest accuracy for cGal-3 (88%), cFN-1 (81%), CK-19 (78%) and cNIS (77%). In the comparison between FA and FTC, moderate accuracies were found for FN-1 (accuracy 71%) and cNIS (accuracy 65%). The distinction between FA and FVPTC had a high accuracy for HBME-1 (89%), PPAR-γ (74%) and FN-1 (74%). HBME-1 also gave a good discrimination between FVPTC and FTC (accuracy 84%).

Clustered expression pattern of Gal-3, FN-1 and cNIS distinguish benign thyroid tumors from thyroid carcinomas

To identify optimal combinations of antibodies, we performed an unsupervised hierarchical cluster analysis including all tissues and all antibodies.

The results of this analysis are given in Figure 2. We found that 3 antibodies, cGal-3, cNIS and cFN-1 had the highest discriminating power to cluster benign and malignant thyroid lesions: all malignant lesions had positive staining for at least 2 of the 3 antibodies cGal-3, cNIS and cFN-1 (Cluster 1), whereas lesions with absent staining for all 3 antibodies were all benign (Cluster 5). All malignancies were combined because the subgroups were too small to allow separate cluster analysis. Initially, there was only one case of FA in cluster 1. Strikingly, after rechecking whole section histological H&E slides, this adenoma was identified harbouring classic focal papillary carcinoma and re-categorized.

Table 4. Proteins differently expressed between thyroid lesions

Diagnosis	**Normal**	**MNG**	**Graves**	**FA**	**PTC**	**FTC**
MNG	nGal-3**					
Graves	cCITED-1* cGal-3* nGal-3** mNIS** PPARγ*	mNIS** PPARγ*				
FA	cFN-1* CITED-1** mNIS**	mNIS* cGal-3*	cGal-3* mNIS* PPARγ*			
PTC	cFN-1** mFN-1** cCITED-1** nCITED-1** CK-19** cGal-3** nGal-3** HBME-1** mNIS** cNIS** PPARγ**	cFN-1** mFN-1* cCITED1** CK-19** cGal-3** nGal-3** HBME-1** cNIS** mNIS** PPARγ**	cFN-1** mFN-1* cCITED-1* CK-19** cGal-3** nGal-3** HBME-1** mNIS** cNIS** PPARγ**	cFN-1** mFN-1* CK-19** cGal-3** nGal-3** HBME-1* cNIS** PPARγ**		
FTC	cFN-1** mFN1* cCITED-1** nGal-3* HBME-1* mNIS** cNIS** PPARγ*	cFN-1** CITED-1* cNIS** mNIS* PPARγ*	cFN-1* mNIS** cNIS* PPARγ**	cFN-1* cNIS*	CK-19** cGal-3** nGal-3** HBME-1**	
FVPTC	cFN1 ** cGAL-3* nGAL-3** HBME1 ** mNIS* CK-19* CITED1* PPARγ*	cFN1** cCITED1** HBME1** cNIS* PPARγ*	cFN-1** cCITED-1** HBME-1** mNIS** PPARγ**	cFN1* CK-19* nGAL3* HBME1** PPARγ*	cFN-1* mFN-1* CK-19** cGal-3* nGal-3* HBME-1* cNIS*	HBME-1*

MNG: Multinodular Goiter; FA: Follicular Adenoma; PTC: Papillary Thyroid Carcinoma;
FTC: Follicular Thyroid Carcinoma; FVPTC: Follicular Variant PTC
Gal-3: Galectin 3 (c=intracellular, n=nuclear); NIS: sodium iodide symporter (m=membranous);
FN-1: Fibronectin1; CK-19: cytokeratin 19

* $p<0.05$ ** $p<0.001$

Table 5. Diagnostic value of proteins differentially expressed in follicular thyroid lesions

I / II	Protein	Cut-off value	FA			PTC			FTC		
			Correct (I)	Correct (II)	Accuracy	Correct (I)	Correct (II)	Accuracy	Correct (I)	Correct (II)	Accuracy
FVPTC	*PPAR γ*	0	100	50	74						
	HBME-1	1	89	90	89	73	11	63	80	89	84
	CK-19	1.5	100	22	63	78	78	78			
	cGal-3	2				94	56	88			
	nGal-3	2	80	44	63	88	56	82			
	cFN-1	2	60	89	74	95	11	81			
	mFN-1	1				40	100	50			
	cNIS	2				80	67	77			
FTC	*cFN-1*	2	60	83	71						
	cNIS	2	60	78	65						

FA: Follicular Adenoma; PTC: Papillary Thyroid Carcinoma; FTC: Follicular Thyroid Carcinoma; FVPTC: Follicular Variant PTC
Gal-3: Galectin 3 (c=intracellular, n=nuclear); NIS: sodium iodide symporter (m=membranous); FN-1: Fibronectin1; CK-19: cytokeratin 19

Table 6. Combined intracellular expression of Fibronectin (FN), Galectin 3 (Gal-3) and NIS in thyroid lesions

		All Malignant Thyroid Tumors						
		One-Antibody			Antibodies Combined			
	Antibody	Sensitivity of Malignancy (%)	Specificity of Malignancy (%)	Accuracy (%)	**co-expression**	Sensitivity of Malignancy (%)	Specificity of Malignancy (%)	Accuracy (%)
All Benign Thyroid Tissues	*cNIS*	72	90	82				
	cFN-1	92	82	86	*Two antibodies positive:*	97	100	99
	cGAL-3	75	94	85				
Follicular Adenoma	*cNIS*	69	88	71				
	cFN-1	92	56	88	*Two antibodies positive:*	97	100	98
	cGAL-3	78	100	81				

Gal-3: Galectin 3 (c=intracellular, n=nuclear); NIS: sodium iodide symporter (m=membranous);
FN-1: Fibronectin1; CK-19: cytokeratin 19

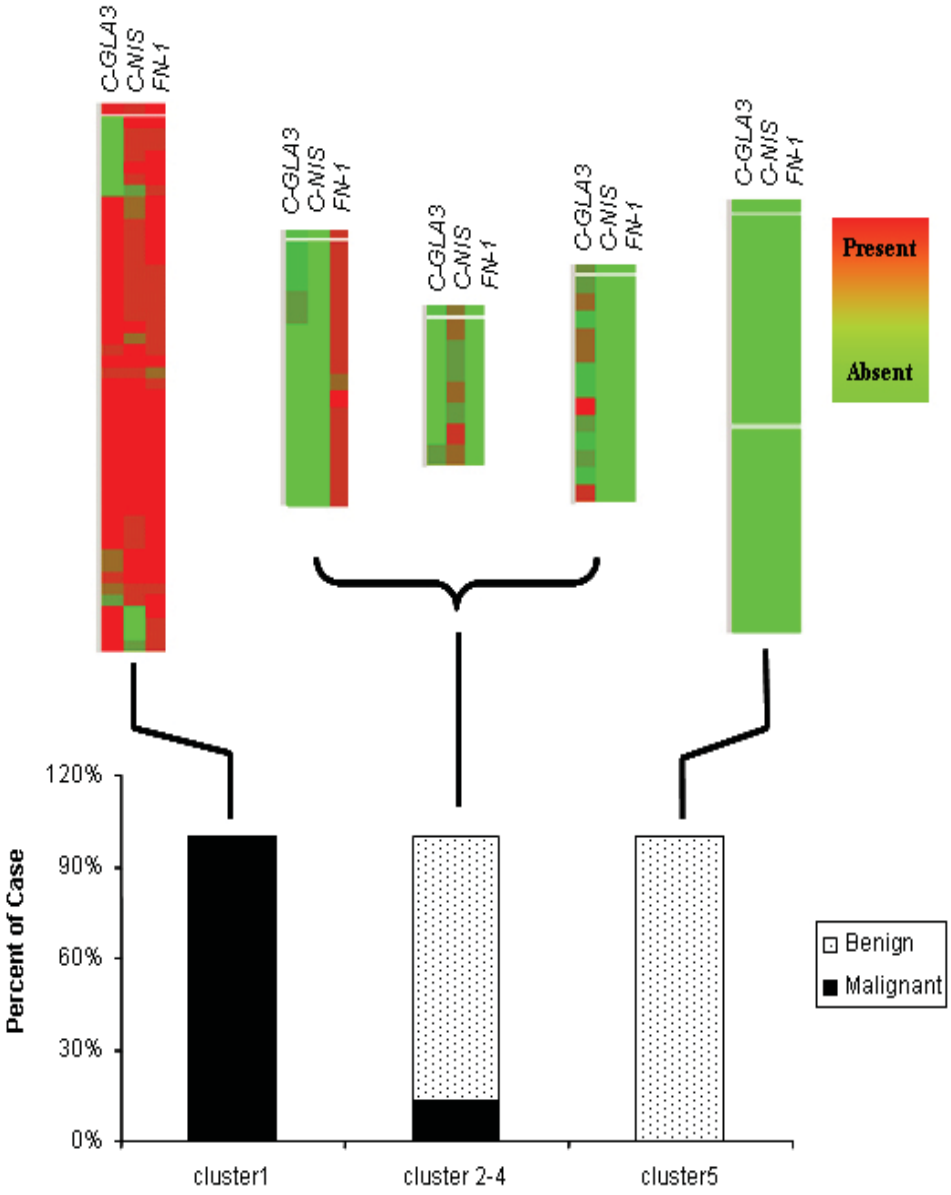

Figure 2. Hierarchical cluster analyses using 7 antibodies in all thyroid tissues. cNIS, FN-1 and Gal-3 were identified as the best predictors of benign or malignant thyroid lesions. Presence of 2 of these antibodies (Cluster 1) gave an almost 100% clustering of malignant thyroid lesions, whereas absence of these proteins (Cluster 5) was suggestive of benign thyroid lesions.

We therefore used the combined staining patterns of these antibodies to discriminate between benign and malignant thyroid lesions and FA and malignant thyroid lesions (Table 6). We found that positive staining for 2 of the 3 antibodies cFN-1, cGal-3 and cNIS had a high sensitivity (97-98%) and high specificity for thyroid carcinoma (100%).

Discussion

The present study was performed to evaluate the diagnostic value of Gal-3, HBME-1, CK-19, CITED-1, FN-1, PPAR-γ and NIS staining in a large panel of thyroid neoplasms, focussing on the differential diagnosis of follicular thyroid lesions.

Our study differed from earlier ones with regard to the identification of optimal semi-quantitative cut-off levels using ROC analysis and the use of hierarchical cluster analysis.

We initially analyzed differentially expressed antibodies comparing all thyroid tissues. In general, we found overexpression of FN-1, CITED-1, Gal-3, CK-19, HBME-1 and cNIS in thyroid carcinomas, whereas membranous NIS and PPARγ showed decreased expression in carcinomas in comparison with benign thyroid tissues.

The most challenging differential diagnosis is between FA and thyroid carcinoma. We found all proteins to be differentially expressed between FA and PTC. The differences between FA on the one hand and FTC and FVPTC on the other hand were less prominent, but we found a differential expression of PPARγ, HBME-1, Gal-3, cNIS and FN-1. We could not confirm the differential expression of CITED-1 and CK-19 between FA, FVPTC and FTC as reported by Prasad et al (24).

CK-19 is the most commonly used cytokeratin in investigating thyroid lesions. We and others found that CK-19 is relatively specific for PTC (16;18;19). However, in our analyses CK-19 has limited use in the differential diagnosis of follicular thyroid lesions. This has also been reported by Sahoo et al (17). In the study of Prasad et al (24), CK-19 had a sensitivity of 64% for thyroid carcinoma.

Several recent studies have reported that HBME-1 expression is a useful diagnostic marker for PTC (23;24). We found HBME-1 expression predominantly in PTC and FVPTC and in a limited number of FA with relatively high accuracy. Therefore, HBME-1 may indeed be useful in the differential diagnosis of FVPTC and FA (accuracy 88%).

We found CITED-1 expression both in benign and malignant thyroid lesions. Although the highest proportion of positive samples was found in PTC (as

previously reported (23;24)) and in FVPTC, the considerable proportion of positive samples in benign lesions makes CITED-1 in our opinion a less attractive marker for differential diagnosis.

Gal-3 was predominantly expressed in PTC (92%) and to a lesser extent in FTC and FVPTC. Other investigators have used Gal-3 in differentiating FTC from FA in fine-needle aspirates (7), however Gal-3 was also reported in benign thyroid lesions (10). We found a reasonable accuracy (88%) in the differential diagnosis between FA and FVPTC for Gal-3. We also found Gal-3 to be a useful marker in a panel of antibodies.

FN-1 was first reported to be overexpressed in PTC (22;23). In a subsequent study, FN-1 appeared to be a valuable marker for the differentiation of FA and thyroid carcinomas (24). The percentage of FA (40%) positive for FN-1 in our study was higher than reported by Prasad et al (24). We found accuracies of 74% for the differentiation between FA and FVPTC and 71% for the differentiation between FA and FTC. Cluster analysis also identified FN-1 as a useful marker.

Although some studies report decreased NIS protein expression in thyroid carcinoma (30), Dohan et al reported cytoplasmic overexpression of NIS in a large series of human thyroid cancers (25). We confirmed cytoplasmic NIS overexpression in PTC (83% of tissues) and FTC (67% of tissues). As we used the same antibody as Dohan et al. it may well be that the differences with other studies are related to differences in antibody specificity. Nevertheless, the differential expression of cNIS between subtypes of thyroid neoplasms makes it a candidate for differentiating between these lesions. The accuracies of 68% (FA vs. FTC) and 77% (PTC vs. FVPTC) however are moderate. Cytoplasmic NIS was also identified by cluster analysis as a potential useful marker in the discrimination between FA and malignant carcinomas.

PPARγ has found to be downregulated in experimental models of thyroid carcinoma (26-28). The importance of the downregulation of PPARγ is also illustrated in the PPARγ/PAX8 rearrangement (31) which was initially observed in a series of FTC. Although the PPARγ/PAX8 rearrangement was therefore considered a specific marker for FTC, later studies also reported the rearrangement in benign thyroid lesions (32;33). We found decreased PPARγ nuclear staining in malignant tumors, whereas in non-malignant lesions, the percentage of positive cells varied from 50-100%. Although our results confirm the decreased expression of PPARγ in thyroid carcinoma, the diagnostic accuracies for the differentiation between follicular lesions were limited.

As no marker in itself has a superior diagnostic value, a combination of markers may be more accurate than any single marker. We performed a cluster analysis including all tissues and antibodies. To our knowledge, this has not been done before for immunohistochemistry in thyroid lesions. In this study, hierarchical clustering analysis on all valid samples confirmed that thyroid carcinomas, FA and benign lesions could

be categorized with high sensitivity, specificity and accuracy. Our study shows that a diagnostic immunohistochemical panel comprising Gal-3 and FN-1 was 97% sensitive for all thyroid carcinomas, whereas specificity was 100%. The diagnostic values of CK-19, CITED-1 and HBME-1 in our series were not sufficient to be included in the panel, which is in line with the results of de Matos et al (19). However, HBME-1 was found to be a useful marker for the differentiation between FA and FVPTC. Because the number of FVPTC was small, hierarchical clustering did not allow a separate analysis of this group of tumors. Prasad et al. also found a limited accuracy for HBME-1, CK-19 and CITED-1 (24).

In conclusion, Gal-3, FN-1 and cNIS is a useful diagnostic panel in the differential diagnosis of thyroid lesions. The absence of Gal-3, FN-1 and cNIS is highly suggestive for a benign lesion. HBME-1 may be useful in the specific differentiation of FVPTC from FA.

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Chapter 3

Serum Thyroglobulin Concentrations Predict Disease-free Remission and Death in Differentiated Thyroid Carcinoma

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Abstract

Objective: Most studies on the diagnostic value of serum thyroglobulin (Tg) concentrations in differentiated thyroid carcinoma (DTC) use fixed cut-off levels in heterogeneous groups of patients with respect to initial therapy and do not provide prognostic data. The objective of this study was to investigate the prognostic values of serum Tg for disease free remission and death, measured at fixed time points after initial therapy using receiver operator curve (ROC) analyses.

Design: Single-centre observational study with 366 consecutive patients with DTC, who had all been treated according to the same protocol for initial therapy and follow-up.

Methods: Tg concentrations were measured at five fixed time points after initial surgery. Tg cut-off values with highest accuracy were calculated with ROC analyses.

Results: During follow-up of 8.3±4.6 years, 84% of the patients were cured.

Pre-ablative Tg levels were an *independent* prognostic indicator for disease free remission (Tg cut off value 27.5 ug/L, positive predictive value 98%). Highest diagnostic accuracies of serum Tg for tumour presence were found during TSH stimulated Tg measurements, 6 months after initial therapy (Tg cut-off value 10 ug/L: sensitivity 100%, specificity 93%).

DTC related mortality was 14%. TSH stimulated Tg levels before ablation and 6 months after initial therapy were independent prognostic indicators for death.

Conclusion: Optimal institutional Tg cut-off levels for diagnosis and prognosis should be defined using ROC analyses for each condition and time point. Tg measurements 6 months after initial therapy during TSH stimulation had an excellent diagnostic value. Tg levels are independent prognostic indicators for disease free remission and death. Using this strategy, high-risk patient groups can be selected based on Tg levels, in addition to conventionally used prognostic indicators.

Introduction

Differentiated thyroid carcinoma (DTC) has an excellent prognosis with 10-year survival rates of 85-93 % (1). The purpose of follow-up protocols in DTC is the early detection of tumour recurrence or metastatic disease in order to optimize additional treatment. Most patients during follow up have been cured definitively, and, as a consequence, have a low pre-test probability for recurrent disease. Therefore, the sensitivity of the diagnostic test must be adequate to detect the few patients with evident thyroid carcinoma, whereas specificity must also be high to avoid unnecessary treatments in patients without recurrent disease. In addition, the burden of diagnostic tests for the patient should be kept at a minimum.

Serum thyroglobulin (Tg) measurements are the cornerstone in the follow-up in DTC. Numerous studies have been performed on the diagnostic value of Tg measurements. We recently published a structured meta-analysis on the diagnostic value of Tg including 46 articles (2). The interpretation of many studies on Tg performed so far is difficult, because in most studies i) Heterogeneous patient groups with respect to initial therapy are included, ii) The time points of Tg measurements after diagnosis are not clearly indicated, and iii) Fixed Tg cut-off levels are used, without receiver operator curve (ROC) analyses. The application of ROC data is essential, as a chosen cut-off level is a subjective choice based on the balance between a desired percentage of missed recurrences versus unnecessary therapies. Therefore, in a recent European consensus paper, it was recommended to define institutional Tg cut-off levels (3). Only a few studies have been published on the interpretation of Tg levels during follow up of DTC using ROC analyses. However, in those studies, heterogeneous patient groups were included and the time-points of Tg measurements were not clearly indicated (4;5;6). In addition, most studies provide data on the diagnostic value of Tg for tumour presence, but do not give data on the *prognostic* significance for disease free remission or death. One large study (7) studied the prognostic significance of 1-month post-surgical Tg levels and found a significant prognostic cut-off level of 10 ug/L. The few studies that were published on the prognostic significance of Tg measurements used fixed cut-off levels, contained selected subgroups of patients and included either Tg measurements at one time point or at undefined time points (8;9;10;11;12).

We therefore performed a study on the diagnostic and prognostic value of Tg in a homogeneous group of DTC patients with respect to initial therapy, using Tg measurements at 5 defined time-points after diagnosis, in combination with ROC analyses. In addition, we studied the diagnostic and prognostic value of Tg antibodies for tumour presence or death.

Patients and methods

Three-hundred-and-sixty-six consecutive patients were included in the study. These patients had received initial therapy for DTC between January 1986 and January 2000. All follow up data were collected until January 1, 2003. January 1986 was chosen as a starting date, because from that date forward, all relevant patient data were registered in a computerized database. Initial surgery and radioiodine ablation therapy were performed at the Leiden University Medical Centre or at one of the connected general hospitals. All hospitals are affiliated in the Regional Comprehensive Cancer Centre, using the same standardized protocol for the treatment and follow-up of DTC (Table 1).

Table 1. Patient characteristics

Parameter	N	Cured Patients N (%)	Patients Relapse after Cure N (%)	Deaths N (%)
Total	366	305 (84)	46 (13)	52 (14)
Gender (Male/ Female)	91 / 275	72 (80) /233 (85)	13 (14)/33 (13)	13 / 39 (14 / 14)
Stages				
T1	22	21 (96)	1 (5)	0 (0)
T2	188	176 (94)	17 (9)	10 (5)
T3	56	51 (91)	9 (16)	8 (14)
T4	96	53 (55) * #	17 (18)	32 (33) * #
T unknown	4	0 (0)	0 (0)	2 (50)
N1	107	76 (71) *	15 (14)	22 (21) *
M1	52	19 (36) * #	6 (11)	27 (54) * #
Histology				
Papillary	203	173 (86)	28 (14)	25 (12)
Follicular	72	58 (81) *	11 (15)	17 (24) *
Follicular variant papillary carcinoma	68	56 (82)	5 (7)	6 (9)
Hürthle Cell	23	18 (78)	2 (9)	4 (17)
Age (continuous)				
< 55 yr	210	221 (95)* #	18 (8)	3 (1)* #
> 55 yr	156	84 (64)* #	28 (21)	49 (31)* #

* Significant at univariate analysis
# Significant at multivariate analysis (see Table 4)

All patients were treated by near-total thyroidectomy, followed by routine radioiodine ablative therapy with 2800 MBq I-131.

Follow-up was performed according to a standard protocol. Serum Tg levels were measured at the following time points: 1) After initial surgery during thyroxin withdrawal just before radioiodine ablation, 2) Six months after initial surgical therapy during thyroxin therapy, 3) Six months after initial surgical therapy after thyroxin withdrawal ("off") and 4) Yearly during thyroxin therapy. Although additional TSH stimulated Tg measurements were performed in selected subgroups of patients at other time points after initial therapy, we did not include those data as these tests were not uniformly done in all patients, and calculations of diagnostic values would thus be biased. Thyroxin therapy was aimed to suppress TSH levels (below 0.1 mU/L). Six months after initial therapy a diagnostic 185 MBq I-131 scintigraphy was performed after thyroxin withdrawal.

Tumour presence during follow-up was defined as histologically or radiologically (X-ray, CT-scan, MRI-scan, FDG-PET scan or I-131 scintigraphy) within a 1-year interval before or after the time of Tg measurements. Although we realize that Tg is considered the best parameter for tumour presence, Tg was not used as a golden standard for tumour presence, as the diagnostic value of Tg was the subject of this study.

Disease free remission was defined as the absence of thyroid carcinoma for a minimum of 3 years according to the above mentioned parameters.

The following data were registered: age at diagnosis, sex, date of diagnosis, histology, TNM stage, date of cure, date of recurrence, tumour localization, death cause, Tg levels, TSH levels, Tg antibody levels and date of last follow up or death. TNM stage was registered according to the 5th edition (13). This was done because most patients were analysed before the latest edition of the TNM classification. We used the following end-points of follow-up: date of death (82 patients), date of emigration (12 patients) and date of most recent contact (272 patients).

Death causes were analysed in all 82 patients who had died during follow-up. Death cause was investigated using medical records, death certificates, enquiries with other physicians involved in the treatment of each patient, enquiries in other hospitals, enquiries with general practitioners and autopsy findings. Death causes were divided into thyroid cancer related death and other causes.

Analyses were performed in evaluable patients defined as patients in whom all of 4 conditions were fulfilled: Alive at the time-point of Tg measurement, documented serum Tg measurements, documented serum TgAb measurements *and* documented golden standard parameters for presence or absence of disease. If Tg-antibodies were present, the Tg measurement at this time was excluded from the calculations because of possible interference with the Tg assay. The numbers of these patients are given in Table 2.

Table 2. Diagnostic Values of Serum Tg Measurements for Active Tumour Calculated with Receiver Operator Curve Analysis. Patients with Tg antibodies were excluded

		Evaluable patients (N) #	Positive TgAb N (% of Evaluable Patients)	Tumor Location	Patients with Tumor N (% of Patients Negative TgAb)	Tg Cut-Off µg/L	Sensitivity (%)± SE	Specificity (%)± SE	PPV (%)	NPV (%)
Pre-Ablation		*304*	82 (27.0)	All	33 (15.1)	27.5	87.9 ± 5.7	90.3 ± 2.2	61.7	97.7
				Distant Metastases	21 (9.6)	27.5	85.7 ± 7.6	85.3 ± 2.5	38.3	98.2
Six Months After Initial Therapy	*Suppressed TSH*	287	79 (27.5)	All	37 (18.0)	2.5	89.2 ± 5.1	93.5 ± 2.0	75.0	97.5
				Distant Metastases	24 (11.7)	2.5	87.5 ± 6.8	87.3 ± 2.5	47.7	98.1
	Stimulated TSH	287	79 (27.5)	All	37 (18.0)	10.0	100.0 ± 0.0	93.1 ± 2.1	76.7	100.0
				Distant Metastases	24 (11.7)	10.0	100.0 ± 0.0	86.0 ± 2.8	48.8	100.0
Two Years After Initial Therapy	*Suppressed TSH*	244	32 (13.1)	All	43 (20.6)	2.0	85.0 ± 5.4	85.7 ± 2.7	60.6	95.7
				Distant Metastases	33 (15.8)	2.0	72.7 ± 7.8	88.6 ± 2.4	54.5	94.5
Five Years After Initial Therapy	*Suppressed TSH*	182	23 (12.6)	All	35 (22.6)	2.5	82.9 ± 6.4	96.7 ± 1.6	87.9	95.1
				Distant Metastases	30 (19.4)	2.5	83.3 ± 6.8	93.6 ± 2.2	75.8	95.9

Patients who were alive at the time points of measurements and in whom both Tg, TgAb and documentation of disease state according to the criteria for golden standard (see Methods) could be evaluated. PPV= positive predicted value, NVP = negative predicted value

Measurements of Tg and Tg-AB

Until January 1997 serum Tg was measured using an immunoradiometric assay (IRMA), the Dynotest TG (Brahms Diagnostica GmbH, Germany) with a sensitivity of 0.3 μg/L. From January 1997, the Dynotest TG-s (Brahms Diagnostica GmbH, Germany) was used, with a sensitivity of 0.05 μg/l. Inter-assay variability of 0.3 μg/l. The comparability of the 2 methods is excellent: R^2: 0.99, slope 0.99, intercept 0.09 (14). Serum Tg-antibodies were also measured at these specific time points by the Ab-HTGK-3 IRMA (DiaSorin Biomedics, Italy).

Statistical analyses

Data are presented as mean ± SD. All statistical analyses were performed using SPSS for windows version 12.0 (SPSS Inc., Chicago, IL). Data are expressed as number of patients (percentages), as mean ± Standard Deviation (SD) or as median (range). Receiver operator curves (ROC) were used to find the cut-off value with highest accuracy. Prognostic indicators for recurrence or death were calculated using univariate- and multivariate Cox-regression analyses: Indicators that were identified as significant for survival in univariate analysis were entered into a stepwise multivariate model. A p-value of < 0.05 was considered significant.

Results

Characteristics of the patients are shown in Table 1. Mean age at time of surgery was 48 ± 18 years. Mean follow-up was 8.3 ± 4.6 years. Significant prognostic factors for disease free remission and death are given in Table 4.

Diagnostic value of Tg

The diagnostic values of Tg measurements at the different time points are given in Table 2.

The diagnostic value of Tg before ablation therapy was reasonable in our analysis, with a sensitivity of 87.9% and a specificity of 90.3% at a cut-off value of 27.5 ug/L.

When a cut-off level of 2 ug/L was used, sensitivity increased to 93.9%, whereas specificity dropped to 45% with a positive predictive value of only 23% instead of 62%, with similar negative predictive value.

The highest diagnostic value of Tg was found during TSH stimulated Tg measurements 6 months after initial therapy (see Figure 1). The Tg cut-off value with highest accuracy was 10.0 ug/L, with sensitivity and specificity of 100.0 and

93.1%, respectively. When the more commonly used cut-off value of 2 ug/L was used, sensitivity remained similar, but specificity dropped to 82% with a positive predictive value of only 54%, instead of 73% (Figure 1). We analysed the course of 9 patients with Tg values > 10 ug/L, 6 months after initial therapy during TSH stimulation: in 3 patients, tumour was detected 2-5 years after initial therapy. In 4 patients Tg became undetectable and they were cured. Two patients had persistent measurable Tg, but no tumour was detectable up to 15 years after initial therapy.

Tg measurements on thyroxin, 2 and 5 years after initial therapy had lower sensitivities, but had comparable specificities and negative predictive values albeit at lower Tg cut-off values.

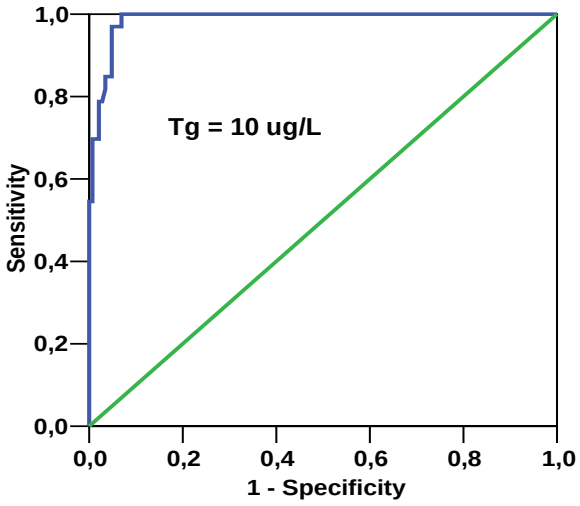

Figure 1. Receiver Operator Curve six months after initial therapy during stimulated TSH to obtain optimal cut-off levels of serum Tg measurements for the diagnosis of active tumour in patients with differentiated thyroid carcinoma.

Prognostic value of Tg

Disease free remission

The prognostic value of Tg for disease free remission is given in Tables 3 and 4. Interestingly, Tg before ablation had a high predictive value of 97.8% for disease free remission at a cut-off value of 27.5 ug/L. Tg appeared to be an independent prognostic marker for disease free remission (likelihood ratio for disease free remission 43.2 for Tg < 27.5 ug/L, $p<0.001$), irrespective of T4, M1 and age.

Thyroid Specific Death

The prognostic values for Tg measurements for DTC related death are given in Tables 3 and 4 and Figure 2. The negative predictive value was high for all time-points of Tg measurements.

Tg was an independent predictor for thyroid related death during TSH stimulation, 6 months after initial therapy (hazard ratio for Tg ≥ 10.0 ug/L 10.9, p=0.008, Table 4, Figure 2), 2 years after initial therapy (hazard ratio for Tg ≥ 2.0 ug/L 12.9, p<0.001) and 5 years after initial therapy (hazard ratio for Tg ≥ 2.0 ug/L 29.1, p=0.001).

Table 3. Prognostic Value of Serum Tg Measurements for Disease free remission and Thyroid Carcinoma Related Death. Patients with Tg antibodies were excluded

		Outcome	Tg µg/L Cut-Off	Sensitivity (%) ± SE	Specificity (%±) SE	PPV (%)	NPV (%)
Pre-Ablation		Disease free remission	27.5	84.4 ± 2.6	88.9 ± 5.6	97.8	49.1
		Death	21.5	66.7 ± 9.6	81.3 ± 2.8	30.2	95.3
Six Months After Initial Therapy	*Suppressed TSH*	Death	2.5	72.0 ± 9.0	85.7 ± 2.6	40.9	95.7
	Stimulated TSH	Death	10.0	85.0 ± 8.0	83.5 ± 2.9	39.5	97.8
Two Years After Initial Therapy	*Suppressed TSH*	Death	2.0	85.0 ± 8.0	85.7 ± 2.5	38.6	98.2
	Suppressed TSH	Death	2.0	82.4 ± 9.2	92.8 ± 2.2	58.3	97.7

PPV= positive predictive value, NVP = negative predictive value

Tg antibodies

The percentage of patients with Tg antibodies dropped from 27% immediately after initial surgery to 12% 5 years after initial therapy (see Table 2). There were no significant differences in tumour presence between patients with and without Tg antibodies: 15 – 23% in patients without Tg antibodies and 16 – 33% in patients with Tg antibodies. The presence of Tg antibodies did not have a significant prognostic for disease free remission or death.

Table 4. Likelihood Ratios for Serum Tg values for Outcome (Disease free remission or Thyroid Carcinoma Related Death) as calculated with Cox-survival Analysis. Patients with Tg antibodies were excluded

		Outcome	Univariate Analysis		Multivariate Analysis		Other Significant Parameters
			p	Likelihood Ratio (CI)	p	Likelihood Ratio (CI)	
Pre-Ablation		Disease free remission	<0.001	43.2 (15.0 – 124.3) #	<0.001	29.9 (5.2 – 171.5) #	Age, M1, T4
		Death	<0.001	8.0 (3.4 – 18.7)*	N.S.	--	Age, M1, T4
Six Months After Initial Therapy	*Suppressed TSH*	Death	<0.001	14.4 (5.7 – 36.7) *	N.S.	--	Age, T4
	Stimulated TSH	Death	<0.001	31.2 (7.1 – 136.7) *	0.008	10.9 (1.9 – 63.5) *	Age, T4
Two Years After Initial Therapy	*Suppressed TSH*	Death	<0.001	30.9 (9.0 – 105.7) *	<0.001	12.9 (3.4 – 49.2) *	Age, M1
	Suppressed TSH	Death	<0.001	24.2 (5.0 – 116.2) *	0.001	29.1 (3.6 – 232.2)*	Age

Likelihood Ratio for Tg value < Cut-Off * Likelihood Ratio for Tg value ≥ Cut-Off
Tg Cut Off values are given in Table 3

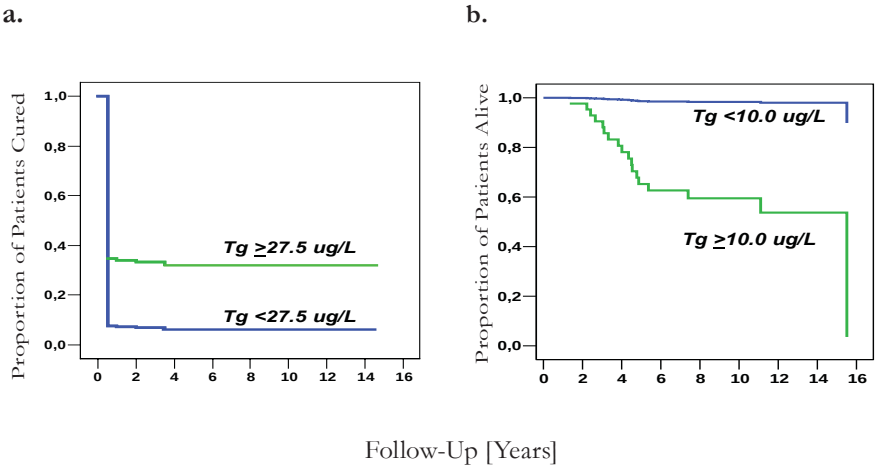

Figure 2. Prognostic value of Tg measurements for Differentiated Thyroid Carcinoma related disease free remission and mortality.
a. Disease free remission, Tg levels pre-ablation, four weeks after surgery; X-axis: years after initial therpy, Y-axis: Disease free remission
b. Survival according to TSH stimulated Tg, 6 months after initial therapy; X-axis: years after initial therapy, Y-axis: cumulative survival with death as endpoint.

Discussion

In the present study we investigated the diagnostic and prognostic value of serum Tg measurements for tumour presence, disease free remission and death in the follow-up of DTC by ROC analysis in a homogeneous group of patients with respect to initial therapy.

The study differed from earlier investigations with respect to the homogeneity of the patient group with respect to initial therapy, the fact that multiple Tg measurements were analysed at fixed time points during follow-up and the use of ROC analyses.

We found an excellent diagnostic accuracy of serum Tg values during TSH stimulation 6 months after initial therapy (sensitivity 100%), with a higher Tg cut-off level (10.0 ug/L) than commonly reported (2;15;16;10;8). When we used the more commonly used cut-off value of 2 ug/L, the specificity and positive predictive values dropped considerably (52% instead of 72%). We also found that Tg cut-off levels are dependant on the time-point of follow-up, which is an important finding, as in most papers on Tg, the time after diagnosis is not considered.

Tg levels are not only diagnostic indicators of tumour presence, but also predict disease free remission or death. We found that serum Tg levels before radioiodine ablation are an independent predictor for disease free remission, irrespective of the classical prognostic indicators. In our series a patient with Tg level pre-ablation of < 27.5 ug/L has an almost 98% chance to be definitely cured irrespective of the prognostic indicators stage T4, follicular histology, metastases and higher age.

TSH stimulated Tg measurements 6 months after initial therapy and at 2 and 5 years after initial therapy were independent predictors of thyroid carcinoma related death. Negative predictive values for DTC related death were high (95.3 – 98.2%) at all 5 time points of follow up, albeit with different Tg cut-off values.

In the discussion about the diagnostic value of Tg, specificity is a controversial issue. It has been argued that the specificity of Tg is per definition 100%. Although from a biological point of view it is undoubtedly correct that Tg is only synthesized by thyroid cells, in the clinical practice, the meaning of measurable Tg levels is not always clear, even more so with the advent of high sensitive Tg assays. A less than 100% specificity of Tg for thyroid carcinoma can be explained by the limitations of current imaging techniques to detect thyroid carcinoma. In this respect, it is advocated to administer a high dose of radioiodine to patients with elevated Tg levels, a policy that we agree with (17;18;19;20). However, we also observed that in only 3 of the 9 patients with TSH stimulated Tg levels > 10 ug/L and without detectable tumour, tumour became apparent during follow up, which is in line with the observation of Baudin et al (8). Therefore, in our opinion, a potential solution to circumvent the debate about specificity of Tg is to consider Tg as a risk indicator. The independent prognostic value of serum Tg values for disease free remission and death are arguments to include Tg in the conventional panel of risk factors. A potential consequence could be to administer higher dosages of radioiodine for ablation in patients with Tg levels higher than the above mentioned thresholds. As such we do not advocate that patients with Tg levels below institutionally defined cut-off levels should not be followed up carefully, but we belief that the elimination of Tg should not be a goal in itself.

Tg cut-off levels are not only influenced by clinical considerations, but also by analytical aspects. Analytical problems include the lack of universal standardisation of the Tg assays (21), intra-assay variability, "Hook" effects and the presence of Tg auto-antibodies(22;23). Another important point, not addressed in this study, is the observation that Tg rises may be more informative than absolute Tg levels (24;8).

The percentage of patients with Tg antibodies (initially 27%) is in line with previous studies (25;23;26). The percentages of active tumour in patients with and without Tg antibodies were comparable, conforming the lack of diagnostic value of Tg antibodies.

Because our study involved a large cohort of patients studied before the introduction of rhTSH, we did not include rhTSH stimulated Tg measurements in our series. However, recent reports indicate that the diagnostic accuracy is comparable (2;15;3). It has been suggested that Tg cut-off levels for rhTSH should be lower than for thyroid hormone withdrawal (27). However, no systematic analyses have been published comparing optimal Tg cut-off levels for both strategies. Furthermore, in a large study, similar Tg cut-off values were used for rhTSH and thyroxin withdrawal (16).

Because our analysis is based on retrospective data, we believe that the prognostic Tg cut-off values as found in our study should be interpreted with some caution, as they should be confirmed in a prospective study. We believe however that the main message, that Tg cut-off values should not be adopted from the literature, that Tg cut-off levels are dependant on the time of follow-up and that Tg has a prognostic value is valid.

In conclusion, our studies illustrate the importance of the definition of institutional Tg cut-off levels. We analysed the diagnostic value of Tg at specific time points and detected an excellent prognostic value 6 months after initial therapy during TSH stimulation. Our analyses allow the definition of groups of patients with an increased risk for residual disease or mortality, in addition to conventionally used prognostic indicators. Based on our analysis we recommend to subject every patient, who has undergone thyroid surgery and thyroid remnant ablation at least once to TSH stimulated Tg measurements.

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Chapter 4

The In Vitro Effects of Triiodothyronine on Iodide Uptake in FRTL-5 Cells

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Abstract

Background: Thyrotropin (TSH) stimulated radioiodide scintigraphy and therapy are important in the clinical care of patients with differentiated thyroid carcinoma (DTC). The introduction of recombinant human TSH (rhTSH) is an attractive alternative for thyroid hormone withdrawal (THW). Some reports suggest however that radioiodide uptake after rhTSH is inferior to THW. One of the explanations is that there is a direct effect of triiodothyronine (T3) on iodide uptake.

Aim: To study the effects of triiodothyronine (T3) on iodine uptake and expression of the sodium iodide symporter (NIS).

Methods: Iodide uptake (both steady state and initial rate) were studied in the rat thyroid cell line FRTL-5. FRLT-5 cells were cultured in medium with stripped serum in the absence or presence of 1pM, 2nM or 50nM T3 and all in presence of 1mU/ml TSH for 72 hours. NIS and TSH receptor mRNA and NIS protein expression were studied by quantitative PCR and Western-Blot.

Results: T3 inhibited iodine uptake both at initial rate and during steady state in a concentration dependent manner at steady state. NIS and TSHR expression at mRNA level were both reduced. Western blot of NIS protein showed a significant reduction of NIS protein after 2 nM.

Conclusion: T3 reduces radioiodine uptake and NIS and TSHR expression in FRTL-5 cells. We speculate that this is not caused by iodide being released from T3, as this amount is negligible, but that these are direct genetic effects, of which the mechanism needs further investigation.

Introduction

The concepts of therapy and diagnostic procedures during follow-up in differentiated thyroid carcinoma (DTC) are based on the responsiveness of thyroid carcinoma cells to thyrotropin (TSH)(1). TSH stimulated radioiodine uptake is important for both the ablation of thyroid hormone remnants during initial therapy and treatment of residual or metastatic DTC. In addition, TSH stimulated serum thyroglobulin (Tg) measurements have superior diagnostic value to detect recurrent DTC (2).

High serum TSH levels can be realized by conventional thyroxin withdrawal or more recently by recombinant human TSH (rhTSH), which has advantages with respect to quality of life (3). rhTSH has initially been used for diagnostic radioiodine scintigraphy and Tg measurements (4-13). In addition, rhTSH has also been used for radioiodine therapy in active DTC (14-18) and for the ablation of thyroid remnants (19-21).

The assumption for rhTSH treatment is that the pharmacodynamic properties of rhTSH and thyroxin withdrawal are comparable and that continuation of thyroxin therapy does not influence iodide uptake and Tg synthesis.

It is generally acknowledged that Tg measurements during rhTSH have comparable accuracy as thyroxine withdrawal (2;7). Some authors, however, have observed a lower sensitivity of diagnostic radioiodine scintigraphies performed after rhTSH (22;23). The efficacy of radioiodine therapy after rhTSH may be comparable with withdrawal, but no randomized studies have been performed to allow a direct comparison (14;24). Efficacy of radioiodine ablation after rhTSH was comparable after thyroxin withdrawal in a recent randomized trial (21), although earlier studies with lower activities of radioiodine showed a lower efficacy (25). One of the possible explanations for the supposedly decreased radioiodine uptake during rhTSH may be that triiodothyronin (T3) directly influences iodine uptake in the thyroid. We therefore studied the in vitro effects of T3 on iodide uptake.

Materials and Methods

Cell culture and cell proliferation assay

The rat thyroid FRTL-5 cell-line derived from the ATCC (ATCC, Manassas USA) expresses endogenously NIS which is subjected to TSH regulation (26). FRTL-5 cells were routinely cultured in Coon's F-12 modification medium (Sigma, Missouri USA) supplemented with 5% of stripped bovine calf serum, 1 mM non-essential

amino acids (Life Technologies, Inc.), 10 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and a six-hormone mixture (6H) containing insulin (1.3 μM), hydrocortisone (1 μM), transferrin (60 pM), L-glycyl-histidyl-lysine (2.5 μM), somatostatin (6.1 nM), and TSH (1 milliunits/ml) as reported previously (27).

For the proliferation assay, 500 cells/well were seeded in 96-well culture plates. T3 was added at concentrations varying from 1 pm to 50 nM. Two nM T3 is the average serum T3 concentration in rats and therefore considered physiological. Cell growth was measured using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay in conjunction with the addition of the electron coupling reagent phenazine methosulfate (PMS) (Promega). Briefly, 1, 3, 6 and 16 days after addition of T3, 180 μl culture medium was replaced by medium containing 10 μl of MTS/PMS mixture for 3 hours and placed at 37°C in a humidified incubator with 5% CO2. The absorbance of each well was measured with a microplate reader (Rainbow reader) at 570 nm wavelengths.

Radioiodide uptake assay

For uptake experiments, FRTL-5 cells were grown in 12-well plates. T3 was added in concentrations ranging from 0, 0.5, 1 and 2 nM for 72 hours prior to the uptake studies. For steady state iodide uptake assessments, cells were also cultured in medium without TSH (5H). The radioiodine uptake was performed as previous described (28). Briefly, the cells were washed 3 times with Hanks Balanced Salt Solution (HBSS) prior to the uptake assay. For the steady state uptake experiments, FRTL-5 cells were incubated with HBSS containing 10 μM $Na^{125}I$ with a specific activity of 50 mCi/mmol for 30 min 37 °C. Thereafter, the radioiodine was washed twice with cold HBSS. Cells were lysed with ice-cold ethanol. Radioactivity was subsequently measured in a gamma emitter counter. The DNA content of each well was subsequently determined after trichloroacetic acid precipitation, by the diphenylamine method (29). Based on the specific activity of the substrates, the efficiency of the γ-counter, and the DNA content of each well, iodide uptake was expressed as picomoles of substrate transported per microgram of DNA or as percentage of control conditions.

In the initial rate experiments, the effect of substrate concentration on uptake was determined by incubating washed FRTL-5 cells for 2 min in HBSS containing NaI from 0.625 to 160 μmol/L. After 2 min, radioiodide uptake was quantified as indicated above.

RNA isolation and real-time quantative PCR

Total RNA of FRTL-5 cells was extracted after 72 hour culturing without or with 1pM, 2 nM or 50 nM T3, using TRIzol LS reagent (Invitrogen Life Technologies, Inc.), followed by RNA cleanup with the RNeasy mini kit (Qiagen, Valencia, CA).

RNA concentrations were determined by measuring the absorbance at 260 nm. RNA was reverse transcribed into cDNA using the SuperScript First-stand Synthesis System for RT-PCR(Gibco BRL).

The following primer sets were used for quantitative PCR (qPCR): TSHR5'-3' TGC TTTCAA TGG AAC AAA GC; 3'-5' GGA AGG AAG AGC AGT AAC GC. NIS 5'-3' GGT TGT GGT AAT GCT CGT TG; 3'-5' GGG TCA AAG TCC ATC AGG TT. beta-actin 5'-3'TCC TTC CTG GGT ATG GAA TC; 3'-5' GCA CTG TGT TGG CAT AGA GG. All PCR amplicons spanned exon-intro boundaries.

The qPCRs were performed in the presence of 5ul Taq Gold buffer, 1.75ul 50mM MgCl2, 1ul 5mM dNTPs, 0.1ul 5U Aplitaq Gold DNA polymerase, 0.25ul 10uM stock solution of sense and antisense primers, 1.5ul sybrgreen and 1ul 5ng/ul cDNA in a final volume of 25ul. Water was used as a negative control. qPCR reactions perform on an iCycler (Biorad, Hercules, CA, USA) using the SybrGreen qPCR core-kit (Eurogentec, Seraing, Belgium). Cycle conditions were: 10 minutes at 94°C followed by 40 cycles of 10 s at 94°C and 1 minute at 60°C. Cycle threshold (Ct) extraction was performed using the iCycler IQ software (version 3, Biorad). The Ct value for NIS and TSHR are subtracted from the Ct values of actin (delta Ct values) (Fig.1). The relative delta Ct was calculated by 2^deltCT. The mean delta Ct value of an individual sample was based on three independent measurements.

Western blot analysis

Western-blot was performed as described previously (30). FRTL-5 cells were grown in the absence or presence of 0.5, 1 and 2 nM T3. Proteins were extracted and quantified using the Lowry method. All samples were diluted 1:2 with loading buffer and heated at 37°C for 30 min prior to electrophoresis.

Western blot analysis was carried out as follows: Twenty-five micrograms of protein per lane were loaded on a 9% SDS polyacrylamide gel and subjected to electophoresis at a constant voltage (150 V). Electroblotting to a nitrocellulose membrane was performed for 1 h. Blocking was done overnight using TTBS/milk (TBS, 1% Tween 20 and 5% milk). The membrane was incubated for 1 hr with a 1:5000 dilution of affinity-purified anti-rNIS antibody (30), which was kindly provided by Dr. Carrasco (Albert Einstein College of Medicine, Bronx, USA) in TTBS/milk. After washing, the membrane was incubated with a 1:5.000 dilution of a horseradish peroxidase-linked donkey anti-rabbit IgG (Amersham) in TTBS/milk. Quantitation of the signal intensity was performed by densitometry (Molecular Dynamics, Inc.). Membranes were also stained with a beta-actin antibody to check the amount of protein loaded.

Results

Cell proliferation assay

The results of the proliferation assay are given in Figure 1. Proliferation was assessed at 1, 3, 6 and 16 days after addition of T3. Addition of different concentrations of T3 (1 pM, 2nM or 50 nM) did not influence the proliferation. It was verified that T3 itself did not directly influence the MTS assay in a separate experiment in which both MTS and DNA concentrations were measured (data not shown).

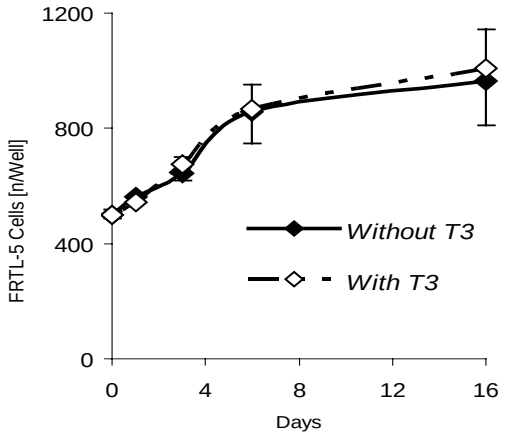

Figure 1. Proliferation of FRTL-5 cells, cultured without or in the presence of 50 nM T3. Cells were cultured in H-6 medium with stripped serum. Proliferation was measured with the MTS assay (see Materials and Methods).

Iodide Uptake

Iodide uptake was measured both in steady state conditions and in an initial rate experiment.

In steady state conditions, as expected, iodide uptake was much higher in the presence of TSH than in FRTL-5 cells without TSH (Figure 2a). Addition of T3 significantly decreased iodide accumulation, in a concentration dependent manner, irrespective whether the cells were cultured in the presence or absence of TSH (Figure 2a and Figure 2b). T3 decreases uptake even with absence of TSH although in a less pronouced level.

In the initial rate experiment, 1 and 2 nM T3 lowered the V_{max} of iodide uptake to about 50% of the control curve, whereas K_m was not influenced (Figure 2b).

NIS mRNA and protein expression

NIS mRNA expression as assessed by quantitative PCR was significantly reduced

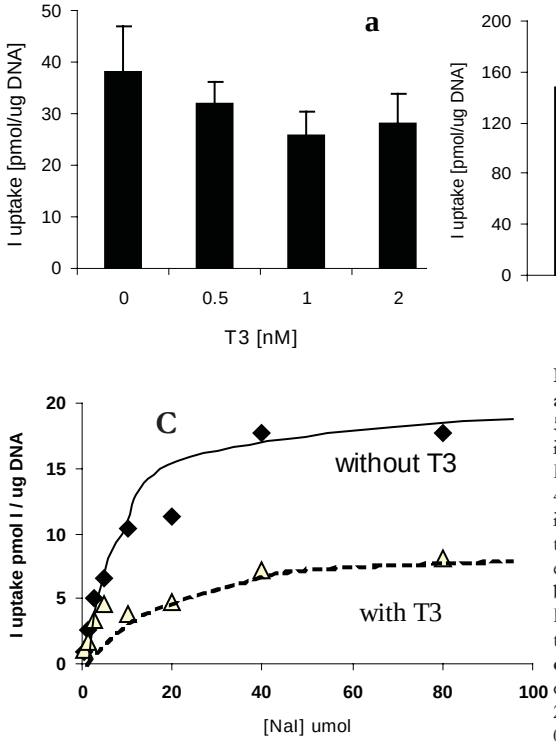

Figure 2.
a. FRTL-5 cells were cultured for 10 days in H-5 medium with stripped serum. T3 was added in indicated concentrations in the presence of H-6 medium. Iodide uptake was measured at 45 min after addition of I-125 (Specific activity 50 mCi/mmol. Activity was extracted from the cells by addition of ethanol. Uptake was expressed as pmol iodide/ug DNA.
b. Same as described in Fig.2a, except that FRTL-5 cells were cultured in H-6 throughout the whole experiment.
c. Initial rate (2 min) iodide uptake by FTRL-5 cells, cultured in H-6 medium with or without 2 nM T3 in a concentration range of NaI of 0.525 – 80 uM, with or without T3.

by the addition of 1 pM, 2 nM and 50 nM T3. The relative concentration of mRNA expression versus control was 0.86 for 2 nM T3 and 0.55 for 50 nM T3.

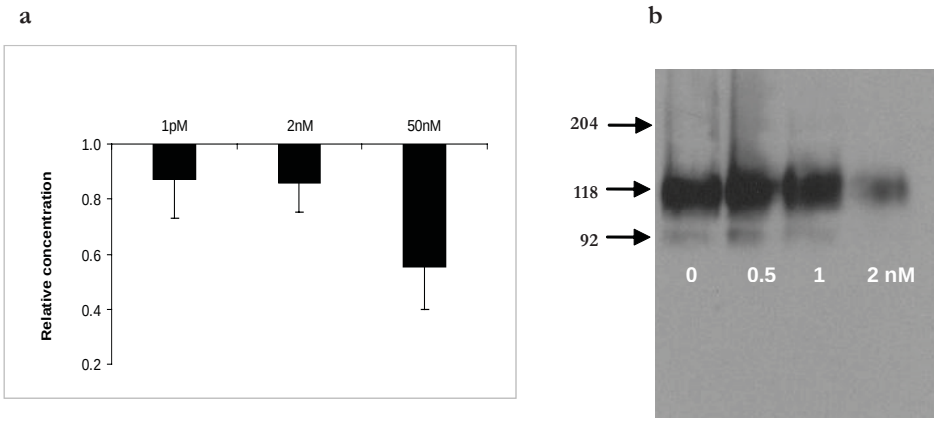

Figure 3.
a. Effects of T3 on NIS mRNA of FRTL-5 cells, cultured in H-6 medium with stripped serum. Proliferation expression as assessed by real time PCR, expressed as relative concentration (2^ delta delta CT)
b. NIS protein expression of FRTL-5 cells cultured in H-6 medium with stripped serum with or without T3.

Western Blot analysis showed that NIS protein expression was significantly reduced in FRTL5 cells cultured in 2 nM T3.

Discussion

The present study was conducted to investigate whether T3 had direct effects on iodide uptake in the thyroid irrespective of presence of TSH. We found indeed a decreased uptake of iodide in the rat cell-line FRTL-5 cultured in the presence of physiological concentrations of T3 even with absence of TSH although in a less pronouced level. Thus we speculate that T3 has TSH idenpendent effects on iodine uptake. This decreased uptake was accompanied by decreased NIS mRNA and protein expression.

The background of this experiment is the advent of rhTSH for the preparation of radioiodide scintigraphy and therapy in DTC (6;21). As patients will continue thyroxin therapy during rhTSH therapy, the question is whether T3 itself may affect iodide uptake as thyroid tissue contains functional T3 receptors (31;32). There have indeed been some suggestions that radioiodide scintigraphies after rhTSH have a lower sensitivity than after thyroxin withdrawal (5;6) and that ablation with 30 mCi radioiodide is less efficient after rhTSH than after thyroxin withdrawal (25). Several explanations for these observations have been proposed.

It has been suggested that the iodide content of levo-thyroxine (T4) therapy during rhTSH may dilute the specific activity of the radioiodide administered. Indeed, 65.4% of the molecular weight of T4 consists of iodide which may result in a net daily supply of 25-60 ug iodide, when taking 100 ug/day. Indeed increased urinary iodide excretion has been observed during rhTSH as compared with thyroxin withdrawal (33;34).

In our study, we found a substantial decrease in iodide uptake of up to 50% after T3. The amount of iodide coming from T3 in our experiment (In case of 2 nM T3: 9 nM of iodide) cannot explain the decrease in iodide uptake, as the steady state experiments were performed in the presence of 10 uM NaI. The resulting dilution of radioactivity may thus only be 0.001, which is negligible.

Another explanation for the diminished quality of radioiodide scintigraphies after rhTSH may be the altered iodide kinetics in euthyroidism as compared with hypothyroidism. Indeed, renal clearance of iodide is higher in euthyroidism, thereby reducing the whole body dose of radioiodine after rhTSH (35;36). In the latter study it was concluded that the effective half life of radioiodide in the thyroid after rhTSH was decreased but that the residence time of radioiodide in the thyroid was longer than after withdrawal. In our study, using an in vitro iodide uptake assay, the influence of whole body iodide kinetics was ruled out.

From our results it seems likely that T3 has effects on NIS gene expression at least in FRTL5 cells, resulting in lower functional NIS protein. It has been debated whether the promoter for NIS contains T3 responsive elements. In one study, it was suggested that T3 in fact stimulates the NIS promoter (37). However, these experiments were not performed with stripped serum. In earlier studies, it has been observed that T3 decreases the mRNA and protein expression of NIS as well as the uptake of iodide (32;38). In several experiments, it has been found that the promoter of the TSHR gene contains T3 responsive elements and that T3 suppresses the expression of the TSHR (39;40) (41). Another explanation for the repression of TSHR gene transcription by T3 has been suggested by Tagami et al (42) who found that unliganded thyroid hormone receptor recruits histone deacetylase (HDAC) from the TSHR promoter, resulting in increased histone acetylation and transcriptional activation of the TSHR. In the presence of T3 HDAC comes available to repress TSHR promoter activity. However, we observed that T3 also decreased iodide uptake in FRTL5 cultured in medium without additional TSH.

In conclusion, we found evidence for a TSH and iodide independent effect of T3 on NIS gene expression. The mechanism remains to be resolved and also the question whether the effect is present and relevant in humans. The clinical relevance of this finding is not clear. Randomized trials with clearly defined endpoints can provide answers to this question. The similar ablation efficacy in rhTSH treated patients and patients undergoing thyroxin withdrawal suggest that the contribution of T3 induced NIS suppression may be limited.

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Chapter 5

Lithium as Adjuvant to Radioiodine Therapy in Differentiated Thyroid Carcinoma, Clinical and In Vitro Studies

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Abstract

Objective: Lithium has been reported to increase radioiodide (RaI) dose in benign thyroid disease and differentiated thyroid carcinoma (DTC). It is not known if lithium influences the *outcome* of RaI therapy in DTC. We therefore studied the clinical effects of RaI without and with lithiumcarbonate in patients with proven metastatic DTC. In addition, controversy exists on the mechanism by which lithium increases RaI dose in DTC. We performed an in vitro study specifically aimed at lithium effects on the sodium iodide symporter (NIS).

Design: Clinical study: 12 patients were selected with metastases of DTC who had received previous RaI therapy without lithium (control) that had not influenced tumor progression, despite RaI accumulation in metastases. The patients received 1200 mg lithiumcarbonate/day followed by 6000 MBq RaI. Outcome parameters were RaI uptake, serum thyroglobulin (Tg) levels and radiological dimensions of metastases as compared between RaI with lithium and control. *In vitro study:* Iodide uptake was studied in the benign rat thyroid cell line FRTL-5, in the polarized non-thyroid MDCK cell-line, stably transfected with hNIS to study lithium effects on NIS in a non-thyroid background and the human follicular thyroid carcinoma cell line FTC133-hNIS to study lithium effects in a background of DTC. Lithiumchloride was added in concentrations up to 2 mM for 0-48 hours. Both steady state iodide uptake (30 min) and initial rate (2 min) were studied using a specific activity of 100 mCi/mmol I, the latter experiment to determine lithium effects on substrate dependency. Iodide efflux studies were performed as well.

Results: Despite an increased uptake of RaI in 7 patients, no beneficial effect of RaI with lithium was observed on the clinical course as assessed by serum Tg measurements and radiographically.

In the in vitro studies, no effects of lithiumchloride on iodide uptake or efflux were observed.

Conclusions: We conclude that the addition of lithium to RaI did not have beneficial effects on the clinical course in 12 patients with metastatic DTC. No beneficial effects of lithium on iodide uptake were observed in vitro. Therefore, the clinical value of lithium in DTC remains subject of debate.

Introduction

Although the role of RaI therapy in recurrent or metastatic thyroid cancer is beyond dispute, the remission rate in metastases treated with I-131 is limited [1-3]. Therefore, strategies to enhance the tumor dose of RaI are worthwhile.

Lithium salts have been introduced decades ago for the treatment of psychiatric disorders [4]. Lithium salts have been associated with an increased trapping of iodide by the thyroid gland [5,6]. This property of lithium led to the assumption that lithium may enhance the dose of RaI in benign and malignant thyroid disorders. Indeed, increased RaI retention by lithium has been confirmed in Graves hyperthyroidism leading to a higher therapeutic efficacy [7-11], although this could not be confirmed in other studies [12,13]. In addition, lithium has been reported to increase tumor dosages of RaI in DTC [5,14-17]. These studies vary in the time course of lithium application: some studies initiated lithium administration 2 days prior to RaI therapy [12,17] whereas others started lithium only at the instant of RaI therapy [5,10,14].

Despite the observation of increased RaI uptake in DTC, no studies have been published to our knowledge in which the effects of the addition of lithium to RaI on the *clinical course* of patients were investigated. We therefore studied the clinical effects of RaI without and with lithiumcarbonate in 12 patients with proven metastatic DTC.

The mechanism of the enhanced RaI trapping by lithium salts in DTC is presently unclear. Thyroid carcinomas that accumulate iodide have in common with benign thyroid diseases that they express the sodium iodide symporter (NIS) that is responsible for iodide uptake [18], whereas thyroid cancer differs from normal thyroid in numerous other aspects, including the loss of follicular architecture and the loss of expression of many proteins involved in thyroid hormone synthesis [19]. Therefore, the most obvious explanation for lithium effects on iodide trapping in benign and malignant thyroid disease would be to enhance NIS function. In the literature however, variable effects of lithium salts on iodide uptake in vitro or in animal studies are reported. Some studies found that lithium salts *inhibit* the uptake of iodide, iodotyrosin coupling and the release of thyroid hormone [20-23]. Other studies found unaltered uptake [8,9,24,25] or increased iodide uptake [26].

As most of these studies were performed before the cloning of NIS, we wanted to study the effects of lithium salts on NIS function in the background of normal thyroid physiology, in a non-thyroid background and in the background of thyroid carcinoma. As this objective cannot be addressed easily in patients, we performed in vitro studies, studying lithium uptake in thyroid and non-thyroid cell lines with endogenous NIS expression or stably transfected with hNIS.

Patients, Materials and Methods

Clinical study

After the publication of the study of Koong et al [17], it was decided to apply the treatment schedule of this study in patients with metastases of DTC that had been scheduled for RaI therapy and who had had an unfavorable response to prior RaI therapy despite the fact that their metastatic lesions accumulated RaI as revealed by whole body scintigraphy (WBS), 7 days after radioiodide therapy. This first RaI therapy served as a control. Patients who were selected had to have undergone total thyroidectomy and RaI ablative therapy. The presence of metastases of thyroid carcinoma was established by measurable serum Tg levels and the presence of metastatic sites at post-therapeutic whole body scintigraphy, X-ray, CT or MRI after prior radioactive iodine therapy.

The objectives of this study were to investigate if addition of lithium to RaI has beneficial effects on radioiodine uptake and the clinical course of the patients. Outcome measures were the uptake of RaI on post-therapy WBS, progression of serum Tg levels after RaI therapy and the change in dimensions of the metastatic sites at X-ray, CT or MRI.

Twelve patients were included in the protocol (2 males, 10 females). Their clinical characteristics are presented in Table 1. The mean age at diagnosis of thyroid carcinoma was 59 years. Most patients had papillary thyroid carcinoma. In 10 of the patients, metastases were already present at the time of diagnosis of thyroid carcinoma, most of them pulmonary. Before the RaI therapy combined with lithiumcarbonate and the control RaI therapy were performed, all patients had received extensive therapies; RaI therapy had been administered in a mean cumulative dosage of 28 GBq (Table 1). Six of the 12 patients had received additional non-RaI therapies during the course of their disease. However, none of these therapies had been applied within a 1-year period prior or after the historical control RaI therapy or the RaI therapy combined with lithiumcarbonate.

Protocol

Four weeks before RaI therapies, patients were routinely switched from T4 to T_3 therapy. T_3 was discontinued two weeks before RaI therapy. A low iodide diet was started 1 week prior to the RaI therapy. RaI was administered orally as an activity of 6000 MBq $Na^{131}I$. Seven days after RaI administration, whole body scintigraphy was performed. During the second RaI therapy, lithiumcarbonate (Litarex, Dumex, Baarn, The Netherlands) was prescribed according to the schedule of Koong [17]. Lithiumcarbonate 564 mg was given twice (bid) with a 12 h interval. Plasma lithium levels were measured by atomic absorption spectrometry. The dose was adjusted if

necessary to achieve a lithium concentration of 0.6-1.2 mmol/L. Lithium was continued during 7 days after the RaI administration. To investigate the effects of lithiumcarbonate on RaI uptake, ideally a randomized crossover design with a washout period should be performed. The crossover design would be necessary to account for the continuing rise in serum TSH levels during the period of T_4 withdrawal. This would have implicated a prolonged period of T_4 withdrawal and consequently high TSH levels. This was considered not ethical because the RaI therapy was scheduled anyway and a prolonged period of increased TSH levels could theoretically have unfavorable effects on tumor progression [27-29].

Table 1. Characteristics of 12 patients with metastases of differentiated thyroid arcinoma

Patients (n)	12
Females / Males (n)	10/2
Age at diagnosis (y)	59 ± 11
Tumor Histology (n)	
Papillary	7
Follicular variant	2
Follicular	3
Stage at Diagnosis (n)	
T 1–3 and M-0	2
T-4 and M-0	0
M-1	10
Metastases at Diagnosis	
Lungs	8
Bone	5
Soft-tissues	2
Cumulative Activity I-131 (GBq)	28.1 ± 11.1
Metastases at Therapy	
Lungs	10
Bone	7
Soft-tissues	2
Additional Therapies	
Surgery	2
Embolization	4
External irradiation	4

$Na^{131}I$ whole-body scintigraphy was performed 7 days after the oral administration of 6000 MBq of ^{131}I (Mallinckrodt BV, Petten, The Netherlands). The run speed of the dual-head gamma camera (Toshiba GCA 7200, equipped with a high-energy collimator) was 15 cm per minute (matrix size 256×256). WBS was followed by anterior and posterior planar images of the head and neck and chest region (matrix size 256×256, preset time 10 min). Quantitative assessment of I-131 uptake was performed by calculating uptake in 2 regions of interest by 2 observers who were unaware of treatment modality. These regions were carefully chosen in such a way that they had not been subjected to other treatment modalities. Two regions were chosen to assess whether a potential effect of lithium was uniform or not. Quantitative uptake on WBS performed after lithium was compared with control in corresponding regions of interest, and expressed as 'increased', 'stable', 'decreased' or 'mixed'. 'Mixed' was used when the result of lithium in the 2 regions of interest differed.

Thyroglobulin increments are expressed as the differences in the natural logarithms (Ln) of the Tg values during suppressive T_4 therapy observed at the end of the observation period after RaI therapy and the last Tg value during T_4 before RaI, divided by the duration of the observation period; in formula: Delta LnTg:

$(LnTg_{end}\text{-}LnTg_{start})$/months.

Radiological measures were scored semi-quantitatively as: 'stable', 'progression', 'regression' or 'cure'. ×256, preset time 10 min).

Laboratory measurements

Serum TSH was determined with on a Modular Analytics E-170 system (Roche Diagnostic Systems, Basle, Switzerland), intra-assay variability: 0.88-10.66%, inter-assay variability: 0.91-12.05%). Serum Tg was measured. Serum Tg was determined with IRMA (Tg kit, Brahms, Berlin Germany) on a Wallac gammacounter (Wallac, Turku, Finland), intra-assay variability: 0.14-13.9%, inter-assay variability: 12.3-17.4 %). Serum Tg antibodies were determined with IRMA (Sorin Biomedica, Amsterdam, The Netherlands) on a Wallac gammacounter (Wallac, Turku, Finland) intra-assay variability: 3.6-4.1%, inter-assay variability: 11.6%).

In vitro studies

Cell lines and culturing conditions

Three cell-lines were studied: The rat thyroid FRTL-5 cell-line derived from the ATCC (ATCC, Manassas, New York) expresses endogenously NIS which is subjected to TSH regulation [30]. FRTL-5 were grown in Ham's F-12 media (Life Technologies, Inc.) supplemented with 5% calf serum, 1 mM non-essential amino acids (Life Technologies, Inc.), 10 mM glutamine, 100 units/ml penicillin, 100 μg/ml

streptomycin, and a six-hormone mixture (6H) containing insulin (1.3 μM), hydrocortisone (1 μM), transferrin (60 pM), L-glycyl-histidyl-lysine (2.5 μM), somatostatin (6.1 nM), and TSH (1 mU/ml) as reported previously [31].

Recent studies suggest striking similarities between polarized protein sorting in thyrocytes and MDCK epithelial cells. We have therefore used MDCK clones stably transfected with hNIS [32](donated by N. Carrasco, Albert Einstein College of Medicine, New York) to study direct effects of lithium on NIS in a non-thyroid background.

To study if lithium influences NIS function in the background of a thyroid carcinoma, the follicular thyroid carcinoma cell line FTC133 was used. FTC133 (kindly donated by Dr. Goretzki and Dr. Simon, University of Düsseldorf, Germany) was derived from a 42-year-old male with metastatic follicular thyroid carcinoma [27]. We have stably transfected this cell line with hNIS [33,34]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and modified HAM-F12 medium 1:1 supplemented with 10% fetal bovine serum, penicillin/streptomycin and geneticin to maintain an advantageous environment for transfected cells, in a humidified incubator at 37ºC and 5% CO_2. Lithiumchloride was added to the culturing fluids in various concentrations and time schedules as indicated.

In vitro iodide uptake

For uptake experiments, cells were grown in 12-well plates. LiCl was added in concentrations ranging from 0 to 2 mM either 48 hours prior to the uptake studies or at the moment of uptake studies (acute). Culturing media were carefully checked for pH after addition of Lithium. Prior to the uptake studies, the cells were washed 3 times in Hanks Balanced Salt Solution (HBSS), buffered with 10 mM Hepes (pH 7.5) Thereafter, HBSS containing 20 μM $Na^{125}I$ with a specific activity of 100 mCi/mmol was added to the cells. Cells were incubated at 37 °C in a humidified atmosphere.

Three types of uptake studies were performed: steady state, initial rate and efflux studies.

In all experiments, reactions were terminated by aspirating the radioactive mixture and washing three times with the ice cold HBSS. Accumulated ^{125}I was determined by permeabilizing the cells with 500 ul ethanol for 20 min at –20 ^{0}C and quantitating the released radioisotope in a γ counter. The DNA content of each well was subsequently determined after trichloroacetic acid precipitation, by the diphenylamine method [18]. Based on the specific activity of the substrates, the efficiency of the γ-counter, and the DNA content of each well, iodide uptake was expressed as picomoles of substrate transported per microgram of DNA or as percentage of control conditions.

For steady state experiments, the radioactive cells were incubated for 30 minutes with the radioactive solutions.

In the initial rate experiments, the effect of substrate concentration on uptake was determined by incubating washed cells for 2 min in medium containing 9 concentrations of iodide, ranging between 0.625 and 160 μmol/L. Uptake reactions were then terminated and substrate uptake was quantitated as indicated above.

Iodide efflux was studied in a subsequent experiment; after addition of HBSS with 20 μM $Na^{125}I$ with a specific activity of 100 mCi/mmol during 30 min in the presence or absence of LiCl in concentrations from 50-2000 umol/L, the radioactive supernatant was removed and HBSS with or without lithium was added to the cells for 5-min intervals up to 30 min after removal of the radioactive supernatant. Radioactivity was counted in all fluids. The sum of all radioactivity counts in all washing fluids was considered the accumulated radioactivity at the beginning of the efflux.

All experiments were performed in hexaplicate.

Immunofluorescence

FRTL-5 cells in the presence of TSH were seeded onto poly-(lysine)-coated coverslips. Cells were cultured with or without LiCl 2 mM for 48 hours. Cells were washed 3× with PBS/CM, fixed with 2% paraformaldehyde in PBS for 20 min at RT, and rinsed with PBS/CM. Cells were permeabilized with 0.1% Triton in PBS/CM plus 0.2% BSA (PBS/CM/TB) for 10 min at RT. Cells were quenched with 50 mM NH_4Cl in PBS/CM for 10 min at RT and rinsed with PBS/CM/TB. Cells were incubated with 8 nM anti-rat NIS antibodies [35](donated by N. Carrasco), washed, and incubated with 1:700 dilution of fluorescein-labeled goat anti-rabbit antibodies (Vector Laboratories). After washing, cells on the coverslips were mounted onto microscope slides using an antifade kit from Molecular Probes. Coverslips were sealed with quick-dry nail polish and allowed to dry in the dark for 2 h at RT and stored at 4 °C. NIS immunofluorescence was analyzed with a Bio-Rad Radiance 2000 Laser Scanning Confocal MRC 600, equipped with a Nikon Eclipse epifluorescent microscope.

Statistical analyses

Continuous data are expressed as mean ± SD when distributed normally, as tested by the Kolmogorov-Smirnov test, otherwise as medians and ranges. Continuous data between groups were compared with a paired Students T-test or the Wilcoxon test, in case data were not distributed normally. Proportional data were compared with the Chi-square test. In the iodide efflux studies, half-life of accumulated radioactivity was calculated by linear regression analysis of the radioactivity-time curve. A p value of < 0.05 was considered significant.

Results

Clinical study

The control RaI therapy and the RaI therapy with lithiumcarbonate were not different with regard to serum TSH and Tg levels during T_4 withdrawal. (Table 2). No adverse events were observed during lithiumcarbonate administration. All 12 patients had lithium levels > 0.6 mmol/L. In two patients, the lithiumcarbonate dose had to be increased to 564 mg three times per day (tid) to achieve these concentrations. The post-therapeutic increments in Tg levels are given in Table 2. Uptake of RaI was increased after addition of lithium in 7 patients. Two patients had a mixed pattern, some lesion showing increased uptake, other stable or decreased uptake. Median increments in the natural logarithm of Tg levels did not differ significantly between the first RaI therapy (0.08 vs. 0.11, p=0.228). The number of subjects with positive or negative Tg increments after RaI therapy did not differ either between RaI therapies without and with lithiumcarbonate. The same pattern was observed for the radiological evaluation of metastatic sites: the number of subjects with stabile, progressive or regressive metastatic sites was not different after the historical control RaI therapy as compared with RaI combined with lithium. Therefore, we were unable to document a benefit of the administration of lithium in these patients.

Table 2. Effects of RaI therapy with 6000 MBq I-131 on clinical course in patients with progressive differentiated thyroid carcinoma without (control) or with addition of lithiumcarbonate (lithium)

	Control	Lithium	p-value [1]
Serum TSH at therapy (mU L^{-1})	96 ± 56	99 ± 87	0.831 [&]
Serum thyroglobulin Levels (µg L^{-1})			
During withdrawal	2320 (104-277960)	3518 (668-1310000)	0.328 [&]
Increment Post-Therapy			
(Delta LnTg(Ln µg L^{-1} $month^{-1}$))	0.08 (-0.08-0.26)	0.11 (-0.19-1.04)	0.228 [&]
Delta Ln Tg positive/negative (n)	9 / 3	10 / 2	0.615 [*]
Whole body scintigraphy			
Iodide uptake in ROI vs. Lithium vs. Control			
Increased (n patients)		5	
Stable 1		1	
Decreased		3	
Mixed [2]		2	
Radiological evaluation (X-ray, CT, MRI)			
Regression (n patients)	3	2	0.091 [*]
Stabile	5	1	
Progression	4	9	

[1]: lithium vs. control; [2] : 2 regions of interest with different effects of lithium; [*]: Chi-square test; [&]: Wilcoxon –test

In vitro study

Steady state Iodide uptake

We studied the 30 min accumulation of iodide in FRTL5, MDCK-hNIS and FTC133 hNIS after acute or 48 hours incubation with LiCl in concentrations of 50, 100, 500, 1000 and 2000 umol/L. We did not observe any effect of either acute or 48 hour addition of lithiumchloride in any concentration on steady state iodide uptake in the 3 cell lines.

The results for 500, 1000 and 2000 umol/L are shown for FRTL5, MDCK-hNIS and FTC133 hNIS (Figure 1a).

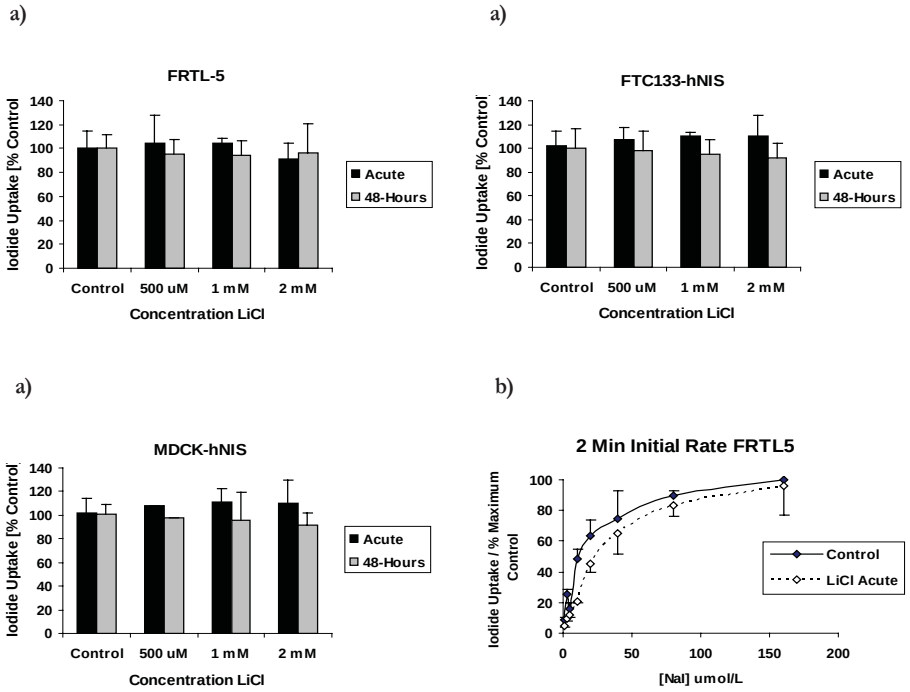

Figure 1. a. Acute and 48 hours effects of 500, 1000 and 2000 uM LiCl on iodide uptake in 3 cell-lines: FRTL-5, MDCK-hNIS and FTC133-hNIS. Incubation media consisted of HBSS with 20 μM $Na^{125}I$ with a specific activity of 100 mCi/mmol. Experiments were terminated after 30 minutes. Iodide uptake was expressed as percentage of control uptake. Mean values for iodide uptake without lithium were: FRTL-5: 12.3±6.6 pmol/ug DNA, for MDCK-hNIS: 49.5±8.3 pmol/ug DNA and FTC133-hNIS: 23.4±0.5 pmol/ ug DNA. **b.** Two minutes iodide uptake by FRTL-5. Incubation media contained $Na^{125}I$ in concentrations from 0.625 uM to 160 uM, all with a specific activity of 100 mCi/mmol. All uptake values were expressed as a percentage of the maximum uptake in FRTL5 without lithium.

Initial rate iodide uptake

We studied the 2 minutes iodide uptake and the effects of substrate concentration in FRTL-5, MDCK-hNIS and FTC133 hNIS after acute or 48 hours incubation with LiCl in concentrations of 50, 100, 500, 1000 and 2000 umol/L. We did not observe any effect of either acute or 48 hour addition of lithium salts in any concentration on initial rate uptake of the 3 cell lines. The results for acute addition of 2 mM LiCl are given in Figure 1b.

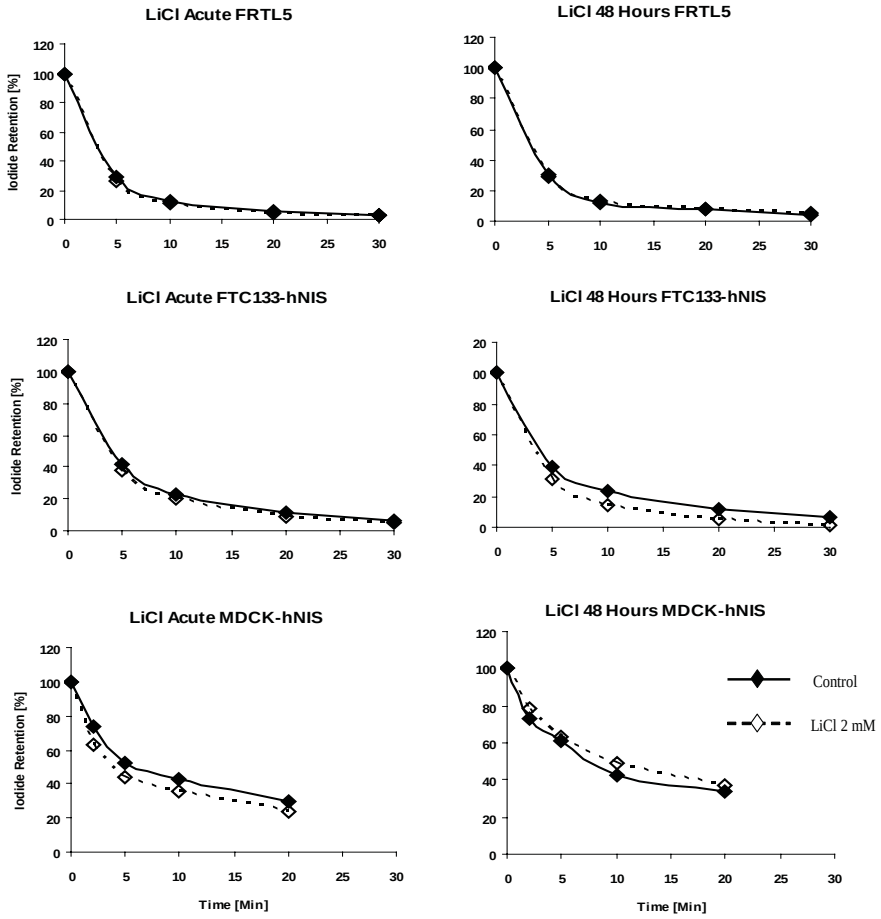

Figure 2. Influence of LiCl 2 mM, added during 48 hours or during the efflux experiment (acute) on iodide efflux in FRTL-5, MDCK-hNIS or FTC133-hNIS. Iodide efflux was studied after addition of HBSS with 20 μM $Na^{125}I$ with a specific activity of 100 mCi/mmol during 30 min in the presence or absence of LiCl. Thereafter, the radioactive supernatant was removed and HBSS with or without lithium was added to the cells for 5-min intervals up to 30min after removal of the radioactive supernatant.

Iodide efflux

To study whether the absence of an effect of lithium salts on iodide uptake may be theoretically explained by an effect of similar magnitude on iodide efflux, we studied iodide efflux or retention in FRTL5, MDCK-hNIS and FTC133 hNIS after acute or 48 hours incubation with LiCl in concentrations of 50, 100, 500, 1000 and 2000 umol/L. We did not observe any effect of either acute or 48 hour addition of lithium salts in any concentration on steady state iodide uptake in the 3 cell lines.

The results for acute and 48 hours addition of 2 mM LiCl for FRTL5, MDCK-hNIS and FTC133-hNIS are shown for FRTL5 (Figure 2).

NIS immunofluorescence

No effects of the addition of 2 mM LiCl for 48 hours on NIS staining were observed (Figure 3).

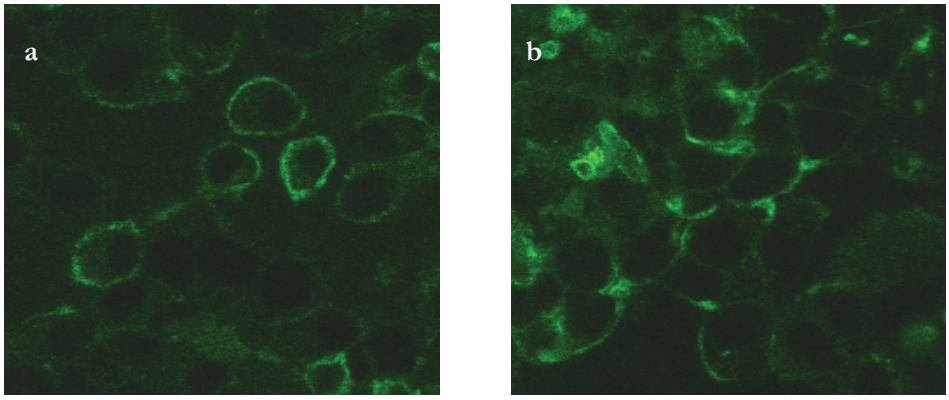

Figure 3. NIS immunostaining of FRTL-5 cells, cultured during 48 hours without (a) or with (b) LiCl 2 mM. No effect on immunofluorescence was seen. Magnification 60 x.

Discussion

We performed the present study to investigate whether the addition of lithium to RaI in patients with metastasized DTC has beneficial effects on the clinical course of the disease. In addition, we studied whether the reported beneficial effects of lithium on RaI uptake in patients with benign or malignant thyroid diseases may be explained by a direct effect of lithium on NIS or alternatively that the effects must be attributed to other mechanisms as suggested in a number of studies [20,21,23].In the clinical study, we did not find any evidence for a positive effect of lithiumcarbonate

on the clinical course of DTC. Several explanations for the lack of success can be hypothesized. First, the category of patients could have been different from the study of Koong et al [36]. However, in both studies patients with iodide accumulating metastases were included. In both studies, papillary carcinomas were predominant and most patients had pulmonary metastases. Second, in our study, the clinical course was compared after two *high dose* RaI therapies with a longer interval than in the study of Koong et al. It can be hypothesized that the longer time interval may have given rise to changes in biological tumor characteristics or alternatively, that the historical control RaI therapy may have selected radioresistant tumor cells [37]. However, in all patients, RaI accumulating lesions were present after the second RaI therapy as well. In addition, although Tg levels were progressive in most patients, their long-term increment rates were not altered substantially after the first RaI therapy, so it is unlikely that this explanation is true. Third, it could be hypothesized that the response of thyroid carcinoma cells to lithium combined with high activities of RaI is different from lithium combined with tracer doses: with high doses of RaI, thyroid cancer cell necrosis could lead to a faster release of radioactivity from the cells [14]: however, if this were true in our patient group, this would have lead to a favorable response on RaI. A fourth explanation could be that even if lithium had led to higher iodide retention in our patients, no additional therapeutic effect of RaI therapy was achieved. Efficacy of RaI therapy is the result of tumor dose on the one hand and radiosensitivity on the other hand. If lithium had resulted in an increased tumor dose, this could still have been insufficient to establish growth arrest of thyroid carcinoma.

In addition, some patients underwent alternative treatments like embolization or external irradiation that might confound the potential effect of lithium. However, we carefully chose indicator metastases that were not subjected to these alternative therapies. In the experimental studies we investigated whether the beneficial effects of lithium salts on RaI uptake in patients with benign or malignant thyroid diseases may be attributed to a direct effect on NIS or that the effects must be attributed to other mechanisms as suggested in a number of studies [20,21,23]. This hypothesis was based on the fact that benign thyroid disease and iodide accumulating thyroid carcinoma have in common the expression of NIS, whereas virtually every other aspect of thyroid hormone physiology is different. In addition, we studied the effects of addition of lithium to RaI therapy on the clinical course in 12 patients with metastatic DTC. In the in vitro experiments, we included 3 cell-lines: FRTL5, in which NIS expression is subjected to normal regulation. MDCK-hNIS is a polarized cell-line in which trafficking of thyroid proteins resembles that in normal thyroid cells [32] but where lithium effects on NIS can be studied in the absence of normal thyroid regulation. FTC133-hNIS is a follicular thyroid carcinoma cell line, stably transfected with hNIS [34], in which effects of lithium salts on NIS in a background of thyroid carcinoma can be studied.

In our experiments, no effects of lithiumchloride were found on iodide uptake, neither when added acutely, nor when added 48 hours before the uptake experiments. Uptake was studied both in steady state and initial rate experiments. To exclude the theoretical possibility that lithium salts may affect uptake and efflux to the same magnitude, efflux studies were performed as well, again with no effect of lithiumchloride. These results are the first reported on in vitro effects of lithium salts on NIS function in a benign or malignant thyroid background and in a non-thyroid background.

Although of course we have not studied all steps of iodide physiology, we believe that an explanation via NIS is highly unlikely and thereby confirm earlier studies in which no effect of lithium salts on iodide uptake were found [8,9,20,22-25]. Haberkorn et al. [38] did not find an effect of lithium salts on iodide trapping in NIS transfected thyroid carcinoma in an animal study. In another study in NIS transfected colon carcinoma cells, even an inhibiting effect of lithium was found [39]. It is suggested that for the enhancement of iodide trapping by lithium intact organification is necessary which then is inhibited by lithium [38]. This may explain the absence of lithium effects in thyroid- or non-thyroid tumors with a short half-life and absence of organification.

In conclusion, our data indicate that if a beneficial effect of lithium in thyroid carcinoma would be present, it would not be by enhancing NIS activity. The clinical data presented in this study raise doubt if there is a beneficial effect at all in enhancing the effects of RaI treatment in thyroid carcinoma. Therefore, the clinical value of lithium in DTC remains a subject of debate.

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Chapter 6

Bexarotene Increases Uptake of Radio-iodide in Metastases of Differentiated Thyroid Carcinoma

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Abstract

Objective: Treatment options of metastases of differentiated thyroid carcinoma (DTC) are limited due to decreased uptake of radioiodide (I-131). Therefore, strategies to improve I-131 uptake are mandatory. It has been suggested that retinoids have beneficial effects on iodide uptake in vitro and in humans. However, to date, only studies with 13-cis retinoic acid have been performed in humans. We therefore decided to study the effects of 6-weeks treatment with the retinoid receptor RXR activator Bexarotene on I-131 uptake in patients with metastatic DTC.

Design: Open prospective intervention study.

Methods: Twelve patients with metastases of DTC, with insufficient uptake of I-131 received 6-weeks treatment with 300 mg Bexarotene/day. Prior to, and after this intervention, I-131 uptake was measured by whole body scintigraphy and single photon emission tomography (SPECT) 3 days after 185 MBq I-131. Diagnostic imaging was preceded by 2 consecutive injections with recombinant human thyrotropin.

Results: Bexarotene treatment induced I-131 uptake in metastases of 8/11 patients (one patient died for reasons not related to the study). However, uptake was only discernable at SPECT and had incomplete matching with metastases as visualized by CT scanning.

Conclusions: Bexarotene partially restores I-131 uptake in metastases of DTC. The clinical relevance of this observation may be limited due to the differential responses of the different metastases within each patient and the low intensity of I-131 uptake.

Introduction

Differentiated thyroid carcinoma (DTC) in general has a favourable prognosis due to the effect of combined treatment of surgery and radioactive iodide (I-131) and the biological behaviour of the tumor (1,2). However, about 50% of patients with distant metastases of DTC die within 10 years after the diagnosis (3). Although the role of I-131 in recurrent or metastatic thyroid cancer is beyond dispute (4 - 6), the efficacy of this therapy is hampered by the decreased expression and/or function of the sodium iodide symporter (NIS) in DTC during the process of dedifferentiation (7 - 9). Therefore, strategies to improve iodide uptake by DTC are mandatory.

Retinoids are derivatives of vitamin A (i.e. retinol). Beneficial effects of retinoids have been reported in promyelocytic leukaemia and several types of carcinoma (10 - 12). In vitro studies have reported that retinoids have beneficial effects in thyroid carcinoma (13 - 16) including increased NIS mRNA expression and iodide uptake in some thyroid cancer cell lines (13). Interestingly, the promoter of the NIS gene has a retinoic acid response element (17). A limited number of human studies have been performed on the effects of retinoids on I-131 uptake. In 4 publications - 3 from the same group - 13-cis retinoic acid therapy increased I-131 uptake in 26-40% of the patients (18 - 21), but failed to do so in another study (22). The only retinoid used so far in human studies in DTC is 13-cis retinoic acid. This compound is a ligand for the retinoic acid receptor RAR. However, 13-cis retinoic acid has a lower affinity for RAR than other retinoids as retinoic acid and all-trans retinoic acid (23). In addition, recent studies indicated a differential expression of both RAR and the retinoid receptor RXR in thyroid carcinoma cell-lines and tissues (24,25), which corresponded to the responsiveness to ligands for these receptors. The importance of RXR expression with respect to responsiveness to retinoid treatment was demonstrated in the latter study (25). We therefore, decided to perform a prospective controlled clinical trial to investigate the efficacy of the novel ligand Bexarotene (Targretin, Ligand Pharmaceuticals, San Diego), in 12 patients with metastases of DTC and decreased or absent I-131 uptake. Bexarotene is an RXR agonist, which also induces RAR by transcriptional activation. The antineoplastic potential has been demonstrated in cutaneous T-cell lymphoma, but also in other malignant tumors (26 - 28).

Patients and Methods

Design

The study was a 6-week open study with 12 patients. Patients underwent diagnostic

I-131 whole body scintigraphy (WBS) before, and after 6-weeks treatment with Bexarotene 300 mg/day. An open study design was chosen, because the study parameters can be assessed by objective criteria. Each patient served as his/her own control. An interval of 6 weeks between the two observations was chosen to allow normalization of serum TSH concentrations after the first application of rhTSH and to enable complete disappearance of the first I-131 dose from the tumor. The objective of this study was to investigate if addition of Bexarotene has beneficial effects on radioiodine uptake in metastatic lesions of patients with DTC.

Patients

The Leiden University Medical Center is a large referral center for differentiated thyroid carcinoma in the Netherlands. With the exception of unifocal T-1,N-0,M-0 tumors, initial therapy consists of near-total thyroidectomy followed by routine I-131 ablative therapy with 3700 MBq I-131. Follow-up is performed according a standard protocol, involving serum thyroglobulin (Tg) measurements, both during Thyroxine suppressive therapy and after Thyroxine withdrawal as well as I-131 scintigraphy after Thyroxine withdrawal. In case of recurrent disease or metastases, surgery will be attempted if the lesion is solitary and accessible, followed by additional radioiodide therapy (7400 MBq).

For the present study, 12 consecutive patients were selected with metastases of DTC as proven by measurable serum Tg levels and the presence of metastases or recurrent disease at post-therapeutic whole body scintigraphy, X-ray, CT or MRI. A CT scan performed < 3 months prior to the study served as anatomical reference for the number, extent and localization of metastases. Patients who were selected had to have undergone total thyroidectomy and I-131 ablative therapy. Uptake of I-131 or effectiveness of earlier I-131 therapies had to be insufficient as indicated by progressive tumor growth despite I-131.

Exclusion criteria were pregnancy, contraindications for the application of recombinant human thyrotropin (rhTSH), contraindications for the use of Bexarotene such as hematological malignancies, leukopenia or coagulopathy, a history of pancreatic disease and severe hypertriglyceridemia (fasting triglyceride levels > 4.5 mmol/l).

The institutional review board approved the study, and all patients gave written informed consent.

Protocol

A CT scan performed < 3 months prior to the study served as anatomical reference for the number, extent and localization of metastases. After inclusion, the patients underwent a first diagnostic scintigraphy 3 days after intravenous administration of 185 MBq I-131. Patients were prescribed a low iodide diet from 7 days prior

to the administration of I-131 (29). The patients received i.m. injections with 0.9 mg rhTSH (Thyrogen®, Genzyme, Naarden) on 2 consecutive days before the I-131 administration. rhTSH instead of Thyroxine withdrawal was used to avoid the methodological and clinical disadvantages of persistent high TSH levels during a long withdrawal period.

The day after the first WBS, patients started treatment with Bexarotene 300 mg/day at the evening meal to prevent interference with Thyroxine absorption.

Six weeks after initiation of Bexarotene therapy, the I-131 imaging study was repeated. Bexarotene was continued until the WBS was performed. Patients visited the hospital every week for a physical examination and assessment of laboratory safety parameters. When the intervention was successful (see below), patients were offered high dose I-131 therapy, again preceded by 6 weeks Bexarotene therapy.

Evaluation of the study objectives

The main outcome parameter of the study is the effect of Bexarotene therapy on I-131 uptake in metastases at WBS. Uptake was investigated as follows: a quantitative assessment of I-131 uptake was performed by calculating uptake in a region of interest using a reference I-131 source (see below). In addition, uptake was compared between the first and the second WBS in comparable regions and expressed as "increased", "stable", "decreased" or "mixed". "Mixed" was used when both lesions with increased, stable or decreased uptake were present. It was studied also if there was a complete or incomplete matching of areas with I-131 uptake at WBS and metastatic locations as visualized by CT scanning.

A "complete response" was defined as increased I-131 uptake in all lesions visible on CT. A "partial response" was defined as increased I-131 uptake as compared with the first WBS, but not in all lesions visible at CT. "No response" was defined as absent or similar I-131 uptake in both WBS. The study was defined as successful when at least 50% of the patients had at least a partial response.

Whole body scintigraphy with 185 MBq I-131

131I whole-body scintigraphy was performed 3 days after the oral administration of 185 MBq of 131I (Mallinckrodt BV, Petten, The Netherlands). The run speed of the dual-head gamma camera (Toshiba GCA 7200, equipped with a high-energy collimator) was 15 cm per minute (matrix size 256×256). WBS was followed by anterior and posterior planar images of the head and neck and chest region (matrix size 256×256, preset time 10 min). Finally, single photon emission computed tomography (SPECT) of the head and neck and chest was performed (128x128 matrix, 6o step angle and 1 min. per step). Two experienced observers visually analyzed all images. A Na131I standard was used to quantify the uptake in the area of interest at WBS.

Laboratory parameters

The following laboratory parameters were assessed: TSH, free-T4, free-T3 and Tg were measured before both injections of rhTSH, before the administration of I-131 and during the WBS. Tg antibodies were measured before both rhTSH injections. Safety parameters were a hematological profile as well as serum levels of sodium, potassium and creatinine, lipids, renal and liver function. They were assessed every week. Urinary iodine excretion was measured to exclude iodine contamination.

Serum TSH was determined with on a Modular Analytics E-170 system (Roche Diagnostic Systems, Basle, Switzerland), intra-assay variability: 0.88-10.66%, inter-assay variability: 0.91-12.05%). Serum Tg was determined with IRMA (Tg kit, Brahms, Berlin Germany) on a Wallac (Wallac, Turku, Finland), intra-assay variability: 0.14-13.9%, inter-assay variability: 12.3-17.4 %). Serum Tg antibodies were determined with IRMA (Sorin Biomedica, Amsterdam, The Netherlands) on a Wallac (Wallac, Turku, Finland) intra-assay variability: 3.6-4.1%, inter-assay variability: 11.6%).

Statistical Methods

Data are reported as mean ± SD. The effects of bexarotene on outcome variables were analyzed using the two-tailed Student's t-test for paired data. Data without normal distribution were analyzed using the Wilcoxon test. Proportional data were analyzed using Chi-square. Differences were considered statistically significant at $P<0.05$. The calculations were performed using SPSS 12.0 for windows (SPSS, Chicago, IL).

Results

Patients

Twelve patients were included in the protocol (5 males, 7 females). Their clinical characteristics are presented in Table 1. The mean age at diagnosis of DTC was 49 ± 11 years. Most patients had papillary thyroid carcinoma. In 3 of the patients, metastases were already present at the time of diagnosis of thyroid carcinoma, most of them pulmonary. Most patients had received extensive therapies; I-131 therapy had been administered in a median cumulative activity of 16 GBq (Table 1). Seven of the 12 patients had received additional therapies during the course of their disease (surgery and/or external radiotherapy).

One patient (nr 3) died during the study. She was admitted to the hospital and underwent acute surgery for intestinal volvulus. This event was considered to have no relation with the study. The other patients tolerated the Bexarotene treatment well. However, in 2 patients (nr. 6 and 9), the dose had to be reduced because

Table 1. Patient Data

Patient	Gender	Age (Diagnosis)	Histology	pTNM (Diagnosis)	Cumulative Activity I-131 (MBq)	Additional Therapy	Disease free interval (years)	Relapse or Metastases
1	M	71	PTC	X-X-1	5640	RT	0	Lungs
2	F	37	FTC	4-0-0	22560	RT, Surgery	6	Local, lungs
3	F	36	PTC	1-0-0	57600	Neck Surgery	24	Lungs
4	F	50	FTC	1-0-1	15416		17	Lungs
5	F	61	Hűrthle cell FTC	4-0-1	9738	RT, Thoracic Surgery	0	Lungs
6	M	52	Hűrthle cell FTC	3-0-0	13893	RT, Thoracic and Neck surgery	4	Lungs
7	M	50	PTC	4-0-0	16500		0	Lungs
8	F	32	FTC	3-0-0	41000	RT, Neck surgery	0	Lungs
9	F	51	FTC	4-0-1	27372	RT	0	Lungs
10	M	47	PTC	3-0-1	13160	Neck Surgery	7	Lungs
11	F	45	PTC	2-0-0	27000		10	Lungs
12	M	59	Hűrthle cell FTC	3-3-0	14100	Neck Surgery	0	Lungs

of hypertriglyceridemia that stabilized after dose reduction. One patient (nr 2), experienced an episode of leucopenia, which also lead to a dose reduction of Bexarotene.

Biochemical parameters

No differences in TSH levels without and after rhTSH stimulation were observed before and after 6 weeks Bexarotene treatment (Table 2). There was a remarkable decrease in serum free T4 and serum free T3 levels after 6 weeks Bexarotene treatment. Serum Tg levels before and after rhTSH were not different before and after Bexarotene therapy. No iodine contamination was observed according to urinary iodine measurements.

Table 2. Biochemical data

	Before Intervention	After Intervention	p
Before rhTSH			
Free Thyroxine (pmol/L)	25.7 ± 6.5	13.2 ± 3.4	<0.001
Free T-3 (pmol/L)	3.6 ±1.3	2.1 ± 1.0	0.016
Thyrotropin (mU/L)	0.025 (<0.005 – 2.18)	0.024 (<0.005 – 1.06)	0.652
Thyroglobulin (ug/L)	108 (2.4 – 880)	158 (3.7 – 1145)	0.892
After rhTSH			
Free Thyroxine 24 h (pmol/L)	25.7 ± 5.6	13.6 ± 3.3	
Thyrotropin 24 h (mU/L)	190.5 (89.2 – 324)	165.6 (100-312)	0.561
Thyrotropin 72 h (mU/L)	17.7 (10.2 – 44.3)	19.6 (12.0 – 56.1)	0.538
Thyroglobulin 24 h (ug/L)	112 (14.7 - 1390)	163 (20.9 – 1905)	0.704
Thyroglobulin 72 h (ug/L)	123 (25.7 – 2650)	165 (45.2 – 1558)	0.747
Cholesterol (mmol/L)	5.4 ± 1.0	7.8 ± 1.2	<0.001
Triglycerides (mmol/L)	1.6 ± 0.7	3.7 ± 1.5	<0.001

Evaluation of the study objectives

The main outcome parameter of the study is the effect of Bexarotene therapy on I-131 uptake in metastases at WBS. No patients with a complete response were observed (Table 3). A partial response was observed in 8 patients. In 7 of these patients, increased uptake was only visible at SPECT, indicating that the accumulation of iodide was low. Scans of 2 of these patients(nr5 and 6) are depicted in Figure 1.

Table 3. Diagnostic Whole Body Scintigraphy 3 Days After 185 MBq I-131

	Before Intervention			After Intervention			Outcome
Patient	WBS	SPECT	Matching	WBS	SPECT	Matching	
1	No Uptake	No Uptake		No Uptake	Superior mediastinal	Incomplete	Partial response
2	No Uptake	No Uptake		No Uptake	Parahilar	Incomplete	Partial response
3	No Uptake	Mediastinal discrete	Incomplete	#			
4	No Uptake	Pulmonary discrete	Incomplete	No Uptake	Pulmonary, increased intensity	Incomplete	Partial response
5	No Uptake	Pulmonary discrete	Incomplete	No Uptake	Pulmonary, increased intensity	Incomplete	Partial response
6	No Uptake	No Uptake		No Uptake	Mediastinal	Incomplete	Partial response
7	No Uptake	Neck, mediastinal discrete	Incomplete	No uptake	Neck, mediastinal discrete (unaltered)	Incomplete	No response
8	Pulmonary	Pulmonary	Incomplete	Pulmonary	Pulmonary, new locations	Incomplete	Partial response
9	No Uptake	No Uptake		No Uptake	No Uptake		No response
10	No Uptake	No Uptake		No Uptake	Pulmonary	Incomplete	Partial response
11	No Uptake	No Uptake		No Uptake	Pulmonary	Incomplete	Partial response
12	No Uptake	No Uptake		No Uptake	No Uptake		No response

Patient died

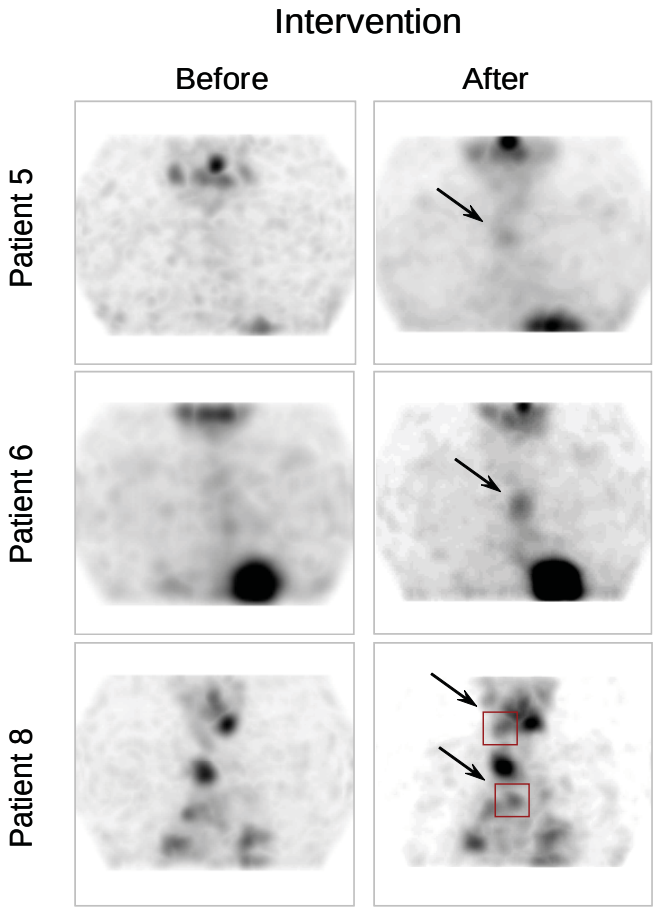

Figure 1. I-131 uptake before and after treatment of 6 weeks with 300 mg/day Beaxoretene in 3 patients with pulmonary metastases of DTC. In all patients, a subtle increase in I-131 uptake was observed after Bexarotene therapy at SPECT imaging, 3 days after 185 MBq I-131. The protocol for image processing is described in the Methods. In patient 8, new lesions (boxes) became apparant after bexarotene therapy.

The number of lesions with increased or visible I-131 uptake was lower than visible at the reference CT scan. In 1 patient, pulmonary metastases were visible at the baseline WBS. Because the matching of these metastases was incomplete, it was decided to include her in the study. After 6 weeks Bexarotene, WBS revealed uptake in additional lesions that were not visible before (Figure 1, patient 8).

Although it was attempted to quantify I-131 by calculating uptake in a region of interest using a reference I-131 source, uptake in regions of interest as visualized by SPECT were too low to allow quantification.

Discussion

The present study investigated the effectiveness of 6-weeks Bexarotene treatment in reinducing I-131 uptake in metastases of patients with DTC with absent or insufficient uptake of I-131 during earlier I-131 therapies. Bexarotene treatment induced I-131 uptake in the majority of the patients (8/11), but the uptake was only discernable at SPECT and not present in all metastases, visualized by CT scanning. Therefore, the clinical relevance of these findings remains to be determined.

All clinical studies performed so far with retinoids in DTC used 13-cis retinoic acid (18 - 22). The study with the best design (22), however, failed to demonstrate any positive effect. Because 13-cis retinoic acid has a limited specificity and affinity for the retinoic acid receptor (23) and the importance of RAR subtypes and RXR have been demonstrated in recent studies (24,25), we hypothesized that a ligand with RXR affinity and also affinity for RAR may have beneficial effects (26 - 28,30).

Several factors may be involved in the partial success of the intervention. I-131 accumulation is not only determined by the trapping of iodide by NIS, but also by the effective half life. The effective half-life of I-131 is diminished in DTC by several factors including decreased organification of iodide due to decreased thyroid peroxidase expression as well as the loss of follicular architecture (31,9). Therefore, enhancing NIS expression may not be adequate to reach sufficient radiation exposure to I-131, even if we used a low iodide diet (29) to increase the specific activity of the I-131 administered. Alternatively, the regulation of NIS may be defective at multiple transcriptional and post-transcriptional levels (32), which can only be partially restored by retinoids.

An interesting observation was that in one patient (nr. 8), a new lesion became apparent after Bexarotene, which did not accumulate iodide earlier. This is an interesting illustration of the heterogeneity in DTC metastases with respect to iodide metabolism.

Free serum Thyroxine and triiodothyronin levels decreased markedly in all patients without increase in TSH levels. Although the effects of Bexarotene on TSH have been well established (33), the fact that Bexarotene decreases thyroid hormone levels in patients in whom thyroid hormone levels are TSH independent suggests an effect on thyroid hormone metabolism. We do not believe that the differences in thyroid hormone levels after Bexarotene have affected the study results, as TSH induction after rhTSH was comparable before and after Bexarotene.

We conclude that Bexarotene treatment may partially restore I-131 uptake in some, but not all, metastases of DTC. The clinical importance of this observation remains to be demonstrated but may be limited by the incomplete matching and the low intensity of I-131.

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Chapter 7

Radioiodine Therapy after Pre-treatment with Bexarotene for Metastases of Differentiated Thyroid Carcinoma

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Submitted

Abstract

Objective: To evaluate the effects of pre-treatment with the RXR agonist Bexarotene on the efficacy of radioiodine therapy of metastases of differentiated thyroid carcinoma (DTC) with limited uptake of radioiodine (I-131).

Design: Open prospective intervention study.

Methods: Eight patients with metastases of DTC, with insufficient uptake of I-131 who showed increased uptake of radioiodine after previous treatment with 300 mg Bexarotene were treated with radioiodine (7400 MBq), preceded by 6 weeks of treatment with Bexarotene 300 mg/day. Outcome parameters were serum Tg levels and dimension of metastases at CT, measured before, and 6 months after, therapy.

Tissue of the primary tumor was stained with antibodies against RAR and RXR subtypes.

Results: Bexarotene pre-treatment induced radioiodine uptake in metastases in all 8 patients, although uptake was only discernable at SPECT and had incomplete matching with the metastases visualized by CT scanning. Six months after radioiodine therapy 6 patients had progressive disease (defined as a >10% increase in serum Tg and/or a >25% increase in tumor dimensions), whereas 2 patients had stable disease. No relation was observed between retinoid receptor staining pattern and the outcome of therapy.

Conclusions: Bexarotene partially restores I-131 uptake in metastases of DTC, but this did not result in susceptibility to radioiodine therapy.

Introduction

The efficacy of radioiodine therapy in metastatic thyroid carcinoma is limited by decreased uptake of radioiodine, which is likely related to decreased expression or function of the sodium iodide symporter (NIS) in DTC during the process of dedifferentiation (1,2,3). Therefore, strategies to improve iodide uptake by DTC are mandatory.

Retinoids are derivatives of vitamin A (*i.e.* retinol). Beneficial effects of retinoids have been reported in *vitro* in thyroid carcinoma (4,5,6,7) including increased NIS mRNA expression and iodide uptake in some thyroid cancer cell lines (4). Interestingly, the promoter of the NIS gene has a retinoic acid response element (8). A limited number of human studies all performed with 13-cis retinoic acid reported variable results (9,10,11,12)(13). The only retinoid used so far in human studies in DTC is 13-cis retinoic acid. As 13-cis retinoic acid has a lower affinity for RAR than other retinoids (14) and the retinoid receptor RXR may also be important in thyroid carcinoma (15,16), we performed a prospective controlled clinical trial to investigate the efficacy of the novel ligand Bexarotene (Targretin, Ligand Pharmaceuticals, San Diego) (17,18,19), in 12 patients with metastases of DTC and decreased or absent I-131 uptake (20). We found increased uptake in metastases in 8 of these patients. Here, we report the results of high dose I-131 therapy after preparation with Bexarotene in these 8 patients.

Patients and Methods

Patients

Patients in whom 6-weeks therapy with Bexarotene 300 mg/day increased radioiodine uptake in metastases of DTC (20) were offered therapy with 7400 MBq radioiodine.

Detailed inclusion criteria and clinical data of the patients in this study are given in our previous study (20) and are summarized in Table 2. In summary, patients were selected with metastases of DTC, who had previously undergone total thyroidectomy and I-131 ablative therapy. Uptake of I-131 or effectiveness of earlier I-131 therapies had to be insufficient as indicated by progressive tumor growth despite I-131.

Exclusion criteria were pregnancy, contraindications for the application of recombinant human thyrotropin (rhTSH), contraindications for the use of Bexarotene such as hematological malignancies, leukopenia or coagulopathy, a history of pancreatic disease and severe hypertriglyceridemia (fasting triglyceride

levels > 4.5 mmol/l). Of the original 12 patients that enrolled in the first study, 8 patients were eligible for treatment with high dose radioiodine.

Protocol

Radioiodine therapy (7400 MBq, Mallinckrodt BV, Petten, The Netherlands) was given after a new 6-weeks treatment with Bexarotene (300 mg/day). A new treatment course with Bexarotene was given because it was not known how long the effects of the first course of Bexarotene would last.

Prior to radioiodine therapy, patients received i.m. injections with 0.9 mg rhTSH (Thyrogen®,, Genzyme, Naarden) on 2 consecutive days before the I-131 administration. rhTSH instead of withdrawal of L-thyroxin substitution was used because Bexarotene is reported to inhibit pituitary TSH production (21). Patients were prescribed a low iodide diet from 7 days prior to the administration of I-131 (22).

Evaluation of the study objectives

The main outcome parameter of the study was the effect of treatment on the progression of metastases of DTC 6 months following I-131 therapy with pretreatment of Bexarotene.

Study objectives were evaluated with CT scans and serum thyroglobulin (Tg) measurements as assessed before Bexarotene therapy and 6 months after radioiodine treatment.

A CT scan obtained before radioiodine therapy served as anatomical reference for the number, extent and localization of metastases. The response was determined as complete response (no disease demonstrable), incomplete response (decrease in Tg $\geq$ 10%, decrease in radiological dimensions of metastases $\geq$ 25%), stable disease (difference between serum Tg levels < 10% and progression in radiological tumour dimensions < 25%) or progressive disease (difference between serum Tg levels $\geq$ 10% or progression in radiological tumour dimensions $\geq$ 25% or the appearance of new metastatic lesions).

Outcome of radioiodine therapy was related to retinoid acid receptor expression in a subset of patients.

131-I whole-body scintigraphy was performed 3.5 and 7 days after the radioiodine therapy. The run speed of the dual-head gamma camera (Toshiba GCA 7200, equipped with a high-energy collimator) was 15 cm per minute (matrix size 256×256). WBS was followed by anterior and posterior planar images of the head and neck and chest region (matrix size 256×256, preset time 10 min). Finally, single photon emission computed tomography (SPECT) of the head and neck and chest was performed (128x128 matrix, 6^{o} step angle and 1 min. per step). Two experienced observers

visually analyzed all images. A $Na^{131}I$ standard was used to quantify the uptake in the area of interest at WBS.

Immunohistochemistry

Immunhistochemstry was performed on tissue blocks obtained from the primary tumors. Tissues from 2 patients, who did not respond to Bexarotene in an earlier study (20), (Pat NI-1 and Pat NI-2), were also included in the staining procedure.

Ten percent formalin-fixed, paraffin-embedded blocks routinely prepared from surgical specimens of primary thyroid tumours were selected for this study. Four μm consecutive tissue sections were cut from each arrayed paraffin block and prepared on pathological slides. The sections were deparaffinised in xylene followed by 0.3% hydrogen peroxide methanol at room temperature for 20 minutes for blocking endogenous peroxidase. After rehydration, antigen retrieval treatment was done for CK-19, HBME-1, FN-1, CITED-1, NIS and PPAR-gamma but Gal-3 immunostaining by microwave treatment in 0.01 M citrate buffer at pH 6.0. After 2 hours cooling down, endogenous avidin activity blocking was performed for NIS immunostaining by incubation with egg-white for 5 minutes followed by biotin for 15 minutes. The sections were incubated with primary antibodies against RAR and RXR (Table 1) in PBS with 1% bovine serum albumin overnight in room temperature. The negative controls were stained with the primary antibody omitted. Next, sections were incubated for 30 minutes with either the biotinylated rabbit-anti-mouse conjugate (Dako, Glostrup, Denmark, 1:200) or goat-anti-rabbit (1:400), followed by incubation for 30 minutes with the streptavidin-biotin-peroxidase conjugate (Dako, Glostrup, Denmark 1:100). This step was by a 10-minute incubation with 3,3'-diaminobenzidinetetrachloride substrate in a buffered 0.05 M Tris/HCl (pH 7.6) solution containing 0.002% hydrogen peroxide. The sections were counterstained with haematoxylin. A semi-quantitative assessment of immunohistochemical scoring was performed according to both the intensity of staining and the percentage of positive cells. Ranging from 1 – 6.

Laboratory parameters

The following laboratory parameters were assessed: plasma levels of TSH, free-T4, free-T3 and Tg were measured before both injections of rhTSH, before the administration of I-131 and during the WBS. Tg antibodies were measured before both rhTSH injections. Safety parameters were a hematological profile as well as serum levels of sodium, potassium and creatinine, lipids, renal and liver function. They were assessed every week. Urinary iodine excretion was measured to exclude iodine contamination.

Serum TSH was determined with on a Modular Analytics E-170 system (Roche Diagnostic Systems, Basle, Switzerland, intra-assay variability: 0.88-10.66%, inter-

Table 1 Antibodies against Retinoic Acid receptors

Primary ntibody	Dilution	Resource	Type	Secondary Antibody	Antigen Retrieval	epitope	Ab clone
RARα	1:3000	Gift[1]	Monoclonal	2	Na-Citrate heating	Ab9α(F)	9 α-9A6
RARβ	1:200	Gift[1]	Monoclonal	2	Na-Citrate heating	Ab8β(F)2	8β-10B2
RARγ	1:350	Gift[1]	Monoclonal	2	Na-Citrate heating	Ab4γ (F)	4γ-7A11
RXRα	1:1000	Gift[1]	Monoclonal	2	Na-Citrate heating	full length	4RX3A2
RXRβ	1:650	Santa Cruz [2]	Polyclonal	1	Na-Citrate heating	6p21.3	sc-831
RXRγ	1:500	Santa Cruz [2]	Polyclonal	1	Na-Citrate heating	1q22-q23	sc-555

Secondary Antibodies

(1) Swine-anti-Rabbit, DacoCytomation, Glostrup, Denmark

(2) Rabbit-anti-Mouse, DacoCytomation, Glostrup, Denmark

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assay variability: 0.91-12.05%). Serum Tg was determined by IRMA (Tg kit, Brahms, Berlin Germany) on a Wallac (Wallac, Turku, Finland, intra-assay variability: 0.14-13.9%, inter-assay variability: 12.3-17.4 %). Serum Tg antibodies were determined with IRMA (Sorin Biomedica, Amsterdam, The Netherlands) on a Wallac (Wallac, Turku, Finland) (intra-assay variability: 3.6-4.1%, inter-assay variability: 11.6%).

Statistical Methods

Data are reported as mean ± SD. The effects of bexarotene on outcome variables were analyzed using the two-tailed Student's *t*-test for paired data. Data without normal distribution were analyzed using the Wilcoxon test. Proportional data were analyzed using Chi-square. Differences were considered statistically significant at $P<0.05$. The calculations were performed using SPSS 12.0 for windows (SPSS, Chicago, IL).

Results

Patients

Eight patients were included in this treatment protocol (4 males, 4 females). Their clinical characteristics are presented in Table 2. The mean age at diagnosis of DTC was 52 ± 10 years. In 2 of the patients, metastases were already present at the time of diagnosis of thyroid carcinoma, most of them pulmonary. Most patients had received extensive therapies; I-131 therapy had been administered in a median cumulative activity of 15 GBq (Table 1). Four of the 8 patients had received additional therapies during the course of their disease (surgery and/or external radiotherapy).

The patients tolerated the Bexarotene treatment well, despite temporary increases in serum triglyceride levels in 5 subjects. In 1 patient the dose of Bexarotene had to be reduced because of an episode of leucopenia.

Evaluation of the study objectives

The main outcome parameters of the study were the treatment effects 6 months following I-131 therapy after preparation with Bexarotene on the progression of metastases of DTC.

No incomplete or complete responses were observed. Six patients had an increase in serum Tg levels of >10% (Table 2). One patient had a relatively low serum Tg level (2.4 ug/L) but this level rose to 64.8 ug/L after TSH stimulation. Three patients had an increase in tumor dimensions at CT of > 25%. In 2 patients, new lesions

Table 2. Patient Data

atient Number	Gender	Age (Diagnosis)	Histology	pTNM (Diagnosis)	Relapse or Metastases	Immunohistochemstry				
						RARα	RARβ	RARγ	RXRα	RXRβ
1	M	71	PTC	X-X-1	Lungs	+	-	-	+	-
2	F	37	FTC	4-0-0	Local, lungs	-	+	+	-	+
3	F	50	FTC	1-0-1	Lungs	NA	NA	NA	NA	NA
4	F	61	Hűrthle cell FTC	4-0-1	Lungs	+	+/-	-	+, very few	+
5	M	52	Hűrthle cell FTC	3-0-0	Lungs	+	+	-	+, few	-
6	M	50	PTC	4-0-0	Lungs	+	-	+?	+	+
7	M	47	PTC	3-0-1	Lungs	+	+	-	+	-
8	F	45	PTC	2-0-0	Lungs	NA	NA	NA	NA	NA

Table 3. Study Outcome 6 Months After 6400 MBq I-131

Patient	Tg (ug/L) Baseline	Tg (ug/L) After rhTSH	WBS Post Therapy	Matching	Tg (ug/L) 6 Months After Radiotherapy	CT	Outcome
1	280	530	Superior mediastinal	Incomplete	456	Progression new lesions	Progression
2	150	203	Parahilar	Incomplete	813	Progression	Progression
3	5	45.2	Pulmonary	Incomplete	5,3	Progression < 25%	Stable disease
4	312	890	Pulmonary	Incomplete	1602	Progression	Progression
5	88.7	321	Mediastinal, *pulmonary* #	Incomplete	300.5	New lesion	Progression
6	14	42,7	Neck, mediastinal discrete	Incomplete	17	Progression < 25%	Progression
7	9.7	29.4	Pulmonary	Incomplete	10.8	Progression < 25%	Stable disease
8	2.4	64.8	Pulmonary	Incomplete	3.2	Stable	Progression

Not visible at diagnostic scintigraphy post-Bexarotene

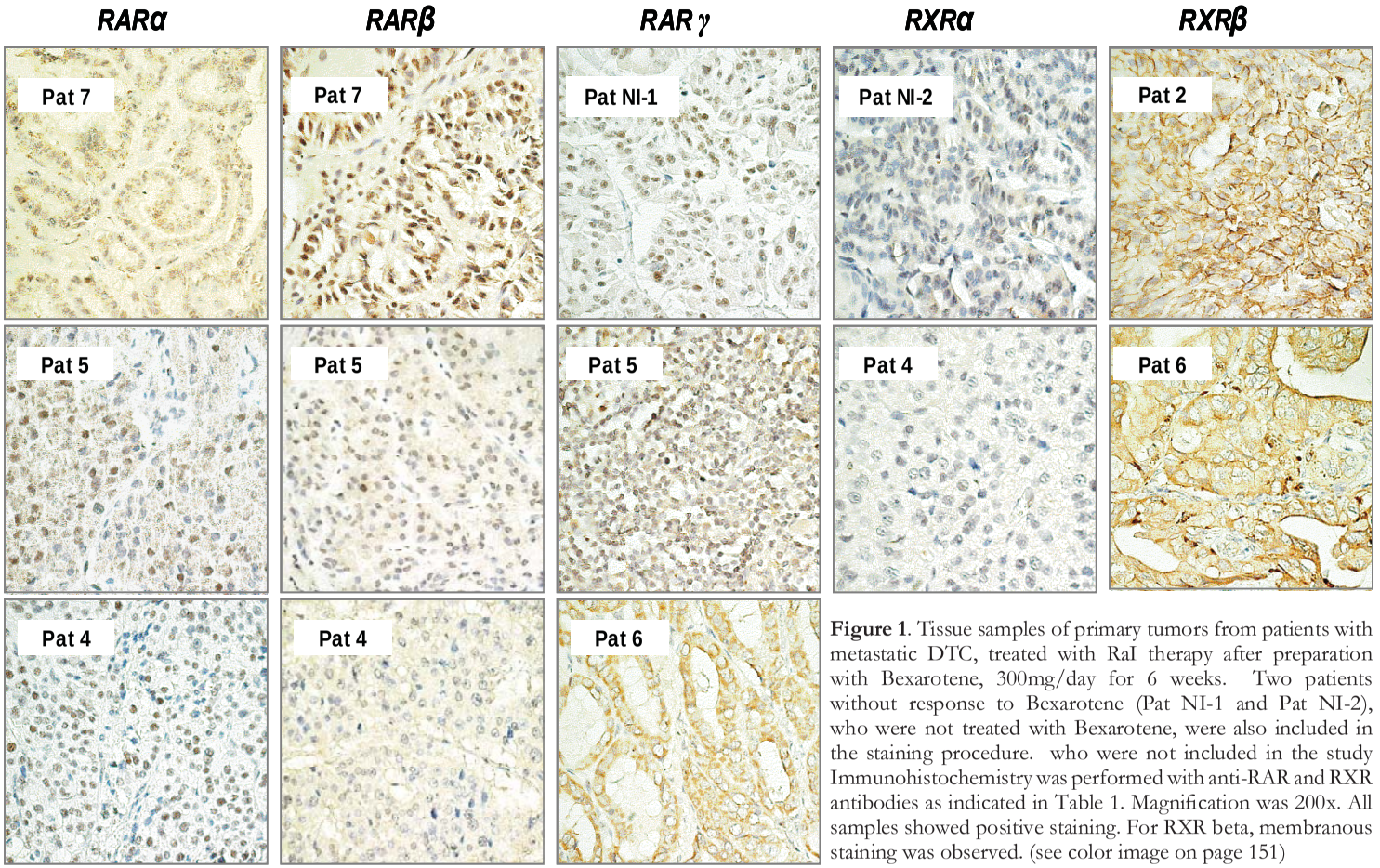

Figure 1. Tissue samples of primary tumors from patients with metastatic DTC, treated with RaI therapy after preparation with Bexarotene, 300mg/day for 6 weeks. Two patients without response to Bexarotene (Pat NI-1 and Pat NI-2), who were not treated with Bexarotene, were also included in the staining procedure. who were not included in the study Immunohistochemistry was performed with anti-RAR and RXR antibodies as indicated in Table 1. Magnification was 200x. All samples showed positive staining. For RXR beta, membranous staining was observed. (see color image on page 151)

appeared. In 3 patients, there was progression at CT but less than 25%.

All patients had received a previous course of Bexarotene to prove increased radio uptake during diagnostic scintigraphy. No differences were observed between diagnostic scintigraphy after this first Bexarotene treatment and the post-therapeutic whole body scans, and, except in one patient (nr. 5) in whom discrete pulmonary lesions became visible at SPECT after post-therapeutic WBS (Table 3). In all patients, there was an incomplete matching of lesions observed at post-therapeutic WBS with the CT scans.

Although it was attempted to quantify I-131 by calculating uptake in a region of interest using a reference I-131 source**,** uptake in regions of interest as visualized by SPECT were too low to allow quantification.

Immunohistochemstry

Data for immunohistochemstry are given in Table 2. Apparently there was no uniform pattern in staining for RAR and RXR subtypes (Figure 1) (see color image on page 151), and no relation was apparent with staining pattern and outcome of therapy.

Discussion

The present study investigated the effectiveness of radioiodine therapy after 6-weeks pre-treatment with the RXR agonist Bexarotene on metastases of patients with DTC with absent or insufficient uptake of I-131 during previous I-131 therapies. Although Bexarotene treatment had shown to induce I-131 uptake in these 8 patients (20), no clinically relevant response to radioiodine was observed. As the initially observed uptake of radioiodine was only discernable at SPECT and not present in all metastases as visualized by CT scanning, the clinical efficacy of Bexarotene therapy is limited.

The background of our study was that the compound used in clinical studies in DTC has been 13-cis retinoic acid (9,10,11,12,13) which had inconsistent effects. Following the observation that the RXR may be important in DTC (15,16), we decided to treat patients with an RXR agonist which also has affinity for the RAR (17,18,19,21).

The lack of success may be explained by several factors. The dose of radioiodine that is realized in a metastatic lesion is determined not only by the trapping of iodide by NIS, but also by the effective half life of the radioisotope, which may be decreased in DTC by decreased organification of iodide due to decreased thyroid peroxidase

expression as well as the loss of follicular architecture (23,3). Alternatively, the regulation of NIS may be defective at multiple transcriptional and post-transcriptional levels (24), which can apparently only be partially restored by retinoids.

An important observation was that there was only incomplete matching between the metastases identified by radiological imaging and post-therapeutic WBS. Although this is an interesting observation, illustrating the heterogeneity of DTC metastases with respect to iodide metabolism, this incomplete matching suggests that the beneficial effects of Bexarotene may be present, at best, in a subset of metastases. However, even if these metastases become susceptible to radioiodine, this does not prevent the progression of other lesions.

The question is whether patients with other characteristics might have a better response to Bexarotene. To investigate this issue, we performed RAR and RXR staining in a subset of patients. However, we did not find a relation between staining pattern and outcome of therapy. A limitation in this respect is that we only had materials of the primary tumor and it may be that the retinoid receptor expression pattern in the metastases was different.

We conclude that Bexarotene treatment partially restores I-131 uptake in some, but not all, metastases of DTC, at least in the patients selected for this study. Due to inhomogeneous effects of Bexarotene on I-131 uptake by the different metastases within each patient and the low intensity of I-131 uptake, the clinical efficacy of Bexarotene pretreatment is limited.

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Chapter 8

Summary & Disscussion

1. Introduction

Differentiated thyroid carcinoma (DTC) has a low incidence and a relatively good prognosis. This relatively favourable prognosis is the result of the biological behaviour of most of these tumors and the efficacy of initial therapy. However, the therapeutic arsenal in DTC is very limited. Once distant metastases have occurred, usually in the lungs or bones, the prognosis is worse, because the results of RaI therapy, which is virtually the only curative treatment, are moderate. A major problem in progressive or metastatic disease is the diminished, or lost, ability of thyroid cancer cells to accumulate RaI, indicated by negative post-therapeutic whole body scintigraphy. In these cases, the prognosis is poor, because alternative treatment options (external radiotherapy or chemotherapy) have only limited success.

Because of the low incidence and favourable prognosis, diagnostic and therapeutic strategies are hard to investigate: the follow-up time is too long and the numbers too small to reach significant endpoints in prospective randomized trials. As a result, many of the current treatment and follow-up protocols are derived from large retrospective studies, mostly from single centers, with many sources of bias. Another aspect is the decentralised approach of the disease. Despite the low incidence, many centers treat patients with DTC. One of the examples of this decentralised approach is the existence of many staging systems, which make comparisons between centers difficult.

DTC is a unique malignant disease in which fascinating biological phenomena are present, like the pathophysiology of iodide transport. This makes DTC an attractive model to study the molecular mechanisms of iodide transport, and to find targets to re-establish iodide transport. This unique position, however, also adds to the somehow isolated situation: DTC is a type of cancer that is less well recognized in the mainstream of novel anti-cancer drugs trials. An example of an unresolved diagnostic dilemma is that the diagnosis of DTC is still largely dependent on conventional histological staining procedures. Despite experimental studies with gene- and protein expression profiles, no important innovation has been introduced in the past decades with respect to diagnosis. This has important implications for the many patients who present with DTC, because in particular the distinction between follicular thyroid carcinoma (FTC) and follicular adenoma (FA) is impossible to make with cytology. As a consequence, many patients will undergo surgery who do not have DTC.

In every follow-up protocol of DTC serum thyroglobulin (Tg) measurements are the backbone of diagnosis of recurrent DTC. However, the many analytical and statistical aspects of Tg measurements are not always reflected in the choice of Tg cut-off values. Indeed, they are defined on some retrospective studies from large centers. An important approach would be to define institutional cut-off values which has been one of the projects in this thesis. Furthermore, the prognostic value of Tg,

in addition to conventional ones like TNM stage, histology and age is an interesting and potentially clinically important issue that has not been addressed extensively in the literature.

A fascinating aspect of DTC is the pathophysiology of iodide transport. The most important molecule for iodide transport, the sodium iodide symporter (NIS) is less functional in DTC. Although decreased expression is important, in this thesis, it was found and confirmed that defective NIS trafficking may be important as well, which may have not only consequences for future research, but also for the diagnosis of DTC.

The introduction of recombinant human TSH (rhTSH) has been important for the patient to avoid the negative aspects of thyroxine withdrawal. The assumption, however, that continuation of thyroid hormone therapy does not directly influence iodide transport has not been properly investigated.

Different approaches to improve iodide transport in DTC can be distinguished. Much attention has been focussed on lithium salts to improve iodide uptake and addition of lithium to radioiodine therapy in metastatic DTC has been recommended in the literature. In the present thesis, these diagnostic and therapeutic dilemmas in differentiated thyroid carcinoma (DTC) are approached from a clinical and experimental perspective.

2. Improving the diagnosis of DTC

The histological diagnosis of DTC, and in particular follicular thyroid lesions, is an important dilemma evaluated in Chapter 2 with huge implications for general health care is. Although the prevalence of DTC is low, that of thyroid nodular disease is not, and improvements in the current practice in which patients with follicular proliferation are referred for surgery could prevent many surgical procedures. Many genetic and immunohistochemical candidate markers have been identified, but none of those has successfully been introduced in routine diagnostic procedures. We chose to investigate the diagnostic value of a panel of proteins (Galectin-3 (Gal-3), HBME-1, CK-19, CITED-1, Fibronectin-1 (FN-1), the sodium iodide symporter (NIS) and peroxisome proliferator activated receptor (PPAR) in 177 benign and malignant thyroid tissues. Our study differed from earlier ones with regard to the identification of optimal semi-quantitative cut-off levels using ROC analysis and the use of hierarchical cluster analysis.

We found all proteins to be differentially expressed between FA and PTC. The differences between FA on the one hand and FTC and Follicular Variant of Papillary Thyroid Carcinoma (FVPTC) on the other hand were less prominent, but we found a differential expression of PPARγ, HBME-1, Gal-3, cNIS and FN-1.

The accuracies of HBME-1, FN-1 and Gal-3 for the differential diagnosis of FVPTC and FA were fair. For FN-1 the accuracy was 71% for the differentiation between

FA and FTC.

We confirmed cytoplasmic NIS overexpression in PTC and FTC. The differential expression of cNIS between subtypes of thyroid neoplasms makes it a candidate for differentiating between these lesions. Cytoplasmic NIS was also identified by cluster analysis as a potential useful marker in the discrimination between FA and malignant carcinomas.

PPARγ has been found to be downregulated in experimental models of thyroid carcinoma. The importance of the downregulation of PPARγ is also illustrated in the PPARγ/PAX8 rearrangement which was initially observed in a series of FTC. We found decreased PPARγ nuclear staining in malignant tumors, but as the percentage of positive cells varied from 50-100% in benign lesions, the diagnostic accuracies for the differentiation between follicular lesions were limited.

Cluster analysis showed that a diagnostic immunohistochemical panel comprising Gal-3 and FN-1 was 97% sensitive for all thyroid carcinomas, whereas specificity was 100%. However, HBME-1 was found to be a useful marker for the differentiation between FA and FVPTC. Because the number of FVPTC was small, hierarchical clustering did not allow a separate analysis of this group of tumors.

In conclusion, Gal-3, FN-1 and cNIS is a useful diagnostic panel in the differential diagnosis of thyroid lesions. The absence of Gal-3, FN-1 and cNIS is highly suggestive for a benign lesion. HBME-1 may be useful in the specific differentiation of FVPTC from FA.

Perspective

The findings of Chapter 2 need to be confirmed in a follow-up study in which the candidate markers are tested in cytological samples. These samples will be scored according to routine criteria and clinical decisions will be based on those. The scores of the candidate markers will be compared with the final histological diagnosis of those thyroid glands that will be removed and a comparison will also be made with the staining patterns of the candidate markers in the surgical samples.

3. The diagnostic and prognostic value of serum Tg in the follow-up of DTC

Serum Tg levels are the most important diagnostic markers in the follow-up of DTC. Recently, guidelines for the follow-up of DTC have been published by the British Thyroid Association (under the auspices of the Royal College of Physicians), the American Thyroid Association and the European Thyroid Association. In the Netherlands, the medical associations involved in DTC and the Dutch Institute for Healthcare Improvement (CBO) have also completed a consensus paper. The cut-off values for Tg levels that are advised in these documents are often not well defined, and based on retrospective studies from a limited number of large

centers. Indeed, it is advised to define institutional cut-off values. The problem with the definition of Tg cut-off values is, as with any diagnostic procedure, the gold standard that is used to the define presence or absence of disease. In DTC, Tg is considered a better marker than for instance radioiodide scintigraphy, so using iodide scintigraphies as a gold standard may lower the specificity for Tg. In addition, the levels of Tg cut-off values are dependent on what is considered the most acceptable ratio between unnecessary therapies or missed recurrent tumors. This is a subjective choice and may be different in different countries or areas. Therefore, insight into the quantitative relation between sensitivity and specificity of Tg is important, which is the base of receiver operator curve (ROC) analysis. Nevertheless, despite the analytical and methodological problems, in Chapter 3 we investigated the diagnostic and prognostic value of serum Tg measurements for tumor presence, cure and death in the follow-up of DTC by ROC analysis in a homogeneous group of 366 patients with respect to initial therapy.

We found an excellent diagnostic accuracy of serum Tg values during TSH stimulation 6 months after initial therapy (sensitivity 100%), albeit with a higher Tg cut-off level than commonly reported. The explanation for this higher cut-off value may be related to a lower initial ablation rate in our institute as compared with others, analytical differences or the use of the ROC technique. We also found that serum Tg levels before RaI ablation are an independent predictor for cure, with a cut-off level of < 27.5 ug/L. TSH stimulated Tg measurements 6 months after initial therapy and at 2 and 5 years after initial therapy were independent predictors of DTC related mortality. Notwithstanding the less than 100% specificity of Tg for DTC, which can indeed be explained by the limitations of gold standards used in our study, we agree with the policy to administer a high dose of RaI to patients with elevated Tg levels. In our opinion, a potential solution to circumvent the debate about specificity of Tg is to consider Tg a risk indicator. The independent prognostic value of serum Tg values for cure and death are arguments to include Tg in the conventional panel of risk factors. The percentage of patients with Tg antibodies (initially 27%) is in line with previous studies. The percentages of active tumor in patients with and without Tg antibodies were comparable, confirming the lack of diagnostic value of Tg antibodies.

In conclusion, our studies illustrate the importance of the definition of institutional Tg cut-off levels. Our analyses allow the definition of groups of patients with an increased risk for residual disease or mortality, in addition to conventionally used prognostic indicators.

Perspective

Given the multiple analytical and methodological aspects that are involved in Tg measurements, we believe that inter-institutional harmonization of Tg measurements should be propagated, preferably in an international context, but certainly at a national

level. In addition, uniformization of treatment protocols and standardized criteria for DTC disease activity should be established, that enable the structured follow up of DTC patients and the definition of Tg cut-off values on a multi-institutional level.

4. Triiodothyronine suppresses in vitro iodide uptake and expression of NIS

The introduction of rhTSH for the diagnosis, initial therapy (ablation) and under certain circumstances the treatment of DTC is without doubt an important innovation for patients with DTC, to avoid the disadvantages of thyroid hormone withdrawal. Although in general the diagnostic properties of rhTSH are similar to thyroxine withdrawal, the iodide uptake kinetics may not be entirely comparable. One of the aspects is that patients with rhTSH are per definition euthyroid. The assumption is that thyroid hormone does not directly affect iodide uptake. We studied in Chapter 4 whether this assumption is correct. We used the rat thyroid cell line FRTL-5 and cultured this cell-line in the presence or absence of physiological concentrations of T3 and studied proliferation, iodide uptake and NIS mRNA and protein expression. We found indeed a decreased uptake of iodide. This decreased uptake was accompanied by decreased NIS mRNA and protein expression.

Although it has been suggested that the iodide content of levo-thyroxine (T4) therapy during rhTSH may dilute the specific activity of radioiodide, and that this may be responsible for decreased radioiodine uptake, we believe that this cannot explain our findings, as the amount of iodide coming from T3 in our experiment is negligible related to the cold iodide concentration of 10 uM. In addition, differences in whole body iodide kinetics, another potential explanation for differences between rhTSH and withdrawal, cannot explain our findings either. From our results, it seems likely that T3 has effects on NIS gene expression resulting in lower functional NIS protein. It has been debated whether the promoter for NIS contains T3 responsive elements. Another explanation can be that the effects of T3 are via repression of the TSHR promoter, but our experiments do not point in that direction.

In conclusion, we found evidence for a TSH and iodide independent effect of T3 on NIS gene expression. The mechanism remains to be resolved and also the question whether the effect exists in humans. The clinical implications of this finding are not clear, but they may add to explanations of the suggested differences in iodide accumulation in rhTSH or conventionally treated patients.

Perspective

We believe that the introduction of rhTSH is an important improvement in the impact of DTC for patients. It is, however, important to study relevant aspects that are involved in the physiology of iodide metabolism, including T3. An interesting development is that there is experimental evidence that T3 may also act as a tumor suppressor. In this perspective, continuation of T3 during diagnosis or radioiodine

therapy may be advantageous.

5. Improving radioiodide therapy in DTC

Therapeutic options for metastases of DTC are limited. RaI may be effective in about half of the patients with pulmonary metastases and a small proportion of patients with bone metastases. However, the efficacy of RaI therapy is often limited by decreased iodide uptake of metastatic DTC. Strategies to improve therapeutic options can be distinguished in therapies to improve RaI therapy or identifying other targets.

Improving RaI therapy is a broad theme. Two main subthemes can be distinguished. First tumors that still accumulate iodide, second tumors in which iodide accumulation is lost.

In tumors that still accumulate iodide, improving radioiodide therapy is essentially aimed at increasing the dose of RaI. The dose of RaI is the result of the amount (activity) of RaI administered to the tumor (specific activity), the rate of uptake, the tumor volume and the effective half life of RaI, which on its turn is determined by the physical half life and the biological half life. All these contributing factors can be optimalized: the amount of RaI presented to the tumor can be improved by a low iodide diet that increases the specific activity. The uptake rate can be influenced by high TSH levels. The half-life of RaI in DTC is an important factor. The loss of follicular architecture and probably decreased activity of thyroid peroxidise (TPO) may contribute to a decreased effective half life and thus by a lower tumor dose. We focussed on this problem and studied the potential effects of lithium salts on iodide accumulation in Chapter 5.

5.1 Effects of lithium on iodide uptake and clinical outcome of radioiodide therapy

The relation between lithium salts and the thyroid has been known for long, as many patients with bipolar depression, treated with lithium salts develop hypothyroidism. The mechanism is not clear but some experimental studies have suggested that lithium inhibits the release of thyroid hormone, or in other words, confines iodide to the thyroid. Because of these properties, lithium has been used to increase the dose of RaI in benign and malignant thyroid disease. The problem however is that it is not clear if lithium influences the outcome of RaI therapy in DTC. We therefore studied the clinical effects of RaI without and with lithiumcarbonate in patients with proven metastatic DTC. In addition, controversy exists on the mechanism by which lithium increases RaI dose in DTC. We performed an in vitro study specifically aimed at lithium effects on the NIS.

We studied 12 patients with metastases of DTC that still accumulate RaI. These patients had received previous unsuccessful RaI therapy without lithium (control) that. The patients received 1200 mg lithiumcarbonate/day followed by RaI therapy.

Despite an increased uptake of RaI in 7 patients, no beneficial effect with lithium was observed on the clinical course as assessed by serum Tg measurements and radiographically. An explanation for the lack of success can be that the longer time interval between the 2 RaI therapies may have given rise to changes in biological tumor characteristics or alternatively, that the first therapy may have selected tumor cells that do not accumulate iodide (radioresistant tumor cells). We think that this is unlikely as in all patients; RaI accumulating lesions were present after the second therapy as well. In addition, although Tg levels were progressive in most patients, their long-term increment rates were not altered substantially after the first therapy. Another explanation may be that tumor cells have become resistant to RaI, in other words that apoptotic mechanisms have become defective a phenomenon that is regularly observed in oncology. Of course, it may also be that lithium did not influence the RaI kinetics at all. We studied the effects of lithiumchloride in different physiological concentrations on iodide uptake in the benign rat thyroid cell line FRTL-5, in the polarized non-thyroid MDCK cell-line, stably transfected with hNIS and in the human follicular thyroid carcinoma cell line FTC133-hNIS. Both steady state iodide uptake, initial rate uptake and iodide efflux studies were performed. The aim of these studies was to study whether lithium salts have a direct effect on NIS. No effects of lithiumchloride were found on iodide uptake or efflux, irrespective of the concentrations. We have not studied all steps of iodide physiology. As it is suggested that for the enhancement of iodide trapping by lithium intact organification is necessary we cannot rule out that in the 3-dimensional context of the thyroid, lithium may indeed inhibit the release of organified iodide, which explains the absence of lithium effects in thyroid- or non-thyroid tumors with a short half-life and absence of organification.

The results of our study challenge the reported beneficial effects of lithium in DTC.

Perspective

We believe that lithium salts have no place in RaI therapy of DTC. Alternatively, other mechanisms to improve RaI half-life should be explored. The concept of organification is interesting and it can be hypothesized that when RaI in coupled to another protein, half life may be positively influenced. However, transfection experiments with TPO may be thought of but are difficult from a clinical perspective. Another option may be the blocking of iodide efflux by using chloride channel blockers.

5.2 Effects of redifferentiation therapy with bexarotene on iodide uptake and clinical outcome of radioiodide therapy

The second subtheme of improving RaI therapy involved tumors in which iodide accumulation is lost. The holy grail here is to re-establish functional NIS expression. The pathophysiology of decreased NIS expression in DTC is complex (as indicated

above) and involves both genetic (transcriptional) defects and post-translational (trafficking) defects. Mutations in the NIS gene have infrequently be reported and the most likely explanation is that the transcription of the NIS gene is hampered by either inactivation of the gene itself or more likely decreased promoter activity. The NIS promoter has multiple responsive elements and absence of one or more activators (like TTF-1 or TSH) will cause decreased transcription. An interesting explanation is epigenetic changes in which methylation or histone deacetylation prevents transcription. Interesting studies have been published using pharmacological substances to revert these gene blockades. We have studied the effects of the retinoid receptor X (RXR) agonist Bexarotene (Chapters 6 and 7) on the reestablishment of RaI uptake in patients with metastatic DTC. Retinoids have been used earlier in DTC, but all clinical studies performed so far used 13-cis retinoic acid which binds only to the RAR subtype. Recent studies have elucidated the importance of other retinoic acid receptor subtypes, like the RXR in DTC. We therefore decided to study the effects of 6-weeks treatment with the retinoid receptor RXR activator Bexarotene 300 mg/day on I-131 uptake in 12 patients with metastatic DTC. Prior to, and after this intervention, RaI uptake was measured by whole body scintigraphy and single photon emission tomography (SPECT) 3 days after 185 MBq I-131. Diagnostic imaging was preceded by 2 consecutive injections with rhTSH. Bexarotene treatment induced I-131 uptake in metastases of 8/11 patients. An interesting observation was that in one patient a new lesion became apparent after Bexarotene, which did not accumulate iodide earlier. This is an interesting illustration of the heterogeneity in DTC metastases with respect to iodide metabolism. However, uptake was only discernable at SPECT and had incomplete matching with metastases as visualized by CT scanning.

Although the amount of RaI uptake was limited in the 8 responders, we decided to give them the benefit of the doubt and offer them therapy with high dose RaI. They received 7400 MBq radioiodine preceded by 6 weeks of treatment with Bexarotene 300 mg/day. Six months after RaI therapy 6 patients had progressive disease, whereas 2 patients had stable disease. The explanation for this partial success may be that Bexarotene did not influence other factors involved in RaI accumulation, like the effective half-life (see above). Alternatively, the regulation of NIS may be defective at multiple transcriptional and post-transcriptional levels which can only be partially restored by retinoids. Another possibility could be that the retinoic receptor expression pattern in the patients was not favorable with respect to Bexarotene therapy. To investigate this issue, we performed RAR and RXR staining in a subset of patients. However, we did not find a relation between staining pattern and outcome of therapy. A limitation in this respect is that we only had materials of the primary tumor and it may be that the retinoid receptor expression pattern in the metastases was different. We observed incomplete matching between the metastases identified by radiological imaging and post-therapeutic WBS. This observation illustrates the heterogeneity of iodide metabolism in DTC metastases and is a limitation for

redifferentiation therapy, because even if a subset of metastases becomes susceptible to RaI, this does not prevent the progression of other lesions.

Perspective

We have shown that redifferentiation therapy in a clinical setting is indeed able to partially restore iodide uptake in DTC. A conceptual problem with redifferentiation therapy is that this therapy is aspecific: also other genes unrelated to the disease may be influenced by these compounds. This may be a serious problem, unless a specific molecular defect in the target pathway is identified, like promyelocytic leukemia, where a translocation in the RAR is the base of retinoid therapy. The advent of designer drugs aimed at specific molecular defects in well dissected pathways, like the class of tyrosine kinase inhibitors, is an important development in cancer, and may prove to be successful in DTC as well.

6. Conclusions of the studies described in this thesis

The low incidence, high survival and the specific clinical and biological aspects of DTC complicate the diagnosis, treatment and follow-up of patients with DTC. This the way in which research and patient care for DTC patients are organized should be seriously evaluated and improved. The recent publication of multi-national guidelines is an important development. The studies in this thesis have addressed some of the clinical and fundamental questions in DTC. Although they have resolved some of the questions, many new questions have risen, warranting ongoing research.

The diagnosis of DTC, which is currently performed using conventional histological techniques, could be improved considerably by applying new markers. Our studies offer interesting perspectives for the introduction of a panel of new immunohistochemical markers that may improve the diagnosis.

Although serum Tg measurements are recommended as the most important diagnostic procedure in the follow-up of DTC, multiple analytical and statistical aspects are involved. Our studies illustrate the importance of defining institutional cut-off levels for Tg, using ROC techniques, to offer insight in the quantitative relationship between sensitivity and specificity. We found Tg to be an independent prognostic marker for cure and mortality as well, which may help the clinician to identify high-risk patients.

The ability of DTC cells to accumulate iodide is the core of radioiodine diagnostic and therapeutic procedures. The introduction of rhTSH has been a great advantage for patients, to avoid thyroid hormone withdrawal. However, among others, the assumption that continuation of thyroid hormone treatment has no effects on iodide metabolism is likely not true. We found that T3 directly influences the expression and function of NIS. Although the clinical implication of this finding is not clear, we believe that a careful evaluation of the effects of any new regimen for radioiodide

therapy on well identified aspects of thyroid physiology should be investigated as good as possible.

The most important problem in the treatment of DTC is the ineffectiveness of radioiodide therapy in a considerable proportion of patients with metastases. Strategies to improve radioiodide therapy should be based on the identification of the molecular defects in iodide physiology in DTC. In patients in whom iodide uptake is still present but ineffective, interventions aimed at improving iodide kinetics are worthwhile. We demonstrated that the frequently propagated strategy to treat patients with lithium salts did not result in a better outcome of radioiodide therapy. Nor did we observe any effect of lithium on the in vitro iodide uptake. Other approaches to improve iodide kinetics are warranted.

In patients in whom DTC metastases do not accumulate radioiodide, research is focusing on the re-expression of functional NIS. We found that the redifferentiating RXR ligand Bexarotene re-established radioiodide uptake in a group of patients. The intensity of radioiodide uptake was however low and only present in a subset of metastases. Subsequent therapy with a high activity of radioiodine was unsuccessful. Many mechanistic and conceptual questions with respect to redifferentiation therapy remain and the advent of a novel class of designer drugs aimed at well identified molecular defects, like the class of tyrosine kinase inhibitors, may prove to be more promising.

Chapter 9

Samenvatting

1. Inleiding

Gedifferentieerd schildkliercarcinoom (DTC) heeft een lage incidentie en een relatief goede prognose. Deze relatief gunstige prognose is het gevolg van het biologische gedrag van de meesten van deze tumoren en de effectiviteit van de initiële therapie. Het therapeutische arsenaal voor DTC is echter zeer beperkt. Wanneer afstandsmetastasen aanwezig zijn, gewoonlijk in de longen of het skelet, is de prognose ongunstig, omdat de resultaten van therapie met radioactief jodium (RaJ), dat de enige therapeutische mogelijkheid is, beperkt zijn. Een belangrijk probleem bij progressieve ziekte of metastasen is de verminderde of afwezige opname van RaJ door schildklierkankercellen. In deze gevallen is de prognose slecht omdat andere behandelingen (uitwendige radiotherapie of chemotherapie) slechts een beperkt succes hebben.

Vanwege de lage incidentie en de goede prognose zijn diagnostische en therapeutische strategieën moeilijk te onderzoeken: de follow-up tijd is te lang en de aantallen patiënten te klein om significante eindpunten te bereiken in prospectieve, gerandomiseerde onderzoeken. Daardoor zijn veel gangbare behandel- en follow-up protocollen gebaseerd op grote retrospectieve series, meestal van enkele centra, met vele bronnen van bias. Een ander aspect is de gedecentraliseerde benadering van de ziekte. Ondanks de lage incidentie behandelen veel ziekenhuizen patiënten met DTC. Een van de voorbeelden van de gedecentraliseerde aanpak is het naast elkaar bestaan van verschillende stageringssystemen, wat vergelijkingen tussen centra moeilijk maakt.

DTC is een bijzondere maligne ziekte met fascinerende biologische fenomenen, zoals de pathofysiologie van het jodide transport. Dit maakt DTC tot een aantrekkelijk model om de moleculaire aspecten van jodide transport te bestuderen, en om nieuwe targets te vinden om het jodide transport bij DTC te herstellen.

Deze unieke eigenschappen dragen echter ook bij aan de wat geïsoleerde positie van deze tumor: DTC is een kankersoort die minder aandacht krijgt in de ontwikkeling van nieuwe anti-kanker medicijnen.

Een voorbeeld van een onopgelost diagnostisch dilemma is dat de diagnose van DTC voornamelijk afhankelijk is van conventionele histologische kleuringen. Ondanks experimentele onderzoeken met gen- en eiwit expressie profielen is er in de laatste decennia geen belangrijke vernieuwing geweest in de diagnostiek. Dit heeft gevolgen voor de vele patiënten die zich presenteren met een schildklierknobbel, omdat in het bijzonder het onderscheid tussen folliculair schildkliercarcinoom (FTC) en folliculair adenoom (FA) onmogelijk te maken is met cytologisch onderzoek. Daarom zullen veel patiënten met een schildklierknobbel een operatie ondergaan die uiteindelijk geen kanker blijken te hebben.

Serum thyroglobuline (Tg) bepalingen staan centraal in elk follow-up protocol van

DTC. Echter, de vele analytische en statistische aspecten van Tg bepalingen worden niet altijd verdisconteerd in de keuze van Tg afkap waarden, die zijn gedefinieerd op basis van de uitkomsten van retrospectieve studies van enkele grote centra. Een belangrijke benadering zou zijn de definitie van afkapwaarden voor een bepaald instituut, hetgeen een van de doelstellingen van dit proefschrift is. Daarnaast is de prognostische betekenis van Tg, in aanvulling op conventionele prognostische factoren zoals TNM stagering, histologie en leeftijd een interessant en potentieel klinisch belangrijk element dat niet uitgebreid in de literatuur aan de orde is gekomen.

Een fascinerend aspect van DTC is de pathofysiologie van het jodide transport. Het belangrijkste molecuul bij het jodide transport is de sodium iodide symporter (NIS) die minder functioneel is bij DTC. Hoewel verminderde NIS expressie belangrijk is, werd in dit proefschrift beschreven en bevestigd dat gestoord transport van NIS naar de celmembraan ook een belangrijk fenomeen is, hetgeen niet alleen consequenties heeft voor toekomstig onderzoek maar ook voor de diagnose van DTC.

De introductie van recombinant humaan thyrotropine (rhTSH) is belangrijk om de bezwaren van schildklierhormoon onttrekking te voorkomen. De aanname dat het voortzetten van schildklierhormoon behandeling het jodide transport niet beïnvloedt is echter niet goed onderzocht.

Verschillende benaderingen om het jodide transport te verbeteren bij DTC kunnen onderscheiden worden. Veel aandacht is gericht op lithium zouten, en het toevoegen van lithium aan RaJ therapie bij DTC wordt aanbevolen in de literatuur. In het huidige proefschrift worden de diagnostische en therapeutische dilemmas in DTC benaderd vanuit een klinisch en experimenteel perspectief.

2. Het verbeteren van de diagnose van DTC

De histologische diagnose van DTC, en in het bijzonder folliculaire schildklieraandoeningen is een belangrijk dilemma dat behandeld wordt in Hoofdstuk 2 en dat grote implicaties voor de algemene gezondheidszorg heeft. Hoewel de prevalentie van DTC laag is, is de prevalentie van schildkliernodi hoog en verbeteringen in de huidige praktijk waarin patiënten met een folliculaire cytologie verwezen worden voor operatie zou veel operaties kunnen besparen. Veel genetische en immuunhistochemische kandidaat markers zijn geïdentificeerd, maar geen van deze is succesvol geïmplementeerd in routine diagnostiek.

Wij hebben de diagnostische waarde bestudeerd van een panel van eiwitten (Galectin-3 (Gal-3), HBME-1, CK-19, CITED-1, Fibronectin-1 (FN-1), the sodium iodide symporter (NIS) en peroxisome proliferator activated receptor (PPAR) bij goedaardige- en kwaadaardige schildklieraandoeningen. Ons onderzoek verschilde van eerdere studies met betrekking tot het gebruik van semi-kwantitatieve

afkapwaarden geïdentificeerd met behulp van receiver operator curve (ROC) analyses en hiërarchische cluster analyse.

We vonden een verschil in expressie van alle eiwitten tussen FA en FTC. De verschillen tussen FA en FTC enerzijds en Folliculaire variant Papillair Schildkliercarcinoom (FVPTC) aan de andere kant waren minder duidelijk, maar wij vonden wel een verschil in expressie van PPARγ, HBME-1, Gal-3, cNIS en FN-1. De accuratesse van HBME-1, FN-1 en Gal-3 voor de differentiaal diagnose van FVPTC en FA waren redelijk. De accuratesse voor het onderscheid tussen FA en FTC voor FN-1 was 71%.

Wij konden de door andere onderzoekers beschreven intracellulaire overexpressie van NIS bij PTC en FTC bevestigen. De verschillende expressie van cNIS tussen subtypes van schildkliertumoren maakt dat dit een kandidaat is voor de differentiaal diagnose van deze aandoeningen. Intracellulaire NIS expressie werd ook gevonden door cluster analyse als een mogelijk nuttige marker voor het onderscheid tussen FA en maligne tumoren.

In experimentele modellen van DTC blijkt PPARγ verminderd tot expressie te komen. Het belang hiervan wordt geïllustreerd in de PPARγ/PAX8 chromosomale herschikking die oorspronkelijk werd waargenomen werd in een serie van FTC. We vonden een verminderde kernaankleuring van PPARγ in maligne tumoren, maar omdat het percentage van positief aankleurende cellen in goedaardige lesies varieerde van 50-100%, is de diagnostische accuratesse voor het onderscheiden van folliculaire aandoeningen beperkt.

Cluster analyse liet zien dat een panel van antilichamen tegen Gal-3 en FN-1 een sensitiviteit heeft van 97% voor alle schildkliercarcinomen, met een specificiteit van 100%. HBME-1 bleek een nuttige marker te zijn voor het onderscheid tussen FA en FVPTC. Omdat het aantal FVPTC tumoren klein was, kon hiërarchische cluster analyse deze groep niet onderscheiden.

Wij concluderen dat Gal-3, FN-1 en cNIS een nuttig panel zijn bij de differentiaal diagnose van schildkliernodi. De afwezigheid van Gal-3, FN-1 en cNIS is sterk suggestief voor een benigne aandoening. HBME-1 kan bruikbaar zijn bij de differentiatie tussen FVPTC en FA.

Perspectief

De bevindingen van Hoofdstuk 2 moeten bevestigd worden in een follow-up onderzoek waarbij de kandidaat markers getest moeten worden op cytologische puncties. Dit materiaal wordt beoordeeld volgens conventionele criteria en klinische beslissingen zullen hierop gebaseerd zijn. De scores van het onderzoekspanel zullen vergeleken worden met de uiteindelijke histologische diagnose van die schildklierlesies die operatief verwijderd zullen worden.

3. De diagnostische en prognostische waarde van serum Tg bepalingen in de follow-up van DTC

Serum Tg bepalingen zijn de belangrijkste markers in de follow-up van DTC. Recent zijn richtlijnen voor de follow-up van DTC gepubliceerd door de British Thyroid Association, De American Thyroid Association en de European Thyroid Association. In Nederland is ook een consensus in wording onder verantwoordelijkheid van het CBO. De afkapwaarden voor Tg in deze documenten zijn vaak niet scherp gedefinieerd en gebaseerd op retrospectieve onderzoeken van een beperkt aantal grote centra. Geadviseerd wordt dan ook om per instituut afkapwaarden te definiëren. Het probleem met de definitie van Tg afkapwaarden is, zoals met elke diagnostische procedure, de gouden standaard om de afwezigheid van ziekte vast te stellen. Bij DTC is Tg een betere marker dan RaJ scintigrafieën, dus waneer RaJ scintigrafieën als gouden standaard worden gebruikt zal de specificiteit van Tg dalen. Bovendien wordt de optimale Tg afkapwaarde bepaald door de meest acceptabele ratio tussen onnodige behandelingen en gemiste recidieven. Dit is een subjectieve keuze die verschillend kan uitvallen in verschillende landen of ziekenhuizen. Daarom is het belangrijk om een inzicht te krijgen in de kwantitatieve relatie tussen sensitiviteit en specificiteit van Tg, hetgeen de basis is van ROC analyses. In Hoofdstuk 3 hebben we de diagnostische en prognostische waarde van serum Tg bepalingen onderzocht in een groep van 366 DTC patiënten die allen dezelfde initiële behandeling ondergingen. Wij vonden een excellente diagnostische accuratesse van serum Tg waarden gedurende TSH stimulatie, 6 maanden na initiële therapie (sensitiviteit 100%), bij een hogere afkapwaarde dan normaal gerapporteerd wordt. De verklaring voor deze hogere afkapwaarde zou gerelateerd kunnen zijn aan het lagere initiële ablatie percentage in het LUMC in vergelijking met andere instellingen, met analytische verschillen of door het gebruik van de ROC techniek. We vonden ook dat serum Tg spiegels voorafgaande aan RaJ ablatie een onafhankelijke voorspeller zijn van genezing. TSH gestimuleerde Tg metingen, 6 maanden na initiële therapie en 2 en 5 jaar na initiële therapie waren onafhankelijke voorspellers van DTC gerelateerde mortaliteit. Hoewel de specificiteit van Tg voor DTC lager is dan 100% (wat verklaard kan worden door de bovengenoemde keuze van de gouden standaard in onze studie), zijn we het eens met het gebruik om een hoge activiteit RaJ toe te dienen aan patiënten met een verhoogde Tg spiegel. Naar onze mening is een goede benadering om de discussie over de specificiteit van Tg te vermijden om Tg als een risico-indicator te beschouwen voor DTC recidief. De onafhankelijke prognostische waarde van serum Tg voor genezing en mortaliteit zijn argumenten om Tg op te nemen in een conventioneel panel van risicofactoren. Het percentage DTC patiënten met antilichamen tegen Tg (27% bij initiële therapie) is vergelijkbaar met andere studies. Tg antilichamen bleken echter geen diagnostische of prognostische waarde te hebben.

Wij concluderen dat ons onderzoek het belang van het definiëren van instituuts gebonden Tg afkapwaarden aantoont en dat serum Tg bepalingen het mogelijk

maken patiënten te onderscheiden met een verhoogd risico voor recidief DTC of overlijden, in aanvulling op conventionele risicofactoren.

Perspectief

Gezien de vele analytische en methodologische aspecten van Tg metingen denken we dat er een harmonisatie moet plaatsvinden van Tg bepalingsmethoden tussen instituten, bij voorkeur in een internationale context. Daarnaast is uniformering van behandelingsprotocollen en de formulering van gestandaardiseerde criteria voor DTC ziekte activiteit van belang, zodat gestructureerde follow-up van DTC patiënten en de definitie van Tg afkapwaarden in regionaal of internationaal verband kan plaatsvinden.

4. Triiodothyronine onderdrukt de in vitro opname van iodide en de expressie van NIS

De introductie van rhTSH voor de diagnose, initiële therapie en (onder zekere voorwaarde) behandeling van DTC is zonder twijfel een belangrijke innovatie voor patiënten met DTC, om de nadelen van schildklierhormoon onttrekking te voorkomen. Hoewel in het algemeen de diagnostische eigenschappen van rhTSH vergelijkbaar zijn met schildklierhormoon onttrekking, is het mogelijk dat de kinetiek van jodide opname niet geheel gelijk is. Een van de aspecten is dat patiënten met rhTSH per definitie euthyroid zijn. De aanname is dat schildklierhormoon de jodide opname niet beïnvloedt. In Hoofdstuk 4 is onderzocht of deze aanname correct is. We gebruikten de ratten schildklier cellijn FRTL-5 en kweekten deze cellijn in de aan- of afwezigheid van fysiologische concentraties triiodothyronine (T3) en bestudeerden proliferatie, jodide opname en NIS mRNA en eiwitexpressie. We vonden inderdaad een verminderde jodide opname tijdens T3 gepaard gaande met een afgenomen NIS mRNA en eiwitexpressie.

Hoewel het is gesuggereerd dat jodide aanwezig in schildklierhormoon de specifieke activiteit van RaJ verdunt en dat dit verantwoordelijk is voor de verminderde opname van RaJ geloven we dat dit niet de verklaring kan zijn voor onze bevindingen, omdat de hoeveelheid jodide in T3 verwaarloosbaar is ten opzichte van de concentratie koud jodide van 10 uM in onze experiment. Ook verschillen in jodide kinetiek op totaal lichaamsniveau, aangevoerd als een andere verklaring voor verschillen tussen rhTSH en onttrekking kunnen deze bevindingen niet verklaren. Onze resultaten wijzen op een effect van T3 op NIS gen expressie resulterend in een verminderde hoeveelheid NIS eiwit.

Er is discussie of de NIS promoter T3 responsieve elementen bevat. Een andere verklaring kan zijn dat de effecten van T3 via repressie van de TSH receptor promoter tot stand komen maar onze experimenten wijzen niet in die richting.

Wij concluderen dat wij aanwijzingen vonden voor een TSH en jodide onafhankelijk effect van T3 op NIS genexpressie. Het mechanisme moet nog opgehelderd worden evenals de vraag of dit fenomeen ook bij mensen een rol speelt. De klinische implicaties van de vinding zijn niet duidelijk, maar deze kan bijdragen aan de gesuggereerde verschillen in jodide accumulatie tussen rhTSH en conventioneel behandelde mensen.

Perspectief

Wij denken dat de introductie van rhTSH een belangrijke verbetering is voor patiënten met DTC. Het is echter belangrijk om relevante aspecten te bestuderen die betrokken zijn bij de fysiologie van het jodide metabolisme, waartoe ook T3 behoort. Een interessante ontwikkeling is de experimentele bevinding dat T3 ook tumor onderdrukkende eigenschappen kan hebben. In dit verband zou het voortzetten van schildklierhormoontherapie gedurende diagnose en behandeling van RaJ voordelen kunnen hebben.

5. Het verbeteren van radioactief jodium therapie bij DTC

Therapeutische opties voor patiënten met metastasen van DTC zijn beperkt. RaJ is effectief bij ongeveer de helft van de patiënten met longmetastasen en een klein percentage patiënten met botmetastasen. De effectiviteit van RaJ is vaak verminderd door een beperkte jodide opname in metastasen. Strategieën om de therapeutische opties te verbreden kunnen onderscheiden worden in behandelingen gericht op het verbeteren van RaJ therapie en de identificatie van nieuwe doelwitten.

Het verbeteren van RaJ therapie is een breed thema. Twee sub-themas kunnen onderscheiden worden. Ten eerste tumoren waarbij nog steeds RaJ opname aanwezig is, ten tweede tumoren waarbij de RaJ opname verloren is.

Bij tumoren die nog RaJ opnemen is de essentie van het verbeteren van RaJ therapie het verhogen van de dosis RaJ. De dosis van RaJ wordt bepaald door de hoeveelheid (activiteit) van RaJ die de tumor bereikt (specifieke activiteit), de mate van opname in de tumor, het tumor volume en de effectieve halfwaarde tijd van RaJ die op zijn beurt bepaald wordt door de fysische halfwaardetijd en de biologische halfwaarde tijd. Al deze factoren kunnen geoptimaliseerd worden: de specifieke activiteit kan verhoogd worden door een jodium beperkt dieet. De mate van opname kan verhoogd worden door hoge serum TSH concentraties. De halfwaarde tijd van RaJ bij DTC is een belangrijke factor. Het verloren gaan van de folliculaire architectuur en mogelijk de verminderde activiteit van thyroid peroxidase (TPO) kunnen bijdragen aan een verminderde halfwaardetijd en dus aan een lagere tumor dosis. We richtten ons op dit probleem en bestudeerden de effecten van lithium zouten op de accumulatie van jodide in Hoofdstuk 5.

5.1 Effecten van lithium op jodide opname en de klinische resultaten van RaJ therapie

De relatie tussen lithium zouten en de schildklier is al lang bekend, aangezien veel patiënten met een bipolaire depressie, die behandeld worden met lithium zouten hypothyreoidie ontwikkelen. Het mechanisme is onduidelijk maar sommige experimentele studies hebben gesuggereerd dat lithium de afgifte van schildklierhormoon uit de schildklier remt, of in andere woorden, jodide in de schildklier opgenomen houdt. Vanwege deze eigenschappen is lithium gebruikt om de dosis RaJ te verhogen in goed- en kwaadaardige schildklierziekten. Het probleem is echter dat het niet duidelijk is of lithium de klinische resultaten van RaJ therapie beïnvloedt. Wij hebben daarom de klinische effecten van RaJ met en zonder lithiumcarbonaat bestudeerd bij patiënten met DTC. Daarnaast is er onduidelijkheid over het mechanisme waarmee lithium de RaJ dosis verhoogd in DTC. Wij verrichtten een in vitro studie om de specifieke effecten van lithium op NIS te bestuderen.

We onderzochten 12 patiënten met metastasen van DTC die nog wel RaJ opnamen maar waarbij eerdere RaJ therapie onvoldoende had gewerkt. Deze eerdere therapie fungeerde als controle De patiënten ontvingen 1200 mg lithiumcarbonaat/dag gevolgd door RaJ therapie. Ondanks een toegenomen RaJ opname bij 7 patiënten werd geen gunstig effect van lithium waargenomen op het klinische beloop, vastgesteld door middel van Tg metingen en met radiologisch onderzoek. Een verklaring voor het gebrek aan succes kan zijn dat in het lange interval tussen de controle RaJ behandeling en de behandeling met lithium biologische veranderingen in de tumor zijn ontstaan of dat de eerste behandeling tumor cellen heeft geselecteerd die radioresistent zijn. Wij denken dat het onwaarschijnlijk is dat dit bij alle patiënten het geval is geweest, aangezien bij de RaJ therapie met lithium ook jodide accumulerende lesies zichtbaar waren. Daarnaast was er na de eerste RaJ therapie geen substantieel verschil in de stijgingssnelheid van Tg serum spiegels zichtbaar. De selectie van radioresistente cellen – kankercellen die niet meer in apoptose gaan na radiotherapie, een bekend fenomeen in de oncologie – is een andere verklaring voor het gebrek aan klinisch effect van RaJ met lithium. We bestudeerden de effecten van lithium in verschillende fysiologische concentraties op jodide opname in de ratten schildkliercellijn FRTL-5, in de gepolariseerde niercellijn MDCK, die stabiel was getransfecteerd met NIS en in de humane, NIS getransfecteerde FTC cellijn FTC133-NIS. Zowel de kinetiek van jodide opname als jodide efflux werden bestudeerd. Het doel van deze studies was om te onderzoeken of lithium een direct effect op NIS heeft. Er werden geen effecten van lithiumchloride op jodide opname of efflux gevonden. We hebben niet alle stappen van de jodide fysiologie onderzocht. Aangezien het gesuggereerd is dat voor het bevorderen van jodide concentraties in de schildklier organificatie nodig is kunnen we niet uitsluiten dat in een 3-dimensionaal model van schildkliercellen lithium inderdaad de afgifte van georganificeerd jodide zou kunnen remmen, hetgeen een ander aspect is van de afwezigheid van een effect van lithium bij schildkliertumoren

met een korte halfwaardetijd en afwezige organificatie.

De resultaten van ons onderzoek relativeren de eerder gerapporteerde gunstige effecten van lithium bij DTC.

Perspectief

Wij geloven dat lithiumzouten geen plaats hebben bij RaJ therapie voor DTC. Daarom moeten andere mechanismen om de RaJ halfwaardetijd te verlengen worden geëxploreerd. Het concept van organificatie is interessant en het kan verondersteld worden dat wanneer RaJ aan een ander eiwit gekoppeld kan worden de halfwaardetijd positief beïnvloed kan worden. Transfectie experimenten met TPO zijn echter vanuit klinisch perspectief moeilijk voorstelbaar. Een andere optie zou het blokkeren van de jodide efflux kunnen zijn door middel van chloride kanaal blokkers.

5.2 Effecten van redifferentiatietherapie met Bexarotene op jodide opname en klinische uitkomsten van RaJ therapie

Het tweede subthema van het verbeteren van radioactief jodium therapie bij DTC betreft tumoren waarbij de jodide opname is verloren. De 'heilige graal' is het bewerkstelligen van functionele expressie van NIS. De pathofysiologie van verminderde NIS expressie in DTC is gecompliceerd (zoals boven beschreven) en omvat zowel genetische (transcriptie) defecten als post-translationele (trafficking) problemen. Mutaties in het NIS gen zijn zelden beschreven bij DTC. De meest waarschijnlijke verklaring is dat de transcriptie van het NIS gen verminderd is door inactivatie van het gen zelf of de promoter. De NIS promoter heeft meerdere responsive elements en afwezigheid van 1 of meerdere activatoren (zoals TTF-1 of TSH) zal verminderde transcriptie veroorzaken. Een interessante verklaring wordt gevormd door epigenetische veranderingen waarbij methylering of histon deacetylering transcriptie verminderen. Er zijn onderzoeken beschreven waarbij de genetische blokkades die hierdoor ontstaan worden opgeheven met behulp van farmaca. In Hoofdstuk 6 en 7 hebben wij de effecten van de retinoid receptor X (RXR) agonist Bexarotene bestudeerd op het herstel van de jodideopname bij patiënten met metastasen van DTC. Retinoiden zijn eerder gebruikt bij patiënten met DTC, maar alle klinische studies tot-nu-toe zijn gedaan met 13-cis retinoic acid, dat alleen aan de retinoiden receptor A (RAR) bindt. Recente studies hebben het belang van andere retinoiden receptor subtypes in DTC aangetoond, zoals RXR. Wij hebben daarom de effecten bestudeerd van een behandeling van 6 weken met 300 mg Bexarotene/dag op de I-131 opname bij 12 patiënten met DTC. Voorafgaande aan en na deze interventie werd de RaJ opname gemeten op diagnostische scintigrafieën 3 dagen na toediening van 185 MBq RaJ. Deze scintigrafieën werden voorafgegaan door 2 injecties met rhTSH. Bexarotene behandeling induceerde RaJ opname bij 8/11 patiënten (1 patiënt overleed door oorzaken die niets met het onderzoek te

maken hadden). RaJ opname was echter alleen zichtbaar met single photon emission tomography en niet alle metastasen die zichtbaar waren bij CT lieten RaJ opname zien (incomplete matching). Een interessante waarneming was dat in 1 patiënt een nieuwe afwijking zichtbaar werd na Bexarotene, die eerder geen RaJ opnam. Dit is een illustratie van de heterogeniteit van DTC metastasen met betrekking tot RaJ opname.

Hoewel de hoeveelheid RaJ opname beperkt was in de 8 responders, besloten we deze patiënten het voordeel van de twijfel te geven, en hen te behandelen met een hoge activiteit RaJ. Zij kregen 7400 MBq RaJ, voorafgegaan door 6 weken Bexarotene 300 mg/dag. Zes maanden na de therapie bleken 6 patiënten progressieve ziekte te hebben, terwijl 2 patiënten stabiele ziekte hadden. De verklaring voor dit gedeeltelijke succes kan zijn dat Bexarotene andere factoren die van invloed zijn op de RaJ dosis (zoals de RaJ halfwaardetijd) niet heeft beïnvloed. Daarnaast kan het zijn dat bij de regulatie van NIS meerdere stoornissen op het niveau van transcriptie en posttranscriptie zijn die slechts gedeeltelijk hersteld kunnen worden door retinoiden. Een andere mogelijkheid is dat het retinoiden receptor expressieniveau niet gunstig is ten aanzien van Bexarotene therapie. Om dit te onderzoeken verrichten we kleuringen van de RAR en RXR bij een aantal patiënten. Wij vonden geen relatie tussen RAR en RXR kleuringspatroon en therapierespons. Een beperking van deze analyse is wel dat we alleen materiaal van de primaire tumor ter beschikking hadden en dat het expressiepatroon in de metastasen anders geweest zou kunnen zijn.

We vonden een incomplete matching tussen de metastasen die zichtbaar waren op een post-RaJ therapie scintigram en CT. Dit betekent dat zelfs als een subgroep van metastasen weer RaJ opneemt na Bexarotene het waarschijnlijk is dat andere, niet RaJ opnemende metastasen progressief zijn.

Perspectief

Wij hebben aangetoond dat redifferentiatietherapie in een klinische context inderdaad kan leiden tot herstel van de RaJ opname bij DTC. Een conceptueel probleem bij redifferentiatietherapie is dat deze aselect is: ook andere genen die niet gerelateerd zijn aan de ziekte kunnen door deze stoffen beïnvloed worden. Dit zou belangrijke bijwerkingen tot gevolg kunnen hebben, tenzij een specifiek moleculair defect zoals in de RAR receptor bij pro-myelocyten leukemie aan de orde is, dat hersteld kan worden met retinoiden therapie. De opkomst van speciaal ontworpen geneesmiddelen die gericht zijn op een specifiek moleculair defect in goed geanalyseerde signaaltransductie routes, zoals de tyrosinekinasremmers, is een belangrijke ontwikkeling bij kanker, die ook zijn vruchten kan afwerpen bij DTC.

6. Conclusies van dit proefschrift

De incidentie, overleving en de specifieke klinische en biologische aspecten van DTC compliceren de diagnose, behandeling en follow-up. Dit wordt nog verder bemoeilijkt door de wijze waarop onderzoek en patiëntenzorg voor DTC is georganiseerd. Dit zou grondig geëvalueerd en verbeterd moeten worden. De recente publicatie van multinationale richtlijnen is een goede, eerste stap. De onderzoeken van dit proefschrift hebben een aantal klinische en fundamentele aspecten van DTC aan de orde gesteld. Hoewel een aantal vragen beantwoord zijn, zijn ook vele nieuwe vragen gerezen die verder onderzoek nodig maken.

De diagnose van DTC, die momenteel met behulp van conventionele histologische technieken wordt gedaan, kan aanzienlijk verbeterd worden met nieuwe markers. Onze studies bieden belangrijke perspectieven voor de invoering van een nieuw panel van immuunhistochemische markers dat de diagnose kan verbeteren.

Hoewel serum Tg metingen aanbevolen worden als de belangrijkste diagnostische procedure in de follow-up van DTC, zijn er vele analytische en statistische aspecten die hierbij een rol spelen. Onze onderzoeken illustreren het belang van het definiëren van institutionele afkapwaarden van Tg, gebruik makend van ROC technieken, om een inzicht te krijgen in de kwantitatieve relatie tussen sensitiviteit en specificiteit. We vonden dat Tg een onafhankelijke prognostische marker is voor genezing en mortaliteit, hetgeen het mogelijk zou kunnen maken voor de arts om hoog-risico patiënten te identificeren.

Het vermogen van DTC cellen om jodide te accumuleren is de kern van diagnostiek en therapie met RaJ. De introductie van rhTSH is een groot voordeel voor patiënten om de ongemakken van hypothyreoidie te voorkomen. Het is echter onwaarschijnlijk dat schildklierhormoon geen effect heeft op het jodide metabolisme. Wij stelden vast dat T3 een direct effect heeft op de expressie en functie van NIS. Hoewel de klinische consequenties niet duidelijk zijn, denken we dat een zorgvuldige evaluatie van de effecten van een nieuwe behandeling op alle bekende aspecten van schildklierfysiologie van belang is.

Het belangrijkste probleem bij de behandeling van DTC is de beperkte effectiviteit van RaJ bij een aanzienlijk percentage patiënten met metastasen. Strategieën om RaJ behandeling te verbeteren moeten gebaseerd zijn op de identificatie van moleculaire defecten in de jodide fysiologie bij DTC. Bij patiënten bij wie er nog wel RaJ opname is – zij het met onvoldoende effect - zijn interventies gericht zijn op het verbeteren van de jodide kinetiek belangrijk. Wie lieten zien dat de vaak gepropageerde strategie om DTC patiënten met lithiumzouten te behandelen niet resulteerde in een verbeterde uitkomst van RaJ therapie. Noch vonden we een effect van lithium op de in vitro jodide opname. Andere benaderingen om de jodide kinetiek te verbeteren zijn nodig.

Bij patiënten bij wie metastasen van DTC geen RaJ accumuleren is het onderzoek gericht op het herstel van functionele NIS expressie. We vonden dat redifferentiatietherapie met de RXR ligand Bexarotene tot herstel van de jodide opname leidde bij een groep patiënten met DTC metastasen. De intensiteit van de RaJ opname was echter laag en niet aanwezig in alle metastasen. Daaropvolgende therapie met een hoge activiteit RaJ was niet succesvol. Vele mechanistische en conceptuele vragen met betrekking tot redifferentiatietherapie blijven over en de opkomst van nieuwe klassen geneesmiddelen die specifiek ontworpen zijn om een goed gekarakteriseerd moleculair defect te herstellen, zoals de klasse van tyrosine kinase remmers, bergen mogelijk grotere beloften in zich.

Epilogue

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Ying-ying Liu

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1. Y.Y. Liu, G. van der Pluijm, M. Karperien, M.P.M. Stokkel, A.M. Pereira, J. Morreau, J. Kievit, J.A. Romijn, J.W.A. Smit. Lithium as adjuvant to radioiodine therapy in differentiated thyroid carcinoma: clinical and in vitro studies. Clin Endocrinol (Oxf). 2006 Jun;64(6):617-24

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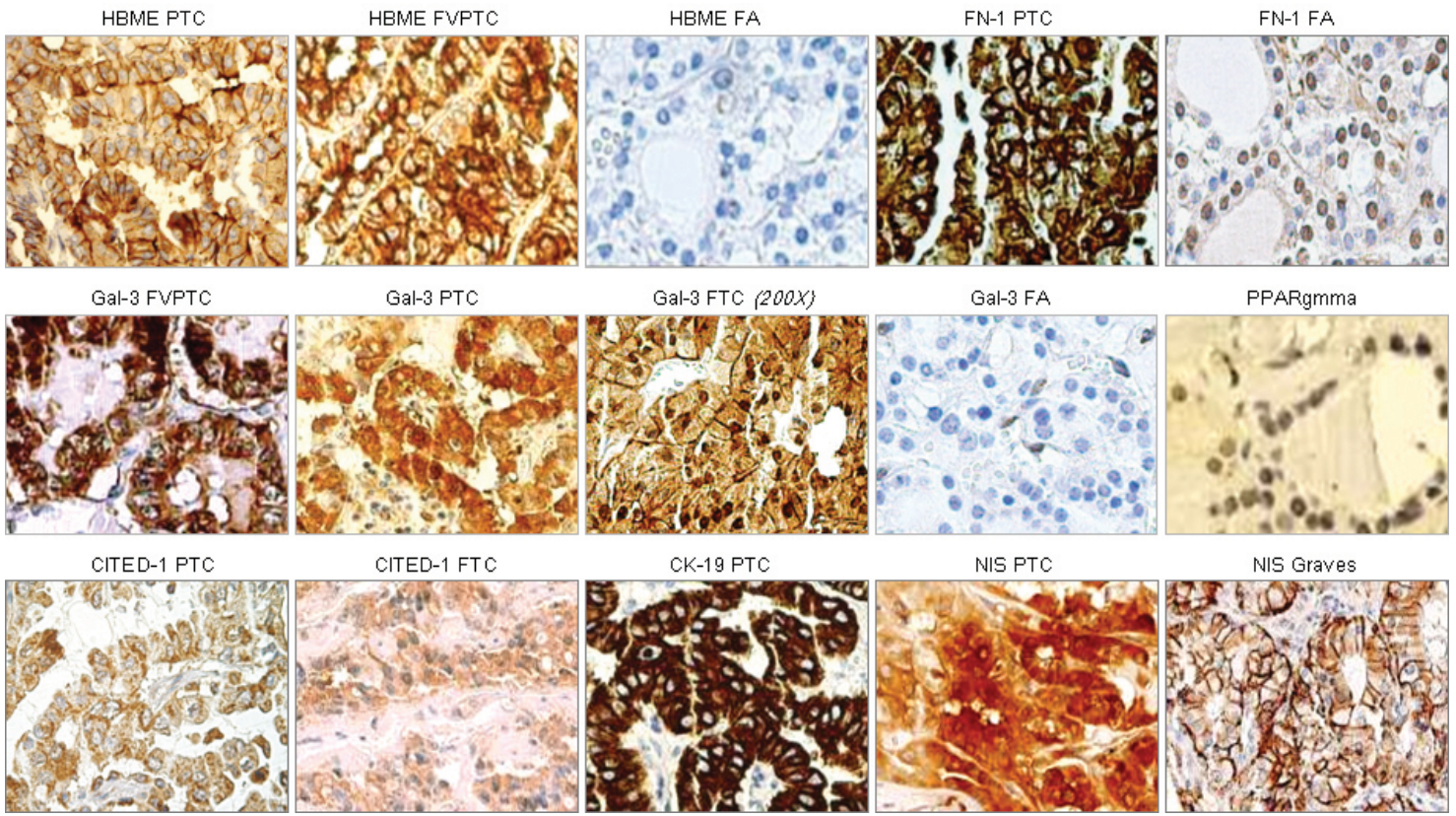
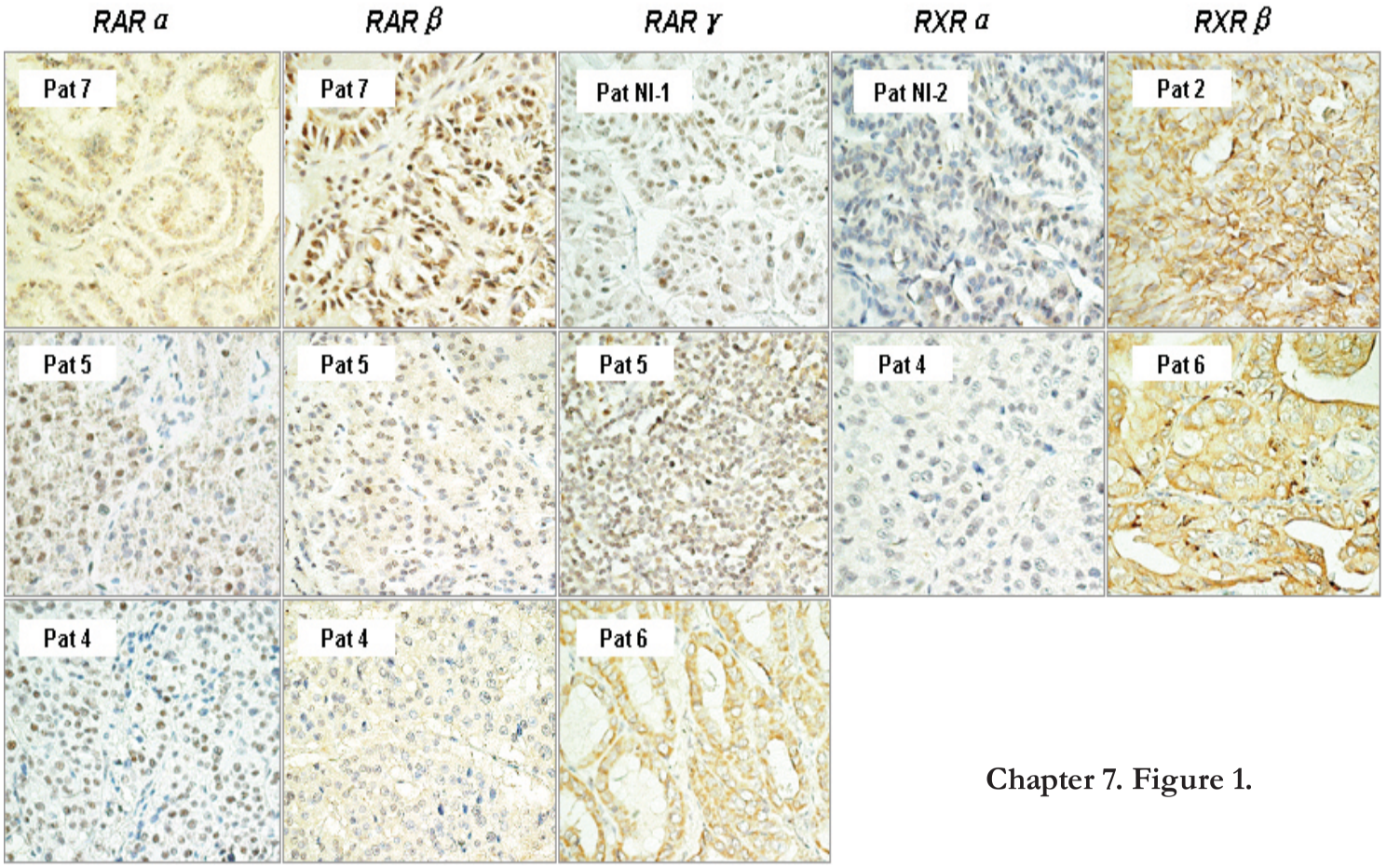
CURRICULUM VITAE

Ying-ying Liu, born in Tianjin of China on 4 March 1969, graduated from Hebei Medical University in 1992 followed by a five year doctoral research in Tianjin Medical University. Granted with medical science of doctoral degree, Dr. Liu worked in the Endocrinology and Metabolism Department in the First Academic Hospital of Tianjin medical University as a physician since 1998. She was initiator of two projects supported by the National Natural Science foundation of China and Outstanding Chinese lecturer foundation of the Ministry of Education of the People's Republic of China respectively. Involved in a cooperative project sponsored by Koninklijke Nederlandse Akademie van Wetenschappen KNAW (Royal Netherlands Academy of Arts and Sciences), Dr Liu worked in the Immunology Department of Erasmus University Medical Center, the Netherlands in 2001. Enrolled in PhD program, Dr. Liu started her PhD studying in Endocrinology and Metabolism Department of Leiden University Medical Center of the Netherlands since 2002 up till now and will contribute rest of her life to find solutions against diseases.

Color Images



Chapter 2. Figure 1.



Chapter 7. Figure 1.