Genetics of thyroid cancer and effects of thyroid hormone on tumor progress *in vivo*

Inaugural-Dissertation

zur

Erlangung des Doktorgrades

Dr. rer. nat.

der Fakultät für

Biologie

an der

Universität Duisburg-Essen

vorgelegt von

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aus Tübingen

August 2017

DOI: 10.17185/duepublico/45360
URN: urn:nbn:de:hbz:464-20190903-100447-4

Die der vorliegenden Arbeit zugrundeliegenden Experimente wurden in der Klinik für Endokrinologie, Diabetologie und Stoffwechsel im Universitätsklinikum Essen durchgeführt.

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Tag der mündlichen Prüfung: 07.12.2017

Publications included in this thesis:

Latteyer S, Tiedje V, König K, Ting S, Heukamp LC, Meder L, Schmid KW, Führer D, Moeller LC. Targeted next-generation sequencing for TP53, RAS, BRAF, ALK and NF1 mutations in anaplastic thyroid cancer. Endocrine. 2016 Dec;54(3):733-741. Epub 2016 Oct 1.

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Latteyer S, Zwanziger D, Führer D, Moeller LC. Thyroxine Promotes Tumor Growth And Angiogenesis In An Orthotopic Lung Cancer Mouse Model. Submitted to Molecular Cancer.

Publications not included in this thesis:

Latteyer S, Tiedje V, Schilling B, Führer D. Perspectives for immunotherapy in endocrine cancer. Endocr Relat Cancer. 2016 Oct;23(10):R469-84. doi: 10.1530/ERC-16-0169. Epub 2016 Aug 2. Review.

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Awards

Latteyer S, Klein-Hitpass L, Khandanpour C, Zwanziger D, Schmid KW, Führer D, Moeller LC. „A 6 bp in-frame germline deletion in exon 7 of RET leads to increased RET phosphorylation, MAPK/ERK activation and MEN2" Echo - Essen conference for hematology and oncology Essen from 10.04.15 till 12.04.15. 1St poster award

Latteyer S, Christoph S, Synoracki S, Zwanziger D, Liao XH, Refetoff S, Schmid KW, Führer D, Moeller LC. Thyroxine Promotes Tumor Growth And Angiogenesis In An Orthotopic Lung Cancer Mouse Model. 16. Tag der Forschung der Medizinischen Fakultät 18.11.2015, Essen, poster award

Latteyer S, Christoph S, Synoracki S, Zwanziger D, Liao XH, Refetoff S, Schmid KW, Führer D, Moeller LC. Thyroxine Promotes Tumor Growth And Angiogenesis In An Orthotopic Lung Cancer Mouse Model. Nordrhein-Westfälische Gesellschaft für Endokrinologie und Diabetologie e. V., Karl-Oberdisse-Preis 2018

Congress contributions

Latteyer S, Tiedje V, König K, Ting S, Heukamp L, Schmid K, Führer D, Moeller LC. Mutations in anaplastic thyroid cancer. Forschungstag des Medizinischen Forschungszentrum Essen 05.11.2015, poster

Latteyer S, Tiedje V, König K, Ting S, Heukamp L, Schmid K, Führer D, Moeller LC. Mutations in anaplastic thyroid cancer. 15. Tag der Forschung der Medizinischen Fakultät 20.11.2014, Universität Duisburg-Essen, poster

Latteyer S, Klein-Hitpass L, Khandanpour C, Zwanziger D, Schmid KW, Führer D, Moeller LC. Novel insights into RET pathway activation in a MEN2 patient. 30th Arbeitstagung Experimentelle Schilddrüsenforschung in Bremen 05.12.2014, oral presentation

Voigtländer R, Moeller LC, **Latteyer S**, Brix K, Biebermann H, Zwanziger D, Fuehrer D. In quest of oral thyroxine resistance (OTR) - analysis of patients with presumed intestinal malabsorption of levothyroxine & normal probands. 1st international conference and thyroid trans action in Bremen 05.12.2014, poster

Latteyer S, Klein-Hitpass L, Khandanpour C, Zwanziger D, Schmid KW, Führer D, Moeller LC. A 6 bp in-frame germline deletion in exon 7 of *RET* leads to increased RET phosphorylation, MAPK/ERK activation and MEN2. 58. Symposium der Deutschen Gesellschaft für Endorkinologie in Lübeck from 18.3.2015 till 21.3.2015, poster.

Latteyer S, Klein-Hitpass L, Khandanpour C, Zwanziger D, Schmid KW, Führer D, Moeller LC. A 6 bp in-frame germline deletion in exon 7 of RET leads to increased RET phosphorylation, MAPK/ERK activation and MEN2. Echo - Essen conference for hematology and oncology Essen vom 10.04.15 bis 12.04.15. 1St poster price

Latteyer S, Tiedje V, König K, Ting S, Heukamp LC, Meder L, Schmid KW, Führer D, Moeller LC. Next generation sequencing reveals novel oncogene candidates in anaplastic thyroid carcinoma. 15th International Thyroid Congress Orlando from 18.10.2015 till 23.10.2015, oral presentation

Latteyer S, Klein-Hitpass L, Khandanpour C, Zwanziger D, Schmid KW, Führer D, Moeller LC. A 6 bp in-frame germline deletion in exon 7 of RET leads to increased RET phosphorylation, MAPK/ERK activation and MEN2. 15th international thyroid congress Orlando from 18.10.2015 till 23.10.2015, oral presentation

Latteyer S, Tiedje V, König K, Ting S, Heukamp LC, Meder L, Schmid KW, Führer D, Moeller LC. Next generation sequencing reveals novel oncogene candidates in anaplastic thyroid carcinoma. 59. Symposium der Deutschen Gesellschaft für Endokrinologie München from 26.05.2016 till 28.05.2016, poster

Latteyer S, Christoph S, Synoracki S, Zwanziger D, Liao XH, Refetoff S, Schmid KW, Führer D, Moeller LC. Thyroxine Promotes Tumor Growth And Angiogenesis In An Orthotopic Lung Cancer Mouse Model. 16. Tag der Forschung der Medizinischen Fakultät 18.11.2015, Essen, poster price

Latteyer S, Christoph S, Synoracki S, Zwanziger D, Liao XH, Refetoff S, Schmid KW, Führer D, Moeller LC. Thyroxine Promotes Tumor Growth And Angiogenesis In An Orthotopic Lung Cancer Mouse Model. Tagung der Sektion Schilddrüse der DGE, Heidelberg from 25.11.2016 till 26.11.2016, oral presentation

Latteyer S, Christoph S, Synoracki S, Zwanziger D, Liao XH, Refetoff S, Schmid KW, Führer D, Moeller LC. Thyroxine Promotes Tumor Growth And Angiogenesis In An Orthotopic Lung Cancer Mouse Model. 32th Arbeitstagung Experimentelle Schilddrüsenforschung in Ratzeburg from 01.12.2016 till 03.12.2016, oral presentation

Latteyer S, Christoph S, Synoracki S, Zwanziger D, Liao XH, Refetoff S, Schmid KW, Führer D, Moeller LC. Thyroxine Promotes Tumor Growth And Angiogenesis In An Orthotopic Lung Cancer Mouse Model. 60. Deutscher Kongress für Endokrinologie in Würzburg from 15.03.2017 ill17.03.2017, oral presentation

Latteyer S, Tiedje V, Muchalla P, Moeller LC, Führer D. A double heterozygous mutation in the proto oncogene-RET leads to constitutive activation of downstream pathways and a MEN2B phenotype although single mutation only cause MEN2A phentotype. 60. Deutscher Kongress für Endokrinologie in Würzburg from 15.03.2017 till17.03.2017, oral presentation

Content

1. Zusammenfassung/Summary

1.1. Zusammenfassung

Schilddrüsenkarzinome sind die häufigsten bösartigen endokrinen Tumore und repräsentieren 1% der malignen Tumorentitäten. Sie lassen sich entsprechend des zugrundeliegenden Zelltyps, Morphologie und Pathologie klassifizieren.

Die Mehrheit der Schilddrüsenkarzinome sind differenziert (>80%) und haben eine exzellente Prognose wohingegen Patienten mit einem gering differenzierten Schilddrüsenkarzinom (<5%) eine eher ungünstigere Prognose haben. Eine weitere kleine Minderheit (<5%) der Schilddrüsenkarzinome zeigt einen rasch progredienten Verlauf mit häufig infauster Prognose bei Diagnose und einer medianen Überlebenszeit von lediglich 4 bis 6 Monate. Dieses sogenannte anaplastische Schilddrüsenkarzinom ist äußerst selten und durch eine hohe Mitoserate, invasives Wachstum, Refraktärität gegenüber Radiojod und eine äußersten Heterogenität auf genetischer Ebene gekennzeichnet. In den letzten 50 Jahren konnte keine signifikante Verbesserung der Behandlung erzielt werden. Morphologisch entspringen die erwähnten Tumore alle aus den Thyreozyten der Schilddrüse und dedifferenzieren bis zu einem Verlust der Fähigkeit Jod zu speichern wie es der Fall beim anaplastischen Schilddrüsenkarzinom ist. Molekularbiologisch ist dies durch eine Akkumulation von Mutationen in unterschiedlichsten Genen zu erklären.

Um den schlechten Verlauf und die schwierige Behandlung des anaplastischen Schilddrüsenkarzinoms besser verstehen zu können, wurde Tumorgewebe von 39 anaplastischen Schilddrüsenkarzinom Patienten mittels zielgerichteter Next-Generation Sequencing untersucht. In 90% der Proben wurden mindestens eine und in 57% Proben mindestens zwei Mutationen der untersuchten Gene gefunden: *TP53* (18/30), *NF1* (11/30), *ALK* (6/30), *NRAS* (4/30), *ATRX* (3/30), *BRAF* (2/30), *HRAS* (2/30), *KRAS* (1/30). Die Daten bestätigen die genetische Heterogenität im anaplastischen Schilddrüsenkarzinom und legen nahe, dass das anaplastische Schilddrüsenkarzinom möglicherweise nicht als eine einzige Entität behandelt werden sollte. Es könnte daher durchaus sinnvoll sein, Patienten nach der zugrundeliegenden Mutation in Untergruppen zu unterteilen und zielgerichtet für diese Mutationen zu behandeln.

Die bereits erwähnten Schilddrüsenkarzinome entstehen alle aus den Thyreozyten der Schilddrüse. Anders verhält es sich beim medullären Schilddrüsenkarzinom, welches sich nicht aus den Thyreozyten, sondern aus den Calcitonin-produzierenden C-Zellen der Schilddrüse entwickelt. Das medulläre Schilddrüsenkarzinom tritt in 25% der Fälle hereditär im Rahmen einer Multiplen Endokrinen Neoplasie Typ 2 A und B auf. Ursächlich sind Mutationen in den Hotspots des Protoonkogenes *RET.* Eine Patientin mit klinischen Symptomen einer MEN2A Erkrankung und einer außergewöhnlichen Konstellation eines zuerst aufgetretenen bilateralen Phäochromozytom gefolgt von einem medullären Schilddrüsenkarzinom wurde in dieser Arbeit untersucht. Trotz mehrfacher genetischer Routinediagnostik, nach den Standards des damaligen Zeitpunktes, in den bekannten Hotspots des Protoonkogenes *RET* konnte keine zugrundeliegende Mutation gefunden werden. Mittels Whole Exomsequencing wurde dennoch eine Mutation außerhalb der Hotspots des Protoonkogene *RET* (c.1512_1517delGGAGGG, p.505_506del) gefunden. Um die pathogene Eigenschaft dieser Mutation zu untersuchen wurde per Mutagenese diese RET-Variante generiert und in zellbiologischen Experimenten mit RET9 Wildtyp verglichen. Mittels Immunoblotting zeigte sich eine (Auto-)phosphorylierung von RET9, ferner eine verstärkte Aktivierung des MAPK/ERK und PI3K/AKT-Signalwegs in der RET9 p.505_506del verglichen mit RET9 Wildtyp. Ein Luciferase-Assay bestätigte die ERK1/2 Aktivierung. Colony Formation Assay zeigte einen signifikanten Selektions- und Proliferationsvorteil gegenüber RET9 Wildtyp exprimierenden Zellen. Die genetische Analyse der Eltern und Geschwister bestätigte, dass kein weiteres Familienmitglied betroffen ist und diese Mutation *de novo* entstanden ist. Obwohl bei der molekularen Diagnostik nach den zu dem Zeitpunkt aktuellen Leitlinien vorgegangen wurde, konnte keine *RET* Mutation gefunden werden. Dieser Fall zeigt, dass die Leitlinien nicht ausreichend waren. Nach Veröffentlich des Fallberichts wurden die Leitlinien angepasst und besagen jetzt, dass bei Patienten mit MEN2 ohne genetischen Befund nach Routinediagnostik die komplette *RET* Sequenz analysiert werden soll.

Schilddrüsenhormone sind wichtig, können aber eventuell in bestimmten Situationen schädlich sein. Studien zeigen, dass eine Hyperthyreose das Tumorwachstum beschleunigt. Der Mechanismus hinter dieser Beobachtung ist noch weitgehend unbekannt. Um dieser Frage nachzugehen wurde in immunkompetenten

C57B6(Cg)-Tyr^{c-2J} Mäusen eine Hypothyreose induziert gefolgt von einer intrapulmonalen Injektion von Lewis Lung Cell Carcinoma. Die Tiere wurden anschließend mit T_3, T_4 und/oder 3,3′,5,5′-Tetraiodothyroacetic acid (Tetrac) behandelt. Mittels *in vivo* imaging wurde das Tumorwachstum über die Zeit analysiert, sowie das Tumorgewicht am Versuchsende. Die Gabe von T_4, nicht T_3, führt zu einem signifikanten Anstieg des Tumorgewichts sowie der Biolumineszenz und wurde in Gegenwart des Integrin ($\alpha_v\beta_3$) Inhibitor Tetrac aufgehoben. Durchflusszyometrische Analysen zeigten keine Änderung der Zusammensetzung von infiltrierenden Immunzellen. Immunhistochemische Analysen der Tumorproben zeigten jedoch eine signifikante Neubildung von Blutgefäßen ($CD31^+$) in T_4 behandelten Tieren, welche in Gegenwart von Tetrac nicht beobachtet wurde war. Die generierten Ergebnisse legen nahe, dass der tumorfördernde Effekt ausschließlich von T_4, nicht T_3, über das Integrin $\alpha_v\beta_3$ vermittelt wird und wahrscheinlich fördernd auf die Neoangiogenese ist.

1.2. Summary

Thyroid cancer is the most prevalent endocrine tumor but represents only 1% of all malignant tumors. Thyroid cancer can be classified according to the underlying altered cell type, morphology and pathology.

Most of the thyroid carcinomas are differentiated (>80%) and have an excellent prognosis whereas patients with a poorly differentiated thyroid carcinoma (<5%) have a rather unfavorable prognosis. A small minority (<5%) of the thyroid carcinomas shows a rapid progression with poor prognosis and a median survival time of only 4 to 6 months. This so-called anaplastic thyroid carcinoma is extremely rare and is characterized by a high rate of mitosis, invasive growth, refractory against radioiodine, and an extreme heterogeneity in the genetic landscape. Furthermore, no improvement in the treatment of patients with anaplastic thyroid carcinoma has been achieved over the last 50 years. Based on the morphology, these tumors originate from the thyrocytes of the thyroid gland and dedifferentiate to a loss of the ability to store iodine as it is the case with the anaplastic thyroid carcinoma. On molecular biology level, this observation is explained by an accumulation of mutations in different genes.

In order to understand the poor course and the difficult treatment of the anaplastic thyroid carcinoma, the genetic landscape was investigated and characterized in tumor tissue of 39 anaplastic thyroid carcinoma patients by next-generation sequencing. In 90% of the samples, at least one mutation and, in 57% at least two mutations of the tested genes were found: *TP53* (18/30), *NF1* (11/30), *ALK* (6/30), *NRAS* (4/30), *ATRX* (3/30), *BRAF* (2/30), *HRAS* (2/30), *KRAS* (1/30). The data confirm the genetic heterogeneity of the anaplastic thyroid carcinoma and show that the anaplastic thyroid carcinoma should not necessarily be treated as a single entity. It could therefore be useful to divide the patients into subgroups regarding the underlying mutation and treat them by targeted therapy.

The thyroid carcinomas mentioned above all arise from the thyrocytes of the thyroid gland. The situation is different in the case of medullary thyroid carcinoma which does not develop from the thyrocytes but from the C-cells of the thyroid gland. The medullary thyroid carcinoma occurs in approximately 25% of cases hereditary as

multiple endocrine neoplasia type 2 A and B. This is caused by mutations in the hotspots of the proto-oncogene *RET*.

A patient with clinical symptoms of MEN2A and an unusual constellation of bilateral pheochromocytoma followed by a medullary thyroid carcinoma will be discussed in this thesis. In spite of several genetic routines according to the current guidelines at that time, no mutation could be found. By means of whole exome sequencing, a mutation was found in the proto-oncogene *RET* (c.1512_1517delGGAGGG, p.505_506del), an exon which is usually not associated with MEN2A. In order to investigate the pathogenic property of this mutation, the RET variant was generated by mutagenesis and compared to RET9 wildtype in cell biology experiments. Immunoblotting revealed an (auto-) phosphorylation of RET9, enhanced activation of the MAPK/ERK, and the PI3K/AKT signaling pathway in the RET9 p.505_506del compared to RET9 wildtype. Luciferase assay confirmed ERK1/2 activation. Colony formation assay confirmed a significant selection and proliferation advantage over RET9 wild-type expressing cells. The genetic analysis of the parents and siblings showed that no other family member is affected and this mutation originated *de novo*. Although molecular diagnostics were carried out according to the current guidelines, no *RET* mutation could be found. This case shows that the guidelines were insufficient and were adapted after the publication of the case report. The guidelines now indicate that the complete *RET* sequence should be analyzed in MEN2 patients without genetic findings after routine diagnosis.

Thyroid hormones are important but may be harmful in certain situations. Studies show that hyperthyroidism increases tumor growth. The mechanism behind this observation is not understood. This question is clinically relevant in the use of modern targeted tyrosine kinase inhibitors, since these can cause hypothyroidism as a side effect, which is compensated by thyroid hormone. To answer this question, hypothyroidism was induced in immunocompetent C57B6(Cg)-Tyr$^{c\text{-}2J}$ mice followed by an intrapulmonary injection of Lewis Lung Cell Carcinoma. The animals were treated with T_3, T_4 and/or 3,3',5,5'-tetraiodothyroacetic acid (Tetrac). By means of *in vivo* imaging, the tumor growth was analyzed over time as well as the tumor weight at the end of the experiment. The administration of T_4, not T_3, results in a significant increase in tumor weight as well as bioluminescence but was gone in the presence of the integrin $\alpha_v\beta_3$ inhibitor Tetrac. Flow cytometric analyses showed no change in the

composition of infiltrating immune cells. However, immunohistochemical analyzes of the tumor samples showed a significantly enhanced formation of blood vessels ($CD31^+$) in T_4 treated animals, which was abolished in the presence of Tetrac. The results suggested that the tumor-promoting effect is mediated exclusively by T_4, not T_3, to the integrin $\alpha_v\beta_3$ and is probably promoted to neoangiogenesis.

2. Introduction

2.1. The thyroid gland as an endocrine organ

The thyroid gland is the largest endocrine organ in the human body with an average weight of about 30g. The butterfly-shaped organ appears from the area of the base of the tongue between 3-4 weeks during embryogenesis. The anlage of the thyroid gland moves caudal till it is localized next to and below the larynx where the thyroid gland appears with a left and a right lobe (Figure 1a). These lobes are connected by a thin isthmus at the height of the third tracheal cartilage. Macroscopically, the thyroid gland mainly contains spherical follicles with variable size. The follicles consist of a polarized epithelial monolayer, which represents the elementary functional unit of the thyroid gland and is also called thyrocyte. The follicles are filled with a slightly acidophilic colloid (Figure 1b). The primary function of the follicles of the thyroid gland is the biosynthesis of the iodine-containing thyroid hormone (TH), 3,3',5-triiodo-L-thyronine (T_3) and 3,3´,5,5'-tetraiodo-L-thyronine (Thyroxine - T_4). Therefore, the thyroid gland has the ability to accumulate and store iodine. The names T_3 and T_4 refer to the numbers of iodine that is bound per molecule T_3 (three atoms of iodine) and T_4 (four atoms of iodine). TH have a wide range of effects in mammals including metabolism, cardiovascular systems and development.

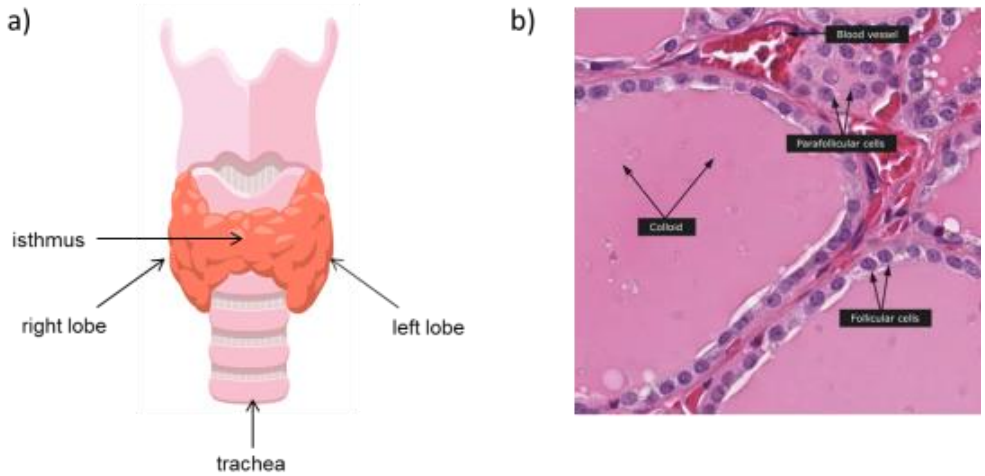

Figure 1: Anatomy of a healthy thyroid gland (a) and histological H&E staining (b). Figure 1a was modified from mindthegraph.com, Figure 1b from proteinatlas.org.

There is a small number of cells located parafollicular and perifollicular, called C-cells, with no direct connection to the follicles. The interfollicular space is strongly vascularized and consists mainly of connective tissue. During embryogenesis, precursor cells of the C-cells migrate from the fifth pouch to the anlage of the thyroid gland. These precursor cells are thought to derive from the neural crest, but recently Johansson et al. published a study indicating that C-cells are likely to be endodermally derived (Johansson et al., 2015). The main function of C-cells is the biosynthesis of the peptide hormone calcitonin. Calcitonin is thought to participate in calcium and phosphorus metabolism. Calcitonin reduces blood calcium by inhibiting osteoclast activity in bones (Belanger L.F., 1968) and inhibits the reabsorption of calcium and phosphate in the renal tubular cell (Quamme, 1980).

2.2. Thyroid hormone (TH)

Thyroid hormone are highly conserved during evolution and have already been found in invertebrate chordates and some plants (Salvatore, 1969). TH are tyrosine-based hormone and are produced by the thyroid gland in two different forms. About 80% of the hormone released by the thyroid gland are 3,3´,5,5'-tetraiodo-L-thyronine (T_4) and around 20% are 3,3',5-triiodo-L-thyronine (T_3). The essential basis for thyroid hormone is iodine comprising 65% of T_4`s weight and 58% of $T_3$`s. The current opinion in textbooks suggests that biological inactive T_4 is a precursor (prohormone) due to the fact that most of the TH bound to receptors is in the form of T_3 derived from the conversion of T_4 to T_3 by deiodinases. After cleavage of an iodine atom by deiodinases, T_4 is converted in its biologically active form T_3 (Siegenthaler, 2006).

2.2.1. Thyroid hormone biosynthesis

Biosynthesis, storage and release of TH are strictly regulated by the hypothalamic–pituitary–thyroid axis. The hypothalamus senses low concentrations of circulating T_3 and T_4 in the bloodstream. The hypothalamic paraventricular nucleus contains a particular subtype of neurons, which produce thyrotropin-releasing hormone (TRH). TRH biosynthesis is negatively regulated by T_3 and T_4 serum concentrations. If the

TH concentration falls below a certain concentration, the pituitary is stimulated to produce and release thyroid-stimulating hormone (TSH).

TSH itself stimulates the thyroid gland to produce TH up to a normal concentration until normal TH concentration is again perceived within the hypothalamus. A negative feedback loop is mediated by the TH via reduction of TRH and TSH by the hypothalamus as well as the anterior pituitary (Figure 2a) (Dietrich et al., 2012). TSH sensing in the thyroid gland is mediated by the TSH receptor (TSHR). The TSHR is a glycoprotein hormone receptor and is expressed at the basolateral membrane of thyroidal follicular cells where it can bind TSH. The TSHR can activate various G protein subtypes (Allgeier et al., 1994; Laugwitz et al., 1996; Laurent et al., 1987) and signaling pathways such as mitogen-activated protein kinase (MAPK) pathway (Buch et al., 2008; Latif et al., 2009).

For the regulation of elementary physiological processes by the thyroid hormone, a daily uptake of ~60 µg iodide is necessary. The accumulation and transport of iodine into the thyroid gland depends on the expression of the sodium iodine symporter (NIS, SLC5A5) on the basolateral membrane of the thyrocytes (Dai et al., 1996) (Figure 2b). NIS transports two Na^+ and one I^- against an electrochemical sodium gradient which is up to 30-fold higher and is co-regulated by the Na^+/K^+ ATPase (Dohan et al., 2003). Within the thyrocyte, iodine is transported by the ion channel transporter pendrin (SLC26A4) and anoctamin-1 (TMEM16A) on the apical (luminal) into the colloid of the follicle. The thyroid peroxidase (TPO) oxidizes iodide to atomic iodine (I) or iodinium (I+) via H_2O_2, which is mainly produced by the dual oxidase-2 (Figure 2b). The oxidized iodine is incorporated into thyroglobulin via reactive iodine species that are released by the TPO (Kessler et al., 2008).

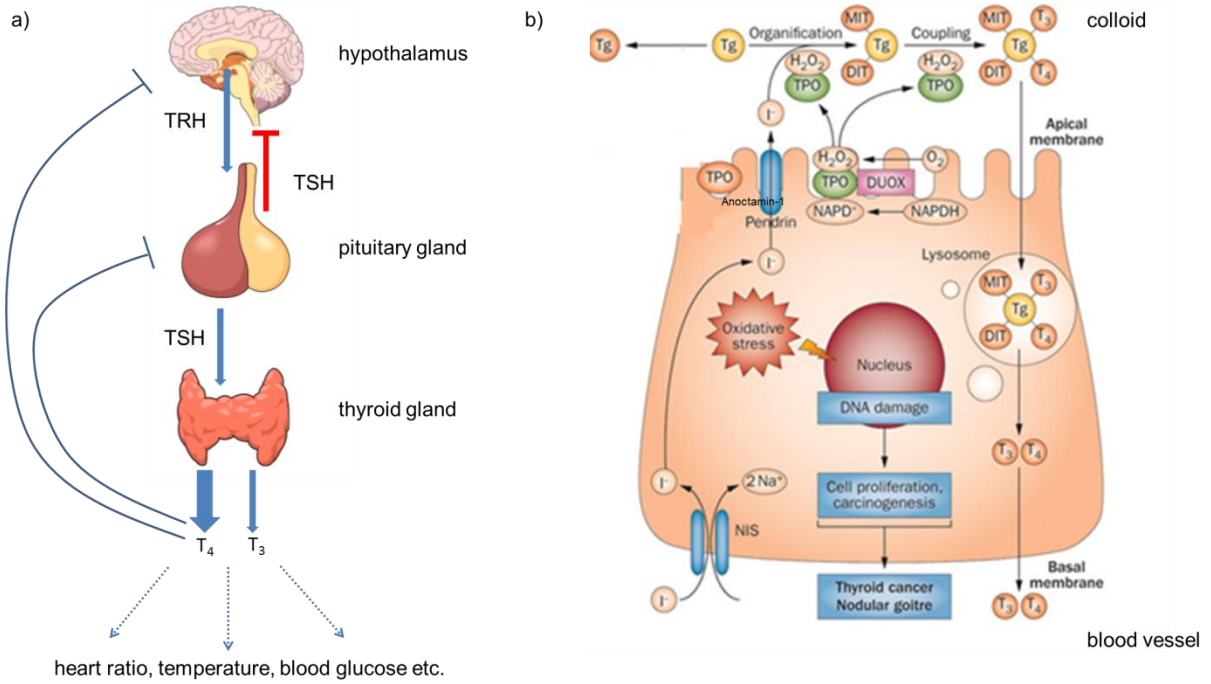

Figure 2: Hypothalamus-pituitary-thyroid axis (a) and thyroid hormone biosynthesis (b). TSH: Thyroid-stimulating hormone, TRH: Thyrotropin-releasing hormone DIT: diiodo-tyrosyl-residue Duox: dual oxidase, MIT: monoiodo-tyrosyl residue, NIS: sodium/iodide symporter, TG: thyroglobulin, TPO: thyroid peroxidase, ((b) modified from (Vitale et al., 2013)

Thyroglobulin is rich in tyrosine residues and serves as a template for the tyrosine-based TH synthesis. Thyroglobulin is secreted and stored in the follicular lumen. The iodination takes place at the phenol rings of thyroglobulin. One and/or both of the carbons beside to the hydroxyl-group are covalently iodinated, yielding monoiodotyrosine (MIT) and/or diiodotyrosine (DIT). In the second enzymatic coupling reaction, T_3 and T_4 are formed from the precursor MIT and DIT by the TPO (Taurog et al., 1996). At the apical surface of the thyrocytes, internalization and proteolysis of thyroglobulin/TH-complex is mediated by proteases such as cathepsins B and L resulting in liberated TH (Brix et al., 1996; Brix et al., 2001; Tepel et al., 2000). After exocytosis of the TH into the blood stream, the majority (>98%) of TH will be bound to transporter proteins like thyroxine-binding globulin or transthyretin and only 2% of TH circulate freely. The half-life of T_4 in the blood is 5-7 days and for T_3 it is only around 1 day (Frolkis and Valueva, 1978).

2.2.2. Inadequate production of thyroid hormone – hypothyroidism and hyperthyroidism

Several thyroid disorders resulting in thyroid malfunction are known. The majority of dysfunction leads to either hypothyroidism or hyperthyroidism. An insufficient production of TH is referred to hypothyroidism, which causes a variety of different symptoms such as fatigue, constipation, depression, and weight gain in humans. The reasons are manifold like Hashimoto thyroiditis due to antibodies directed against TPO, aplasia or dysplasia of the thyroid gland, mutations in key enzymes that are involved in the biosynthesis or uptake of TH, resistance to TH or insufficient iodine uptake. Hypothyroidism is treated with thyroid hormone replacement therapy (thyroxine - T_4). In hypothyroidism, the low concentrations of T_3 and T_4 are sensed by the hypothalamus resulting in increased release of TRH. TRH stimulates the pituitary to release a higher concentration of TSH (Figure 2). A prolonged high TSH concentration leads to proliferation and increase of the follicles leading to hyperplasia which is also named goiter. It is estimated that 90% of all goiters are associated with iodine deficiency (Hörmann, 2005).

In contrast, the excessive release of TH is called hyperthyroidism causing different symptoms like weight loss, tachycardia, diarrhea, heat intolerance, and trembling. The increased TH production can be caused by autoimmune disease (Graves' disease), toxic nodular goiter, toxic thyroid adenoma or thyroiditis (inflammation). Therapy is disease-related. Anti-thyroid drugs are used to reduce the biosynthesis of TH temporarily by inhibition of key enzymes in TH biosynthesis like the TPO with methimazole (MMI; (Nagasaka and Hidaka, 1976)) or competitive inhibition of iodine uptake by NIS with perchlorate (Crooks and Wayne, 1960). Other treatment options are radioiodine therapy and thyroid surgery. In general, hyperthyroidism is 2 to 10 times more often present in women and starts between 20 to 50 years but is more common over 60 years (Garmendia Madariaga et al., 2014). An overview of TH concentrations in euthyroidism, hypothyroidism and hyperthyroidism is provided in table 1. It is important to pay attention to these serum concentrations which are very close, above or below to the reference range, since they are not comparable between individuals. It is still a matter of debate what defines healthy TH serum concentrations between the ages and especially between men and women. In 2014,

a publication in Nature pointed out that there must be a greater awareness of the effects of sex and age on the treatment of thyroid diseases and in animal experiments (Clayton and Collins, 2014).

Table 1: Reference range of human thyroid hormone concentrations in euthyroidism, hypothyroidism and hyperthyroidism

	Euthyroid	**Hypothyroid**	**Hyperthyroid**
TSH [mU/dl]	0.5 - 3	>3	<0.5
TT_3 [ng/dl]	100 - 200	<100	>200
TT_4 [µg/dl]	4.5 – 11.2	<4.5	>11.2

2.3. Thyroid hormone signaling

The signaling pathways activated by TH are complex and tightly regulated depending on the target cells. The expression of cell and tissue specific TH transporters and thyroid hormone receptors (TR) are crucial and specific for adequate responses.

The basic idea regarding the effect of thyroid hormone is that they are actively transported into the cell and inside the nuclei where they bind the TR and regulate gene expression. This pathway is called Type 1: “TR dependent signaling of TH with direct binding to DNA” (Figure 3a) (Flamant et al., 2017). A decade ago, evidence was published showing that TH might also act through a different mechanism by activating the phosphatidylinositol-3 kinase (PI3K) pathway, called Type 3: “TR dependent signaling of TH without DNA binding” pathway (Figure 3b) (Cao et al., 2005; Flamant et al., 2017; Lei et al., 2004; Simoncini et al., 2000). Both pathways, Type 1 and Type 3, require binding of TH to the TR. In 2005, it was shown by Bergh *et al.* that TH not only interacts with TR but also with a purified plasma membrane protein: the integrin $\alpha_v\beta_3$. This pathway is referred to Type 4: “TR independent TH signaling” (Flamant et al., 2017) pathway. Up to now, $\alpha_v\beta_3$ is therefore the only known plasma membrane receptor that transmits TH signaling from the extracellular compartment into the cell (Figure 3c+d) (Bergh et al., 2005).

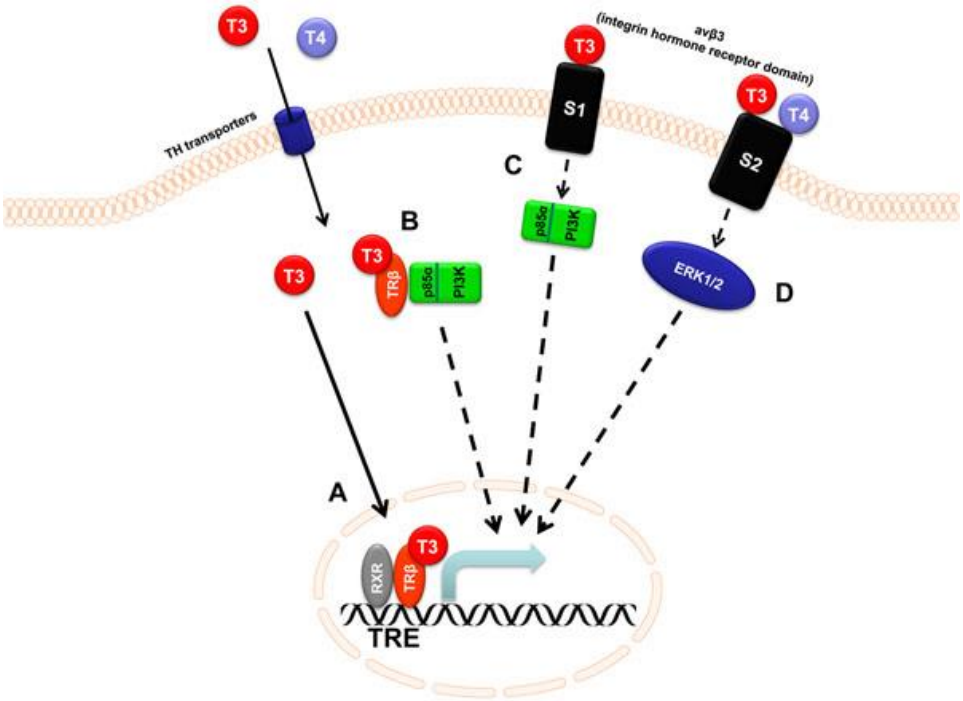

Figure 3: Mode of action by TH signaling (modified from (Lin et al., 2009; Moeller and Broecker-Preuss, 2011; Moeller et al., 2006)**.** (A) Type 1 pathway of gene expression; (B) activation of PI3K by ligand bound TRβ (Type 3); (C) activation of PI3K via $\alpha_v\beta_3$/S1 (Type 4); (D) activation of ERK1/2 via $\alpha_v\beta_3$/S2 (Type 4). PI3K: Phosphoinositide 3-kinase, ERK: extracellular regulated kinase.

2.3.1. Signaling of thyroid hormone by the thyroid hormone receptors

In the year 1986, the groups of Evans and Vennstrom were able to clone two different TR isoforms which are known as *THRA* and *THRB* encoded for TRα and TRβ on human chromosomes 17 and 3, respectively (Sap et al., 1986; Weinberger et al., 1986). However, alternative splicing transcript variants were demonstrated for TRα and TRβ resulting in several different short isoforms (Bigler et al., 1992; Chassande et al., 1997; Williams, 2000; Wrutniak et al., 1995)

These isoforms are expressed differentially during embryogenesis and are tissue-specific. The proteins encoded by *THRA* and *THRB* share a highly homologous structure but are different in length at N- and C-termini. They consist of a DNA binding domain (DBD) and a C-terminal ligand binding domain (LBD). The LBD of the TR binds T_3 with a 10- to 15-fold higher affinity than T_4. The transcription of target genes is inhibited in the absence of TH due to co-repressors with histone deacetylase activity. If T_3 is bound to the LBD of the TR, this leads to a conformational change which releases the co-repressor and recruits the co-activator.

The THs act as enhancers for the regulation of their target genes. The TR/TH complex binds to TH response elements within the promoter region of their target genes and engages the RNA polymerase II.

The TR isoforms seem to have a different affinity to the same promoter and therefore result in different gene expression depending on the predominantly expressed isoform (Chan and Privalsky, 2009; Chiamolera et al., 2012). The activation of the Type 1 pathway usually takes several hours since transcription and translation are complex mechanisms taking time. In the Type 3 pathway, the effects occur much faster. The ligand binding TRβ seems to be able to activate the PI3K pathway via phosphorylation within minutes (Cao et al., 2005; Lei et al., 2004; Simoncini et al., 2000).

Nevertheless, TRβ activates expression of certain target genes e.g. ZAKI-4α, via PI3K. To demonstrate that the gene expression of ZAKI-4α is mediated by the PI3K, human fibroblast were stimulated with either T_3 or with T_3 and the tyrosine kinase inhibitor LY290402. In the presence of the tyrosine kinase inhibitor LY290402, the gene expression of ZAKI-4α was absent with similar results regarding TRα (Cao et al., 2005; Moeller et al., 2005). Furthermore, to validate the existence of the Type 3 pathway, Shibusawa *et al*. mutated the zinc-finger domain within the DBD of the TRβ. Without the ability of the TRβ to bind to the DNA *in vitro,* all effects mediated by the Type 3 pathway are still preserved but the effects of the Type 1 pathway are disappear (Shibusawa et al., 2003).

2.3.2. Epidemiology suggesting tumor promoting effects of thyroid hormone

Hyperthyroidism has long been suspected being a risk factor in the context of tumor progression. In a cohort of 767 ovarian cancer patients and 1367 control patients, it was found that patients with hyperthyroidism possess a higher risk to develop ovarian cancer compared to healthy patients. The probability of ovarian cancer in the hyperthyroid group was nearly twofold higher compared to healthy donors (Ness et al., 2000). Similar results were obtained for pancreatic (Ko et al., 2007) and prostate cancer (Lehrer et al., 2001; Lehrer et al., 2002). Vice versa, hypothyroid men had a lower risk of prostate cancer compared to the healthy control group (Mondul et al., 2012) and women for breast cancer (Cristofanilli et al., 2005).

Furthermore, hypothyroid women are more likely to be diagnosed in an earlier stage of breast cancer and without spreading of the tumor cells to the lymph node indicating a slower progression (Cristofanilli et al., 2005). In contrast, elevated T_3 concentration in a study of 2696 women showed that the highest quartile of T_3 concentration possessed a higher risk developing breast cancer (Tosovic et al., 2010). Contrarily, a control study showed that only in women, not in men, with hypothyroidism a 3-fold increased risk for hepatocellular carcinoma insists. Notable, the patients TH concentrations were not measured. The calculation was based on self-reported information of thyroid disorders by the patients' (Hassan et al., 2009; Moeller and Fuhrer, 2013).

Such epidemiological studies demonstrate a potential role of TH in context of tumorigenesis in humans. The first data showing tumor-promoting effects of TH were made in rodent models in the late 1970s. Shoemaker and his colleagues induced hypothyroidism with a competitive inhibitor of the TPO in mice and inoculated mammary adenocarcinoma cells. They observed reduced tumor size and prolonged survival in hypothyroid mice compared to euthyroid mice (Shoemaker et al., 1976). Nonetheless, TH concentrations were not measured in these experiments so it is unknown if the induction of hypothyroidism in those mice was able to abolish endogenous expression of TH.

Further experiments in rodents with hepatic cancer cells (Mishkin et al., 1981), prostate adenocarcinoma cancer cells (Theodossiou and Schwarzenberger, 2000), and lung adenocarcinoma cancer cells (Theodossiou et al., 1999) revealed similar results regarding tumor growth. An increased tumor weight and size after TH treatment was observed by Kumar *et al.* (Kumar et al., 1979) and Kinoshita *et al.* (Kinoshita et al., 1991). They inoculated sarcoma cancer cells (S1), fibrosarcoma cells (T241) or Lewis Lung Cell carcinoma (3LL) subcutaneous into mice and observed a dose dependent increase of tumor weight only in T_4 treated mice, not T_3, indicating T_4 as the tumor promoting TH in those models. For both experiments, TH concentrations were available and induction of hyperthyroidism in those mice was confirmed.

The mode of action that is mediated by the TH in the context of tumorigenesis was unknown when those experiments were performed but the existence of TRs was known. To answer the question whether TRs are responsible for the tumor promoting

effects, cell lines were used lacking TRs. When those cell lines lacking TRs were transplanted into hypothyroid nude mice, no significant alterations of the previously obtained results regarding tumor growth were observed (Moeller and Fuhrer, 2013; Theodossiou and Schwarzenberger, 2000; Theodossiou et al., 1999). This was the first hint that the effects of TH during tumorigenesis are not predominantly mediated by TRs.

2.3.3. Signaling of thyroid hormone by the integrin $\alpha_v\beta_3$

The first idea that thyroid hormone also act in the extracellular compartment arose when agarose was linked to T_4 so that the agarose-linked T_4 is not able to be transported into the cells. It was observed that the agarose-linked T_4 was able to activate intracellular signaling via the MAPK-pathway on HeLa cells. When co-incubated with an inhibitor (pertussis toxin) of pERK1/2, phosphorylation of pERK1/2 mediated by agarose-linked, T_4 was gone (Lin et al., 1999).

Moreover, stimulation experiments in CV-1 monkey fibroblast and HeLa cells that also lack functional TRs also show a rapid phosphorylation of ERK1/2 upon T_4 stimulation (Lin et al., 1999) indicating TR independent signaling.

A plasma membrane receptor that is able to bind T_4 was identified by Bergh *et al.*. Bergh *et al.* could show that a heterodimer integrin named $\alpha_v\beta_3$ (consisting of αv and β3 subunits) activates signaling pathways upon TH stimulation. They incubated [^{125}I]T_4 together with purified $\alpha_v\beta_3$ and non-radioactive labeled T_4 in a radioligand binding assay. The complex was then separated by a non-denaturing polyacrylamide gel electrophoresis. They observed a dose-dependent diminishing of radioactive signals by unlabeled T_4. T_3 was much less effective at displacing [^{125}I]T_4.

Additionally, preparation of whole cell lysates revealed that only one protein was capable of binding [^{125}I]T_4, which was isolated by immunoprecipitation and this was $\alpha_v\beta_3$. Hence, to study T_3 and T_4 binding to $\alpha_v\beta_3$ pharmacokinetics and pharmacodynamics, investigations were performed and revealed that $\alpha_v\beta_3$ contains two binding sites (Figure 4) (Lin et al., 2009). One binding site (S1) of $\alpha_v\beta_3$ binds exclusively T_3 and activates the PI3K pathway by activating Src kinase and PI3 kinase. However, cell surface action of T_3 results in driving TRα amongst other

proteins from cytoplasm to the nucleus where TRα acts as a transcription factor for different genes but does not contribute to cell proliferation (Figure 4) (Blum et al., 2006; Kim et al., 2008; Monferran et al., 2008; Yu et al., 2008).

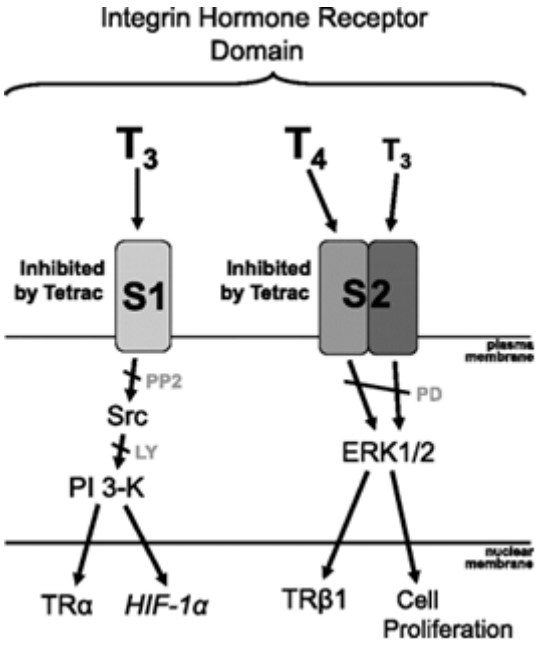

Figure 4: Proposed pathways and mode of action by TH regarding proliferation and intracellular trafficking TR. PD: PD-032590, LY: LY290402 (modified (Lin et al., 2009))

The other receptor binding site (S2) of $\alpha_v\beta_3$ is capable of binding T_3 and T_4 and activates a different pathway, the MAPK/ERK-pathway. In contrast to the S1 binding site, S2 recruits TRβ1 amongst other proteins from cytoplasm into the nucleus allowing the transcription of target genes (Lin et al., 2009; Lin et al., 2003; Yu et al., 2008). A specific inhibitor (3,3′,5,5′-Tetraiodothyroacetic acid (Tetrac)) of the integrin $\alpha_v\beta_3$ can block signaling via S1 and S2 (Bergh et al., 2005). Tetrac is a natural deaminated T_4 derivative which is catalyzed in low concentrations by deiodinases in different organs (Rajabi et al., 2016) .

2.3.4. The natural inhibitor 3,3′,5,5′-Tetraiodothyroacetic acid (Tetrac) of $\alpha_v\beta_3$ counteracts T_3 and T_4 mediated signaling and shows anti-tumor activity

Before Tetrac was identified as an inhibitor of $\alpha_v\beta_3$, it was only known that Tetrac is taken up by pituitary cells which secrete TSH. Besides that finding, it was known that Tetrac competes with TH on binding to transthyretin but not thyroxine-binding globulin. It was assumed that Tetrac contributes to the endogenous human TSH release by TSH producing cells in the pituitary gland (Burger et al., 1979; Davis et al., 1983). The physiological role of Tetrac is still not fully understood.

However, the activation mediated by S1 and S2 of $\alpha_v\beta_3$ upon binding of T_3 and T_4 is prevented in the presence of the specific inhibitor Tetrac. The deaminated T_4 derivative Tetrac (Bergh et al., 2005) acts as an antagonist and inhibits the trophic effect of T_3 and T_4 at the integrin $\alpha_v\beta_3$ in e.g. thyroid cancer (Lin et al., 2007) and lung adenocarinoma (Lin et al., 2009; Lin et al., 2008; Meng et al., 2011). The conversion of T_4 to Tetrac by deiodinase I is mainly performed in the liver and by deiodinase II predominantly found in the brain (Rajabi et al., 2016).

Tetracs affinity towards S1 and S2 binding sites in $\alpha_v\beta_3$ is higher. Tetrac is therefore able to displace T_3 and T_4 from S1 and S2 binding sites (Bergh et al., 2005). Consequently, Tetrac blocks all actions that are mediated by S1 and S2 upon T_3 and/or T_4 binding. The effects of Tetrac in the context of apoptosis and angiogenesis on tumor cells are well studied *in vitro*. Treatment of human breast cancer cell line (MDA-MB-231) showed an upregulation of pro-apoptotic genes like Caspase 2 or X-linked inhibitor of apoptosis protein when incubated with Tetrac. Furthermore, angiogenesis promoting genes like thrombospondin 1 are downregulated (Bergh et al., 2005).

Observations of decreased tumor growth, anti-angiogenesis and pro-apoptotic effect upon Tetrac treatment led to the first xenografts. Human solid cancer cell lines like medullary carcinoma cells (Yalcin et al., 2010b), follicular thyroid cancer cells (Yalcin et al., 2010a), pancreatic cancer cells (Yalcin et al., 2013) and human renal carcinoma cells (Yalcin et al., 2009) were transplanted into non-immunocompetent mouse strains and treated with Tetrac. The results showed a delay in tumor growth

and vascularization showing the potential role of Tetrac in the context of tumorigenesis.

Beside tumor reduction, Tetrac can also decrease the hemoglobin content within tumors indicating lower vascularization (Carmona-Cortes et al., 2014). Several reasons are assumed for the lower vascularization in tumors when $\alpha_v\beta_3$ is blocked by Tetrac. There are indications that TH have a pro-angiogenic effect on endothelial cells by releasing growth factors like basic fibroblast growth factor (bFGF) (Bergh et al., 2005; Davis et al., 2004; Mousa et al., 2006; Mousa et al., 2005). Furthermore, in the absence of TH, Tetrac is capable to inhibit the expression of crucial proteins for neovascularization like vascular endothelial growth factor (VEGF) and bFGF on protein level (Mousa et al., 2008) and mRNA level (Yalcin et al., 2010b). When TH binds to $\alpha_v\beta_3$, the observed effects suggest to favor proliferation of tumor cells and increase vascularization in non-immunocompetent mice.

However, signaling via $\alpha_v\beta_3$ seems to be more complex. $\alpha_v\beta_3$ not only activates downstream signaling pathways but also interacts with other plasma membrane receptors like VEGFR, EGFR and bFGFR which are clustered on the cell surface (Mousa et al., 2008; Shih et al., 2004). Tetrac can universally inhibit all downstream effects in this cluster. Binding of TH to $\alpha_v\beta_3$ seems to favor activation of MAPK/ERK signaling pathway resulting in phosphorylation of ERK1/2. Phosphorylated ERK1/2 activate specific serine phosphorylation of certain cytosolic proteins like signal transducer and activator of transcription-1α (Lin et al., 1998), estrogene receptor α (Tang et al., 2004), TRβ (Davis et al., 2000) and tumor suppressor gene 53 (Shih et al., 2001). Those proteins translocate to the nuclei where they act as transcription factors of target genes. The exact effects of the target genes are not yet fully understood.

$\alpha_v\beta_3$ is not only expressed on endothelial cells and tumor cells but also on immune cells where the function is not yet fully understood (Antonov et al., 2004). Kinoshita *et al.* revealed that isolated NK cells showed an immunosuppressive mode of action only mediated by T_4 but not by T_3 and furthermore an increased cytotoxicity of T_3 and T_4 treated alveolar macrophages. Similar results regarding immunosuppressive function of T_4 is shown by Gupta et al. in an allogenic skin graft mouse model (Gupta et al., 1983).

2.4. Thyroid cancer

Thyroid cancer is the most common malignant endocrine tumor but represents only 1% of malignant cancer. The incidence of thyroid cancer is estimated at 5000-7000 new cases per year in Germany according to the Robert-Koch-Institute. The incidence for women is higher (9.3 / 100.000 new cases per year) whereas only 3.8 / 100.000 men suffer from thyroid cancer (Reiners C, 2005; Robert-Koch-Institut, 2015). The incidence has increased 3-fold within the last 30 years with no alteration of the mortality. One reason probably is the improved diagnostic and therefore the possibility to identify micro tumors (Chen et al., 2009).

The tumor entities are classified according to morphology and immunohistochemistry and differ in aggressiveness, capability of invasion, and expression of certain thyroid specific proteins. Four major thyroid cancer subtypes are known: differentiated thyroid carcinoma (DTC), mainly comprised of papillary thyroid carcinoma (PTC), and follicular thyroid carcinoma (FTC), poorly differentiated thyroid carcinoma (PDTC), anaplastic (undifferentiated) thyroid carcinoma (ATC), and medullary thyroid carcinoma (MTC). Differentiated thyroid carcinoma accounts for ~80% of all thyroid cancer and originates from the follicular cells of the thyroid gland. The PDTC (<5%) and ATC (<5%) also arise from the follicular cells but are much more aggressive and account for the majority of thyroid cancer related deaths.

The only cancer entity which does not arise from the thyrocyte is the MTC (~10%). The MTC originates from the C-cells of the thyroid gland. The overall survival and the prognosis of thyroid cancer patients mainly depend on thyroid cancer type and “Union Internationale Contre le Cancer” (UICC) stage (Table 2). Stage I usually means that a cancer is small in size (<2 cm) and contained within the organ it started in. In stage II, the tumor is larger (2-5cm) but is still within the organ it developed in. When a tumor reaches stage III, the tumor is larger than 5 cm, and in stage IV, the tumor also started to metastasize.

Known risk factors for benign and malignant thyroid cancer are X-ray during childhood in the head and neck area, genetic predisposition (mainly for MTC), high exposure to radioactive substances, iodine deficiency, and elevated TSH. Non-heredity differentiated thyroid cancer usually occurs in the group aged from 30 to 50 years whereas ATC usually occurs in older age (>65 years) (Pfannenstiel P, 1991).

Table 2: 5-year survival of thyroid cancer patients (Barbet et al., 2005; Ibrahimpasic et al., 2014; The.American.Cancer.Society)

Thyroid cancer Type	Stage I	Stage II	Stage III	Stage IV
PTC	100%	100%	93%	51%
FTC	100%	100%	71%	50%
PDTC	94%	94%	65%	45%
ATC	Always stage IV			7%
MTC	100%	98%	81%	28%

2.4.1. Molecular pathogenesis of thyroid cancer

Over the past two decades, the understanding of the underlying molecular pathogenesis in thyroid cancer increased tremendously. Genetic analysis of tumor tissue by high throughput sequencing improved dramatically, the knowledge of the mutational landscape in thyroid cancer. Sequencing results indeed showed a different mutational landscape in DTC compared to PDTC and ATC (Figure 5). The majority of mutations found in thyroid cancer is either involved in MAPK/ERK-pathway (*BRAF, RET/PTC, ALK, RAS)*, PI3K-pathway (*RAS, ALK, RET/PTC, PI3KCA* or tumor suppressor genes (*TP53, NF1, PTEN).*

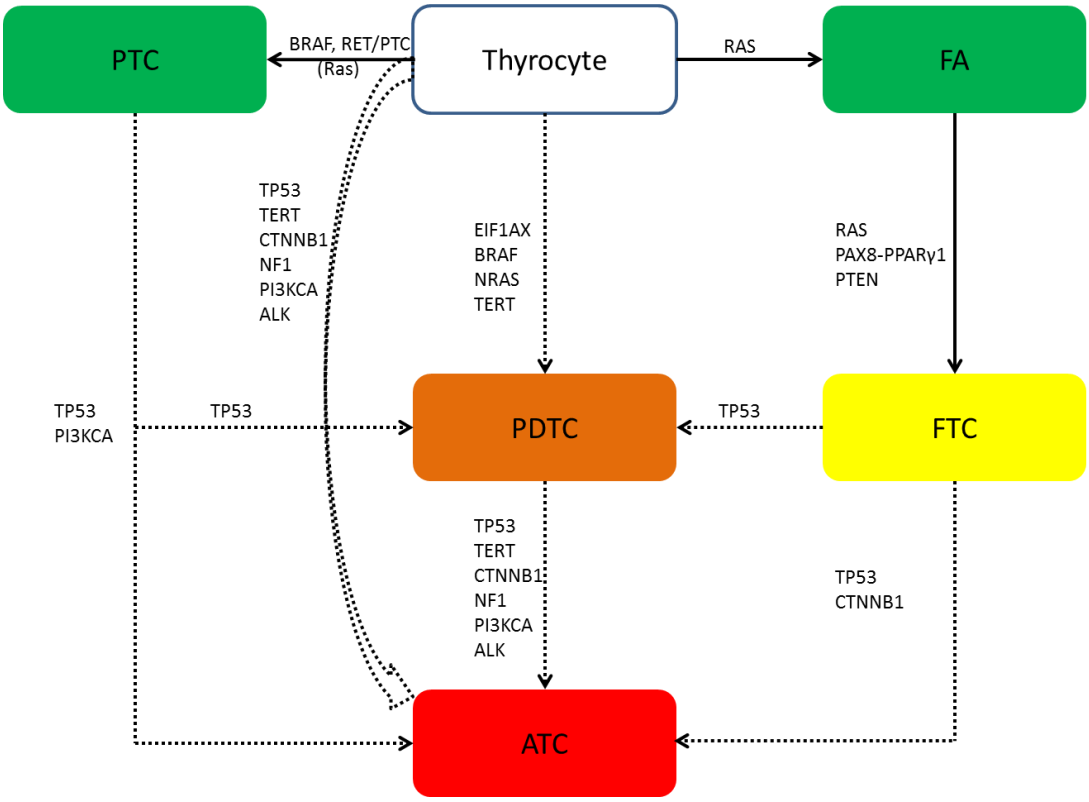

Figure 5: Molecular evolution of thyroid cancer. Progression model of thyroid cancer including pathways involved in pathogenesis thyroid cancer. *ALK*: Anaplastic lymphoma kinase *BRAF*: serine/threonine-specific protein kinases RAF, *CTNNB1*: β-catenin, *EIF1AX*: Eukaryotic translation initiation factor 1A, X-chromosomal, *NF1*: Neurofibromin 1, *PAX8-PPARγ1*: Paired-Box-Protein 8 - Peroxisome proliferator-activated receptor gamma, *RET*: Rearranged during transfection, *PI3KCA*: Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha, *PTEN*: Phosphatase and Tensin homolog, *RAS*: Rat sarcoma, *TERT*: Telomerase reverse transcriptase, *TP53*: Tumor suppressor p53.

The mutation load and hotspots in PTC, follicular adenoma (FA) and FTC are generally low and limited to few genes. PDTC are more heterogeneous regarding the underlying somatic mutation. They usually display similar mutations like the DTC but additionally have more frequent mutations in the tumor suppressor gene *TP53* and *telomerase reverse transcriptase* (TERT). The ATC is even more heterogeneous in terms of somatic mutation and histology compared to DTC and PDTC. Interestingly, mutations in *TP53* and *TERT* genes are crucial during dedifferentiation of the thyrocyte. *TP53* can activate DNA repair proteins, can arrest the cell cycle at G1/S regulation point, initiate apoptosis, and activate senescence when telomeres are too short. Therefore, TP53 is also called “the guardian of the genome” (Carr and Jones, 2016; Isobe et al., 1986; Kern et al., 1991; Matlashewski et al., 1984). *TERT* is the catalytic subunit of the enzyme telomerase that maintains telomere ends during mitosis. Together with the telomerase RNA component (TERC), the telomerase

forms the telomerase complex which prolongs the telomeres after replication especially in stem cells (Kirkpatrick and Mokbel, 2001; Weinrich et al., 1997). *TERT* mutation might be one mechanism to avoid senescence for cancer cells.

It seems that the higher the mutational load is the more undifferentiated is the thyroid cancer. Interestingly, many ATC seem to coexist with DTC and even PDTC can be found in the same patient. Consequently, the question arose whether an undifferentiated thyroid cancer develops *de novo* or as a consequence of dedifferentiation from DTC. At the beginning of this thesis, the heterogeneity of the ATC has not yet been properly characterized. In order to improve the knowledge about ATC, the ATC has been investigated since there is still no established treatment available and it still has a poor prognosis.

2.4.2. Differentiated thyroid carcinoma (DTC) – papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC)

The majority of all thyroid cancers are differentiated thyroid cancers subdivided into papillary thyroid carcinoma and follicular thyroid carcinoma (~80%). PTC comprise several subtypes of different variants but they are characterized by predominantly follicular growth pattern, slow-growing and localized, although they can metastasize. They represents 60-70% of all thyroid cancers. PTCs differ based on their histology but are mostly characterized by papillae with fibrovascular cores, increased mitochondria number, orphan annie eye nuclear inclusions, psammoma bodies, groove formation, optical clearing, eosinophilic inclusions, overlapping of nuclei, and expressing TG. In the majority of cases, the prognosis is excellent. Treatment of PTC usually includes surgery (lobectomy or thyroidectomy), in some stages radioiodine treatment and TSH-suppressive therapy.

Before 2014, it had been known that genetically PTCs are characterized by somatic alterations of genes involved in the MAPK signaling pathway, mainly *BRAF* and *RAS* mutations (Cohen et al., 2003; Kimura et al., 2003; Lemoine et al., 1988; Suarez et al., 1988) but also fusion proteins like RET/PTC (Grieco et al., 1990). In 2014, a milestone study was published by The Cancer Genome Atlas Research Network (TCGA). The Cancer Genome Atlas Research Network performed whole exome

sequencing, RNA sequencing, miRNA sequencing, SNP arrays, DNA methylation arrays, and reverse phase protein arrays on 496 PTC and were therefore able to characterize the genomic landscape of PTC.

Most frequent mutations were $BRAF^{V600E}$ (60%), 11% *RAS* mutations and 7% RET fusions proteins. The Cancer Genome Atlas Research Network also observed a clustering of mutations characterized by a similar activation of signaling pathways and for that reason divided the PTC into two groups. Depending on the underlying mutations, the PTCs were subdivided into so called BRAF-like signaling PTCs and RAS-like signaling PTCs.

Whereas BRAF-like mutations predominantly activate only the MAPK-pathway, the RAS-like PTCs activate MAPK-pathway and the PI3K-pathway. Furthermore, the TCGA observed evidence that potential driving mutations (e.g. *RET*, *BRAF*) are clonal events. The majority of tumor cells harbored those mutations.

Follicular thyroid carcinomas represent 5%-10% of all thyroid cancer and are defined as "A malignant epithelial tumor showing follicular cell differentiation and lacking the diagnostic nuclear features of PTC" by the WHO Classification of Tumours of Endocrine Organs (Sobrinho Simoes M, 2004). FTCs are usually encapsulated single solid tumors, minimally or widely invasive. The treatment comprises surgery, in some cases radioiodine therapy and TSH-suppression. Nearly 50% of all FTCs display a mutation in the *RAS* subfamily. Mutations in the tumor suppressor gene *PTEN* as well as fusion proteins of paired box gene 8 (PAX-8) and peroxisome proliferator-activated receptor γ 1 (PPARγ1) are known.

2.4.3. Less differentiated thyroid carcinoma – poorly differentiated thyroid carcinoma (PDTC) and anaplastic thyroid carcinoma (ATC)

Poorly differentiated thyroid carcinoma (PDTC) was first described in 1984 by Carcangiu (Carcangiu et al., 1984). According to the Turin proposal, PDTC is classified by the presence of insular/solid trabecular growth pattern, the absence of nuclear features of PTC and at least one of the following features: convoluted nuclei, mitotic index >3/10 mitoses pro high power field, and necrosis (Volante et al., 2007). Controversial discussion has since been carried out due to the intermediate

histopathological patterns between differentiated and undifferentiated thyroid cancers and therefore the criteria might be inappropriate. Since the histopathology differs further, criteria like the Memorial Sloan Kettering Cancer Center (MSKCC) were raised (Hiltzik et al., 2006). The currently used therapy includes thyroidectomy (debulking) if applicable lymph node dissection, radioactive iodine and/or radiation therapy as well as TSH-suppression. However, there is a very high risk that the PDTC is already radioiodine refractory. If this is the case, patients are treated with tyrosine kinase inhibitors Sorafenib [Nexavar®] and Lenvatinib [Lenvima®]).

Nevertheless, the mutational load in PDTC is higher compared to DTC but still lower than in ATC (Landa et al., 2016). The anaplastic thyroid carcinoma is a very rare and lethal disease. It represents less than 5% of all thyroid cancer but accounts for the majority of thyroid cancer related deaths. The median survival when diagnosed with ATC is 4-6 months and so far no treatment has conclusively been shown to improve overall survival (O'Neill and Shaha, 2013; Pinto et al., 2014). ATCs are histopathologically characterized by rapidly growing tumor cells, solid mass composed of large pleomorphic and giant cells, necrosis, high macrophage infiltration, hemorrhage, spindle cells, high mitosis rates, and loss of thyroid specific markers like TTF-1, TPO or TG.

Most patients suffer from rapidly growing tumors of the neck and hoarseness. Around 50% of all patients have distant metastases at diagnosis in lung (71%), intrathoracic lymph nodes (59%), neck lymph nodes (51%), pleura (29%), adrenal glands (24%), liver (24%) brain (18%) and/or bones (13%). Invasive growth pattern in the neck area, trachea and/or esophagus also occur frequently (Besic and Gazic, 2013).

The treatment of ATC patients requires a multimodal therapy including surgery, external beam radiation (EBRT), and chemotherapy (Wendler et al., 2016). No standardized treatment could significantly improve progression free survival (PFS) and the overall survival (OS) in ATC patients in the last 50 years. Even multikinase inhibitors fail to achieve improvement in ATC patients (Bible et al., 2012; Savvides et al., 2013). Nevertheless, a multimodal therapy slows down tumor growth to a certain extent.

The reason for the aggressiveness and the resistance to different treatment options might be due to the genomic instability of ATC cells. In DTCs, the mutation load is

usually limited to one to three “driver mutations”. Pathophysiologically, the ATC development is mainly based on uninhibited activation of various cellular signal transduction pathways, in particular of the PI3K and MAPK signal transduction pathway. In ATC, the mutation load shows a higher diversity and mutations in *TERT* (73%), *TP53* (73%), *BRAF* (45%), *NRAS* (18%), *PIK3CA* (18%), *PTEN* (15%), *EIF1AX* (9%), *HRAS* (6%) (Landa et al., 2016). ATC are heterogeneous involving many genes participating in the follicular epithelial cell dedifferentiation process where an effective therapy is still lacking. In 2013, for the first time, a case report was been published in which targeting the classic $BRAF^{V600E}$ mutation with a specific BRAF inhibitor (Vemurafenib [Zelboraf®]) in a patient with ATC derived from PTC has showed impressive tumor response demonstrating the benefits of targeted therapy (Rosove et al., 2013). One year later, the case of an ATC patient with ALK/EML4 fusion protein was reported, in which targeted ALK inhibition reduced the size of the metastases by up to 90% and a practically complete restoration of the quality of life (ECOG 0) for over 2 years after the initial diagnosis of the ATC was achieved (Godbert et al., 2015). In the same year, a case report of a patient with Tuberin 2 and mammalian target of rapamycin (mTor) mutations was reported. Those mutations resulted in a constitutive activation of the AKT/mTor pathway where a treatment with an mTor-inhibitor (Everolimus) resulted in a stable disease for 18 months (Wagle et al., 2014). Those case reports demonstrate that the investigation of the molecular pathogenesis followed by targeted therapy in ATC patients can indeed improve survival.

2.4.4. Medullary thyroid carcinoma (MTC)

Medullary thyroid carcinoma (MTC) is derived from C-cells of the thyroid gland. The MTC was first described as “malignant goiter with amyloid” in the year 1906 by Jaquet (Jaquet.AJ, 1906). MTC differ in its basic biology from other thyroid cancer types (Tiedje et al., 2015). In contrast to other thyroid cancer types, 25% of all MTCs arise hereditary and 75% occur sporadically (Schmid, 2010). MTC patients usually are asymptomatic and often complain of fatigue and exhaustion during initial diagnosis. Some patients can already feel small palpable nodes in the thyroid gland. In the late stage, MTC patients suffer from flushing, dyspnea, diarrhea, and itching.

The reason for the fatigue and exhaustion is altered calcitonin biosynthesis due to one highly specific mutation in a certain gene.

In more than 95% of all hereditary MTC patients, it is possible to identify a germline mutation in the proto-oncogene REarranged during transfection (*RET).* Not only in hereditary MTC but also in 60% of all sporadic MTC, it is possible to identify a somatic mutation in *RET*.

2.4.4.1. REarranged during transfection (RET)

The human *RET* gene is localized on chromosome 10 (10q11.2) and contains 21 exons (Ceccherini et al., 1993). RET has three domains consisting of the cadherin-like domain and cysteine rich domain in the extracellular compartment and the tyrosine kinase domain in the intracellular compartment (Figure 6). RET is expressed in cells derived from the urogenital system, the branchial arches, and the neural crest (Pachnis et al., 1993; Zordan et al., 2006).

Mutations in *RET* result in gain of function leading to ligand independent constitutive activation of RET. Constitutive activation of RET activates several intracellular signaling pathways like the PI3K-pathway and the MAPK-pathway leading to increased cell proliferation and survival (Cerrato et al., 2009; Drosten and Putzer, 2006).

RET signaling requires homodimerization, which is mediated by a complex of *gliant cell line-derived neurotrophic factor* (GDNF) and receptor-α (GFRα) protein family as well as the GDNF-family ligands (GFLs). The receptors and ligands have different affinities to each other. GFRα-GFL-complex recruits two molecules of RET and brings the tyrosine kinase domain in close proximity. Transautophosphorylation occurs within the tyrosine kinase and initiates downstream signaling (Airaksinen et al., 1999). Three different isoforms of RET exist due to natural alternative splicing: RET 9, RET43 and RET51. The numbers represent the amount of amino acids in their c-terminal tails behind the tyrosine kinase domain. Homozygous *RET* knockout mice die within hours after birth. They exhibit abnormalities in development of kidney/urinary and peripheral nervous system development. Furthermore, abnormal

enteric neural crest cell migration demonstrate a significant role of RET during development (Arighi et al., 2005; Schuchardt et al., 1994).

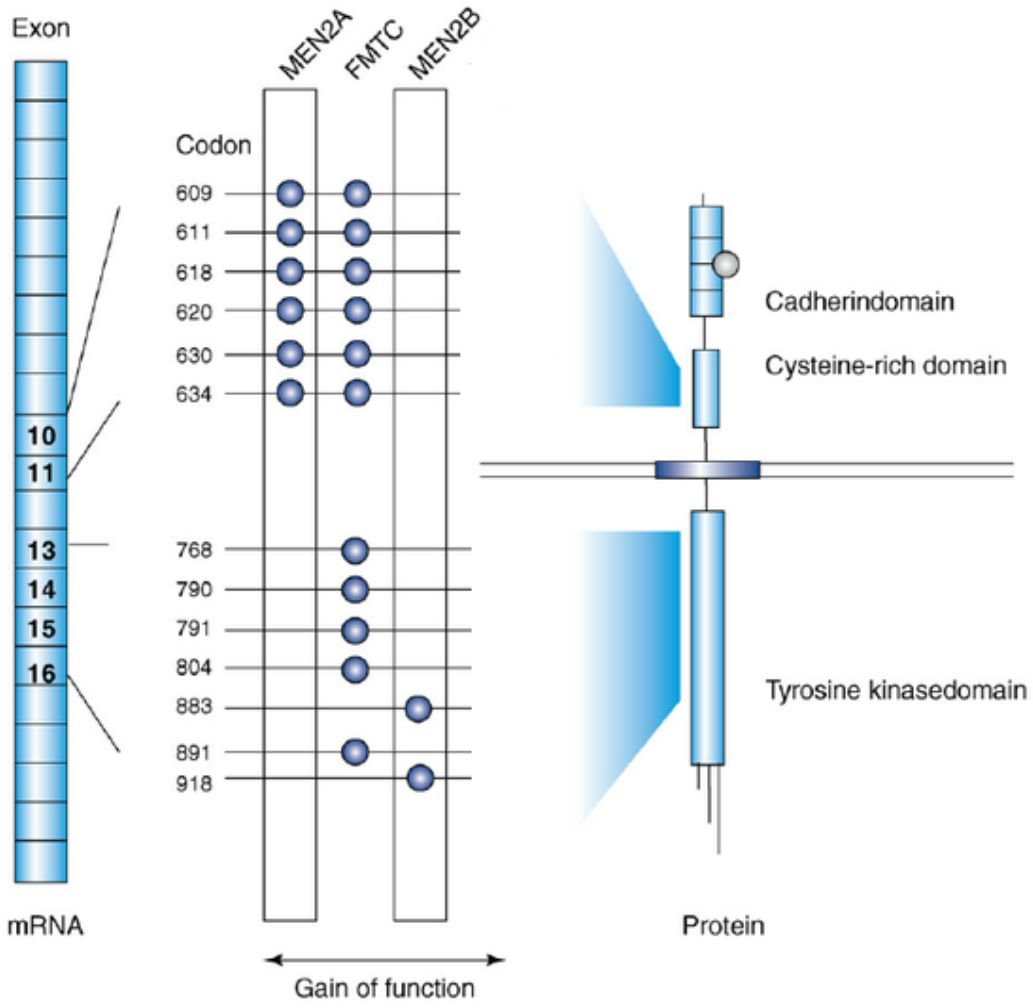

Figure 6: Schematic representation of the RET receptor with mutation hotspots in MTC. In the extracellular part, of RET is the cadherin domain located, the ligand binding domain as well as the cysteine-rich domain. In the intracellular part, is the Tyrosine kinase domain located. FMTC: familial MTC. Modified (Plaza-Menacho et al., 2006).

2.4.4.2. Hereditary MTC

Germline *RET* mutations are autosomal, dominantly inherited and cause a number of endocrine tumors resulting in multiple endocrine neoplasia type 2 disease. MEN2 patients are subdivided into MEN2A and MEN2B according to phenotype (Table 2) (de Groot et al., 2006). In 2015, the American Thyroid Association (ATA) integrated the former separated subgroup of familial MTC (FMTC) to the MEN2A. Patients were

regarded as FMTC when only a MTC and no additional symptoms like pheochromocytoma and hyperparathyroidism was detectable. Since the term FMTC is still frequently found in the literature, it is included separately in these chapters.

Table 3: Clinical syndromes associated with hereditary *RET* mutations (de Groot et al., 2006)

	FMTC	**MEN2A**	**MEN2B**
MTC	100%	100%	100%
Pheochromocytoma	-	10-60%	60%
Hyperparathyroidism	-	10-25%	-
Marfanoid habitus	-	-	100%
Mucosla neuromas	-	-	70-100%
Intestinal ganglioneuromatosis	-	-	60-90%
RET mutations hotspots	Exons:10-11 and 13-16	Exon: 5, 8, 10, 11	Exon: 15-16

The ATA Guidelines Task (Wells et al., 2015) subdivided hereditary MTC patients according to the underlying mutation of *RET* into risk groups. Risk does not mean whether patients with a germline mutation in *RET* develop a MTC but when. Group 1, with mutations in exon 16 of *RET* (M918T), is at “highest risk”. Group 2, with mutations in exon 11 and 15 of *RET* (C634R and A883F), is at “high risk”. Group 3, with any other mutations in *RET,* is at “moderate risk”*.* Patients belonging to group 1 show a remarkably unfavorable clinical course of the disease as compared to the other groups (Morrison and Marley, 1976; Schilling et al., 2001; Wohllk et al., 1996). The ATA guidelines recommend sequencing exon 5, 8, 10, 11, 13-16 of *RET* in patients with red flags for a genetic cause. However, the genetic basis for MEN2 can only be solved in 95% of the patients by sequencing the hotspots of *RET*. No further recommendations are available for the remaining 5% of MEN2 patients.

In general, there seems to be a close genotype-phenotype correlation between the *RET* germline mutation and the clinical outcome. The ATA summarized the relationship of common *RET* mutation, aggressiveness as well as the incidence of pheochromocytoma and hyperparathyroidism (Figure 7) (Wells et al., 2015).

In nearly all hereditary MTC patients, it is possible to identify a *RET* mutation. When parents are affected by a germline *RET* mutation, it is recommended to sequence and closely monitor the descendants. If the descendants are also affected by a *RET*

mutation, a prophylactic thyroidectomy is recommended as well as regularly control for pheochromocytoma and hyperparathyroidism.

RET *mutation*[a]	*Exon*	*MTC risk level*[b]	*Incidence of PHEO*[c]	*Incidence of HPTH*[c]
G533C	8	MOD	+	–
C609F/G/R/S/Y	10	MOD	+/++	+
C611F/G/S/Y/W	10	MOD	+/++	+
C618F/R/S	10	MOD	+/++	+
C620F/R/S	10	MOD	+/++	+
C630R/Y	11	MOD	+/++	+
D631Y	11	MOD	+++	–
C634F/G/R/S/W/Y	11	H	+++	++
K666E	11	MOD	+	–
E768D	13	MOD	–	–
L790F	13	MOD	+	–
V804L	14	MOD	+	+
V804M	14	MOD	+	+
A883F	15	H	+++	–
S891A	15	MOD	+	+
R912P	16	MOD	–	–
M918T	16	HST	+++	–

Figure 7: Close genotype-phenotype correlation between *RET* mutation and clinical outcome. a) Common *RET* mutations b) Risk of aggressive MTC: MOD, moderate; H, high; HST, highest. c) Incidence of PHEO (pheochromocytoma) and hyperparathyroidism (HPTH): +=~10%, ++= ~20-30%, +++=~50%. (Modified: (Wells et al., 2015))

The initial treatment of any MTC patients is normally a thyroidectomy to remove the primary tumor as well as to decrease the elevated calcitonin concentration. If a pheochromocytoma is also detectable in the adrenal medulla, the tumor should also be removed prior to the MTC. In the case that a systemic therapy is necessary due to metastases or progression according to RECIST (Response Evaluation Criteria in Solid Tumors) (Schlumberger et al 2012), two tyrosine kinase inhibitors (TKI) are currently approved by the European Medicines Agency (EMA). In two randomized double-blind phase III studies Vandetanib (Caprelsa® (February 2012)) and Cabozantinib (Cometriq® (March 2014)) were approved by the EMA (Elisei et al., 2013; Wells et al., 2012). Vandetanib primarily inhibits RET and the endothelial growth factor receptor (EGFR) pathway, Cabozantinib also inhibits vascular endothelial growth factor (VEGF) and hepatocyte growth factor receptor (c-MET).

2.4.4.3. Sporadic MTC

Sporadic MTC occurs in around 75% of all MTCs. In 40-60% of the patients, somatic *RET* mutations are detected in the tumor (Eng et al., 1994). The most prevalent somatic *RET* mutation (65%) is RET M918T in exon 16 which is associated with poor prognosis (Moura et al., 2009) which was shown in a cohort of 34 MTC patients (Schilling et al., 2001) and 100 MTC patients (Elisei et al., 2013). Schilling *et al.* and Elisei *et al.* could show that RET M918T mutations in sporadic MTC indeed resulted in a significantly higher risk of distant metastases and significantly more frequent lymph node metastases. Moreover, the overall survival was significantly lower in the group harboring a *RET* mutation compared to no *RET* mutation. Interestingly, in sporadic MTC there seems to be no significant difference regarding prognosis and survival between distinct *RET* mutations like it is known for hereditary MTCs (Moura et al., 2009).

In general, the primary therapy also includes thyroidectomy with central lymph node dissection which can result in a complete cure depending on the tumor stage during initial presentation.

3. Principal issues addressed in this thesis

This thesis answers the following open questions in thyroidology:

1) Which mutations occur in anaplastic thyroid carcinoma and might be relevant for targeted therapy?

At this time, the largest cohort of 30 ATC patients was analyzed by targeted next-generation sequencing. The results significantly increased the knowledge of the mutational landscape of ATCs and will help to develop targeted therapy for ATC patients.

2) What should be done when routine diagnostic according to the ATA guidelines fails to detect a germline mutation in MEN2 patients?

In 95% of all MEN2 patients, the underlying mutation can be found when routine diagnostic is performed according to the ATA guidelines at this moment. But how should the physician proceed to identify a mutation in the remaining 5% of patients? It was possible to detect the germline mutation in a patient with MEN2 outside the commonly sequenced exons. Therefore, this thesis demonstrates that in those cases the complete *RET* gene needs to be sequenced, because pathogenic mutations can occur in exons not yet associated with MEN2. After publication of this work, the ATA guidelines were adjusted accordingly.

3) Can TH promote tumor growth?

The adequate concentration of TH is crucial for normal physiology. However, TH is suspected to influence solid tumor growth in the periphery. In this thesis, it could be shown that TH indeed promotes tumor growth in a murine model. Furthermore, the mode of action by TH in the context of tumor progression was characterized. These results are highly relevant for those tumor patients that are currently supplemented with a TH that could support tumor growth.

4. Publications

Publication 1

Targeted next-generation sequencing for *TP53*, *RAS*, *BRAF*, *ALK* and *NF1* mutations in anaplastic thyroid cancer

S. Latteyer, V. Tiedje, K. König, S. Ting, L.C. Heukamp, L. Meder, K. W. Schmid, D. Führer and L. C. Moeller

Endocrine. 2016 Dec;54(3):733-741. doi: 10.1007/s12020-016-1080-9.

Contribution

Herein I performed, designed and analyzed all bioinformatic results obtained by Targeted Next-generation Sequencing. I contributed to writing of abstract, introduction, methods, results and discussion of the publication.

Endocrine
DOI 10.1007/s12020-016-1080-9

ORIGINAL ARTICLE

Targeted next-generation sequencing for *TP53*, *RAS*, *BRAF*, *ALK* and *NF1* mutations in anaplastic thyroid cancer

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Received: 12 April 2016 / Accepted: 4 August 2016

Abstract Anaplastic thyroid carcinoma (ATC) is the most aggressive thyroid cancer with a median survival of 4–6 months. Identification of mutations contributing to aberrant activation of signaling cascades in ATC may provide novel opportunities for targeted therapy. Thirty-nine ATC samples were studied by next-generation sequencing (NGS) with an established gene panel. High quality readout was obtained in 30/39 ATC. Twenty-eight ATC harbored a mutation in at least one of the studied genes: *TP53* (18/30), *NF1* (11/30), *ALK* (6/30), *NRAS* (4/30), *ATRX* (3/30), *BRAF* (2/30), *HRAS* (2/30), *KRAS* (1/30). In 17/30 ATC (54 %) mutations were found in two or more genes. Twenty-one of the identified variants are listed in COSMIC as somatic mutations reported in other cancer entities. In three ATC samples no mutations were detected and none of the ATCs was positive for $BRAF^{V600E}$. The most frequent mutations were found in *TP53* (60 %), followed by NF1 (37 %). *ALK* mutations were detected in 20 % of ATC and were more frequent than *RAS* or *BRAF* mutations. *ATRX* mutations were identified in 10 % of the ATC samples. These sequencing data from 30 ATC samples demonstrate the accumulation of genetic alterations in ATC because in 90 % of samples mutations were already found in the investigated nine genes alone. Mutations were found with high prevalence in established tumor suppressor and oncogenes in ATC, such as TP53 and H/K/NRAS, but also, although less frequent, in genes that may harbor the potential for targeted treatment in a subset of ATC patients, such as *ALK* and *NF1*.

Keywords Anaplastic thyroid cancer · ATC · Next-generation sequencing · BRAF · ALK · NF1 · ATRX · RAS

Electronic supplementary material The online version of this article (doi:10.1007/s12020-016-1080-9) contains supplementary material, which is available to authorized users.

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Introduction

Anaplastic thyroid carcinoma (ATC) is a very rare and fatal disease. It represents less than 5 % of all thyroid cancers but accounts for the majority of thyroid cancer-related deaths. Median survival is 4–6 months [1, 2] and so far no treatment has conclusively been shown to improve survival [3].

A better understanding of the molecular pathogenesis of ATC is urgently required to explore new treatment options. It is assumed that ATC can occur de novo, but can also evolve from differentiated thyroid cancer. Several genetic alterations have been identified in ATC during the past years, most prominently the inactivation of the p53 tumor

Published online: 01 October 2016

suppressor gene (*TP53*), found in up to 80 % of ATC [4–8]. In addition, constitutive activation of the PI3K pathway has consistently been demonstrated [9, 10] and is caused, for example, by *PIK3K* [11] or *RAS* mutations [9], PTEN inactivation [12] or AKT1 overexpression in ATC [13]. Constitutively active RAS mutations have been identified in 20–30 % of ATCs [4]. In contrast, mutations in the *BRAF* gene have been detected in rare ATC that arise from papillary thyroid cancer (PTC) [14] and in single cases targeted treatment has been reported [15, 16]. Furthermore, genetic alterations in the promoter of the telomerase reverse transcriptase (*TERT*) gene [17, 18] have recently been identified with higher mutation frequency (43–50 % in different series) compared to poorly and differentiated thyroid cancers [17, 19–21].

To better understand the molecular drivers contributing to the aggressive nature of ATC, genetic alterations in ATC need to be further studied. Next-generation sequencing (NGS) is a reliable method for high throughput parallel screening for mutations in several genes in tumor tissues [22]. In the present study, we investigated 39 ATC samples with an oncogene panel established and routinely used in our Department of Pathology. This panel was chosen because it includes genes with established relevance in ATC (*TP53*, *H/N/KRAS*, *BRAF*) and other genes, for which a role in ATC pathogenesis is either not known or not yet fully understood including anaplastic lymphoma kinase [*ALK*], neurofibromin [*NF*]1, alpha thalassemia/mental retardation X-linked [*ATRX*] and protein tyrosine phosphatase non-receptor type 11 [*PTPN11*]).

ALK encodes a transmembrane tyrosine kinase receptor that is expressed at high levels in the nervous system, especially during embryogenesis. Outside the nervous system, ALK is expressed in the intestine, skin, skeletal muscle and in thyrocytes [23]. Constitutively active ALK mutations have been found in neuroblastoma and their oncogenic role is mediated by activation of the PI3K/AKT and MAPK pathways. Furthermore, activating mutations in exon 23 of *ALK* gene have very recently been reported in ATC [24] and *ALK* and *striatin* (*STRN*) fusion genes have been found in PTC, poorly differentiated thyroid cancer and ATC [25]. These findings led to targeted treatment of a patient with ATC positive for the *ALK/STRN* rearrangement with an ALK inhibitor, resulting in an impressive clinical response [26]. NF1 is a GTPase activating protein (GAP) and thought to act as an inhibitor and regulator of RAS. Germline mutations in *NF1* with loss of function lead to neurofibromatosis 1, a syndrome with development of benign and malignant tumors, for example, soft tissue sarcomas and an increased risk of breast cancer.

Loss of ATRX expression changes the pattern of DNA methylation and mutations have been described in glioblastomas, medulloblastomas and, recently, in anaplastic thyroid cancer [27]. PTPN11 mutations with loss of function increase the susceptibility to DNA damage and development of malignancies, for example, leukemia, but the mechanism is not well characterized.

Material and methods

Tissues

Formalin-fixed paraffin embedded tissue (FFPE) samples of 39 ATCs that had been sent for second opinion to the Institute of Pathology at the University Hospital in Essen (reference center of thyroid pathology in Germany) were available for analysis. The diagnosis of ATC was confirmed by two independent board certified pathologists of the institute. Hematoxylin and eosin-stained slides were examined and DNA was isolated only from ATC areas. Sex and age at diagnosis, but no data on the clinical course of the patient, were available for 37 patients.

DNA isolation and sequencing

Genomic DNA was extracted from FFPE tissue with QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany), quantified with Qubit dsDNA HS Assay Kit (Life Technologies, Darmstadt, Germany) on the Qubit 2.0 Fluorometer and amplified with Ion AmpliSeq™ Custom DNA Panels (30 cycles with 50 ng extracted DNA) (LifeTechnologies). Overall, 371 amplicons of nine different genes were amplified with two primer pools (supplementary Table 1). Libraries were prepared as previously described [28], quantified with Qubit dsDNA HS Assay Kit on the Qubit 2.0 Fluorometer, diluted to 10 nM and pooled in equimolar amounts. Pooled libraries were then spiked with 5 % PhiX DNA (Illumina, San Diego, CA, USA) and sequenced paired end with the "MiSeq reagent Kit V2 (300-cycles)" (Illumina). FastQ files generated by the MiSeq Reporter were used as data output.

Generation of variant lists and alignment of raw sequencing reads

After removing adaptor sequences, raw reads (FastQ files) were aligned against human references (NCBI build 37, human genome 19) and only against the chromosomal regions covered by our Custom Panel using Burrows Wheeler Alignment. Remaining unmapped reads were then realigned using BLAT [29, 30] to capture longer insertions or deletions. After combining all alignments into a single BAM file, genomic variants were called as described before [28, 31]. BAM files were viewed in the Integrative Genomics Viewer (IGV; http://www.broadinstitute.org/igv/) [32, 33].

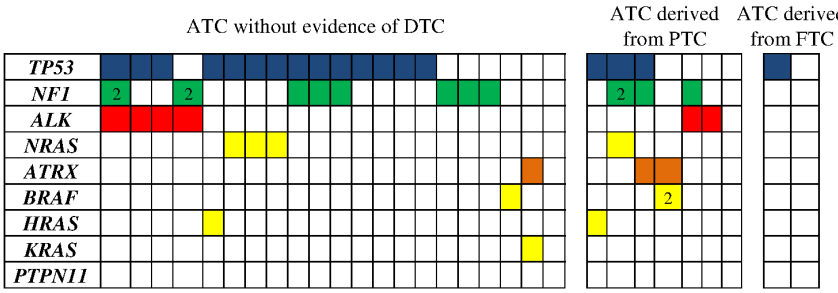

Fig. 1 Distribution and prevalence of mutations found in 30 ATC FFPE samples

Variants were compared to dbSNP (http://www.ncbi.nlm.nih.gov/SNP/), Exome Aggregation Consortium (ExAC) (exac.broadinstitute.org/), 1000 genomes (http://browser.1000genomes.org/index.html) and COSMIC (http://cancer.sanger.ac.uk). The NGS panel was established with more than 180 lung adenocarcinoma samples that were first analyzed by NGS and then validated by Sanger sequencing. Accordance was 100 % [28]. Variants with an allelic frequency ≤5 % and coverage ≤75 were excluded from analysis.

Results

Sequencing yielded high quality readouts for 30 of 39 tumor samples. In nine of 39 ATC samples the amplicon distribution was too heterogeneous to analyze and data quality was low, probably due to the fact that DNA was isolated from FFPE material and may have been degraded. Median age at ATC diagnosis was 68 years for males (55 %) and 72 years for females (45 %). Almost one-third of the ATCs showed residual tissues of differentiated thyroid carcinomas (9/30, 30 %), with seven samples showing PTC and two samples showing follicular thyroid cancer (FTC) tissue parts.

Twenty-seven of 30 tumors harbored genetic alterations in at least one of the genes studied (Fig. 1 and Table 1): *TP53* (18/30), *NF1* (11/30), *NRAS* (4/30), *HRAS* (2/30), *KRAS* (1/30), *BRAF* (2/30), *ATRX* (3/30), *ALK*(6/30), *ATRX* (3/30), *PTPN11* (0/30). Overall, 45 distinct genetic variations were found in these 30 ATC samples. All genetic variants were compared to the databases of COSMIC, dbSNP, ExAC and 1000 genomes and were not described as polymorphisms. Forty-four per cent (20/45) of the identified variants are listed in COSMIC as somatic mutations in other cancers (Table 1). However, since genomic DNA samples other than those from the ATCs were not available, it cannot be clarified whether the identified mutations are somatic or germline mutations. Eight mutations had an allelic fraction of more than 80 %, suggesting loss of heterozygosity (Table 1). In three ATC samples, no mutations were detected with our gene panel. Table 1 summarizes all mutations identified in our ATC cohort sorted by genes and Table 2 provides an overview of the mutations identified in each ATC sample with further information on sex and age of the patient as well as coverage and allelic fraction for the single mutant nucleotides. The average coverage across the cohort, according to the nucleotide in which the mutation occurred was 6027. In 16/30 (53 %) of ATC samples, mutations were found in more than one gene: two mutations in 12/30 samples, three mutations in 3/30 and four mutations in 2/30 samples (Fig. 1 and Table 2).

The most frequent mutations in this ATC series were found in *TP53* (60 %), with a high variability of affected codons, consisting of amino acid changes as well as insertions and deletions leading to frameshifts. The three *NRAS* and *HRAS* mutations, all affecting amino acid 61, were found only in association with a *TP53* mutation. A *KRAS* mutation was found in one ATC together with an *ATRX* mutation (Fig. 1, Table 2).

BRAF mutations were detected in only two ATC samples and one of these samples contained compound-heterozygous *BRAF* mutations (ATC 26, G469[a] and G596A). Of course, the nonsense mutation G469[a] is unlikely to account for constitutive BRAF activation. None of the ATC samples harbored a $BRAF^{V600E}$ mutation (Table 2). Six *ALK* mutations were found, five associations with *TP53* and/or *NF1* mutations (Table 2).

Interestingly, we found a high prevalence of *NF1* variants (11/30, 37 %). The majority of *NF1* variants (8/11) occurs together with mutations in other genes: in two samples with an *ALK* variant, in six samples together with a *TP53* mutation and in three of these with an additional *ALK*, *NRAS* or *ATRX* variant (Fig. 1). Three ATC samples harbored two *NF1* mutations within the same tumor: S644F and L1562F, Q2324H and S575P, and A1872V and T990P. None of the *NF1* mutations was located in the GAP-related domain.

Seven of 30 ATC samples that were derived from PTC (TP53 (3/7), NF1 (2/7), ALK (2/7), ATRX (2/7), BRAF (2/7), NRAS (1/7), HRAS (1/7, overall 13 mutations in seven samples) were harboring higher mutation load compared to 2/30 ATC derived from FTC (TP53 (1/2)) or ATC (TP53

Table 1 Summary of identified mutations sorted by genes, coverage and allelic fraction in ATC

TP53	*Coverage*	*Allelic fraction (%)*
p.W91fs	594	50
p.C135F	2833	57
p.R158L	948	50
p.Y163C	226	83
p.H179L	5846	12
p.H179Y (2x)	9229/9155	29/64
p.S183fs	4416	15
p.R209fs	1013	47
p.F212fs	4470	42
p.R213[a]	13911	61
p.G245D	6161	94
p.R248L	18803	26
p.V272L	2853	85
p.V272E	2160	9
p.R273C	4705	63
p.E285V (2x)	3048/6794	10/42
NF1	*Coverage*	*Allelic fraction (%)*
p.L499R	860	77
p.L552R	1250	13
p.S575P	1052	29
p.S644F	277	67
p.T990P	1363	32
p.F1103fs	182	54
p.R1176I	4536	20
p.L1562F	292	57
p.G1620S	432	27
p.A1872V	891	43
p.T2284fs	4536	20
p.Q2324H	407	36
p.Q2531[a]	982	54
c.3975_splice	279	54
ALK	*Coverage*	*Allelic fraction (%)*
p.L63H	2540	59
p.W290[a]	328	97
p.L890F	170	80
p.Q1364[a]	187	13
p.L1424F (2x)	287/164	97/12
NRAS	*Coverage*	*Allelic fraction (%)*
p.Q61R (3x)	1113/1188/413	48/27/16
p.Q61K	266	6
ATRX	*Coverage*	*Allelic fraction (%)*
p.Q182[a]	8268	26

Table 1 continued

ATRX	*Coverage*	*Allelic fraction (%)*
p.Q929E	92	49
p.G1038R	981	60
BRAF	*Coverage*	*Allelic fraction (%)*
p.G469V	2502	41
p.G469[a]	2407	13
p.G596A	1462	56
HRAS	*Coverage*	*Allelic fraction (%)*
p.Q61R (2x)	5679/1199	60/87
KRAS	*Coverage*	*Allelic fraction (%)*
p.D47N	129528	9
PTPN11	*Coverage*	*Allelic fraction (%)*
p.H114Y	628	70

Italic mutation is listed in cosmic (as of June 2015), *bold* loss of heterozygosity)

fs frame shift

[a] Change to a stop codon

(14/21), NF (8/21), ALK, (4/21) NRAS (3/21), KRAS (1/21), BRAF (1/21), ATRX (1/21), overall 33 mutations in 21 samples) without further evidence of differentiated thyroid cancer (DTC).

Only five identical mutations were identified in more than one ATC sample, TP53 (H179Y and E285V), ALK (L1424F), NRAS (Q61K) and HRAS (Q61R), illustrating the genetic heterogeneity of ATC.

Discussion

We report results from NGS of 39 ATC samples, using an established panel of nine candidate genes [28]. Thirty of 39 ATC samples yielded high quality readouts and mutations were identified in all analyzed genes (*TP53,NF1*, *ALK*, *NRAS*, *ATRX*, *BRAF,HRAS*, *KRAS and PTPN11*).

As expected, *TP53* mutations represented the most frequent mutational event in this ATC series with a prevalence of 60 %, which is slightly lower than the prevalence of 70–80 % *TP53* positive ATC samples reported previously [4, 8, 34, 35], but higher than the reported frequency of 27 % in a recent whole exome analysis of ATC by Kunstman et al.

A BRAF alteration was found in two of 30 ATC samples (6 %) in the present cohort. This is within the reported

Table 2 Overview of the mutations found in the ATC tissue samples sorted by each single case

Age at diagnosis/ sex	Gene	Changed AA	Gene coverage	Allelic faction (%)	Average coverage	Median coverage	Diagnosis
84/F	*ALK*	p.W290[a]	328	97	2525	2074	ATC
	NF1	p.S644F	277	67	6530	5898	
	NF1	p.L1562F	292	57	3099	2841	
	TP53	R158L	948	50	2468	2224	
46/M	*ALK*	p.Q1364[a]	187	13	216	191	ATC
	TP53	p.S183fs	4416	15	4632	4528	
?/?	*ALK*	p.1424F	287	97	81	65	ATC
	TP53	p.R213[a]	13911	61	14391	11228	
58/F	*ALK*	p.L890F	170	80	1429	1140	ATC
	NF1	p.Q2324H	407	36	1302	1065	
	NF1	p.S575P	1052	29	2358	2037	
79/F	*ALK*	p.L63H	2540	59	12500	8921	ATC with PTC
	NF1	p.Q2531[a]	982	54	7143	4889	
64/M	*ALK*	p.L1424F	164	12	81	65	ATC with PTC
66/M	*HRAS*	p.Q61R	5679	60	1450	1247	ATC with PTC
	TP53	p.H179L	5846	12	7626	6929	
54/M	*HRAS*	p.Q61R	164	12	1450	1247	ATC
	TP53	p.V272L	2853	85	7626	6929	
63/M	*NRAS*	p.Q61K	266	6	1546	1342	ATC
	TP53	p.E285V	3048	10	7724	7010	
78/F	*NRAS*	p.Q61R	1118	27	1456	1234	ATC
	TP53	p.G245D	6161	94	10906	10955	
75/M	*NRAS*	p.Q61R	413	16	1456	1234	ATC
	TP53	p.R209fs	1013	47	13644	10255	
73/F	*NF1*	p.A1872V	891	43	14964	13063	ATC with PTC
	NF1	p.T990P	1363	32	17358	17163	
	NRAS	p.Q61R	413	16	1456	1234	
	TP53	p.V272E	2160	9	7626	6929	
76/M	*ATRX*	p.Q1038R	981	60	1851	1571	ATC with PTC
	NF1	p.R1176I	4536	20	8162	7568	
	TP53	p.C135F	2833	57	2841	2284	
62/M	*NF1*	p.F1103fs	182	54	95	84	ATC
	TP53	p.F212fs	4470	42	14647	11468	
78/M	*NF1*	p.L552R	1250	13	1751	1252	ATC
	TP53	p.H179Y	9229	29	4491	4488	
82/F	*NF1*	p.G1620S	432	27	7461	7422	ATC
	TP53	p.R248L	18803	26	10240	9737	
81/F	*TP53*	p.E285V	6794	42	7724	7010	ATC
45/F	*TP53*	p.Y163C	226	83	2700	2557	ATC
54/F	*TP53*	p.H179Y	9155	64	4491	4488	ATC
75/M	*TP53*	p.W91fs	594	50	721	537	ATC

Table 2 continued

Age at diagnosis/ sex	Gene	Changed AA	Gene coverage	Allelic faction (%)	Average coverage	Median coverage	Diagnosis
56/F	*TP53*	p.R273C	4705	63	8655	7128	ATC with FTC
68/F	*NF1*	p.T2284fs	4536	20	1406	1050	ATC
62/M	*NF1*	p.L499R	860	77	2382	2134	ATC
60/F	*NF1*	c.3975_splice	279	54	1692	1522	ATC
80/M	*BRAF*	p.G469V	2502	41	2423	2221	ATC
62/F	*ATRX*	p.Q182[a]	8268	26	19871	21178	ATC with FTC
	BRAF	p.G596A	1462	56	1429	1266	
	BRAF	p.G469[a]	2407	13	2963	2620	
84/M	*ATRX*	p.Q929E	92	49	1362	876	ATC
	KRAS	p.D47N	129528	9	20800	15191	
71/F	No mutation	–	–	–	–	–	ATC
75/F	No mutation	–	–	–	–	–	ATC with PTC
66/M	No mutation	–	–	–	–	–	ATC with FTC

(*M* male, *F* female, *fs* frame shift, [a] amino acid change to a stop codon

range, as the prevalence of BRAF mutations in ATC has been found to range from 0 to 50 % in different series [14, 36–41]. The Cancer Genome Atlas (TCGA) screening of more than 400 PTC showed that the majority of PTC samples (~60 %) harbored a $BRAF^{V600E}$ mutation [42]. In our cohort only one ATC that appeared to derive from a PTC (1/7) harbored a *BRAF* mutation. Interestingly, the mutation burden in ATC derived from PTC (13 mutation in seven samples) was higher than in ATC without evidence of DTC (33 mutations in 21 samples), suggesting a higher accumulation of mutations in ATC that arose from PTC.

In the present series, one-third of ATC samples harbored mutations in the tumor suppressor gene *NF1*. NF1 is highly expressed in thyrocytes [23], suggesting that defects in NF1 may also contribute to development of thyroid cancer. Indeed, patients with neurofibromatosis 1 were reported to have a significantly higher risk, about fivefold, to develop thyroid cancer compared to a control cohort [43]. *NF1* mutations in ATC were recently also reported by Kunstman et al., Landa et al. and Sykorova et al. (2/22, 3/33 and 2/5, respectively) [19, 44]. In contrast to these reports and the present series, *NF1* mutations were rarely found in the TCGA study PTC samples (0.5 %). The fact that ATC is not a typical malignancy in neurofibromatosis and the moderately increased risk of thyroid cancer suggest that NF1 mutations are not the sole driver mutations for aggressive forms of thyroid cancer. However, the higher prevalence of *NF1* mutations in ATCs, between 10 and 37 %, compared to PTCs may indicate that additional loss of this tumor suppressor facilitates progression of thyroid carcinogenesis. This could be reflected in the ATC samples that apparently were derived from PTCs: 3/7 (43 %) of these samples harbored an *NF1* mutation, more than found in regular PTC and possibly contributing to progression from PTC to ATC.

Pathogenic mutations in ALK have been described in neuroblastoma and some ATC [24, 45] and are especially interesting because ALK inhibitors have been approved for other human malignancies. The prevalence of *ALK* mutations in an initial study by Murugan and Xing was 11 % (two out of 18 ATC samples), which is similar to our results. We found six ALK variants in 30 ATC samples, but these include two nonsense mutations that are unlikely to lead to constitutive activation. The other four ALK variants include a missense mutation, L1424F, which is located close to the tyrosine kinase domain and was found in two samples. Mutations of the same position, although to a different amino acid, have been described in lung adenocarcinoma (http://cancergenome.nih.gov). *ALK* mutants and fusion proteins have been shown to activate signaling pathways, especially the PI3K/AKT and the MAPK pathways, in different tumors [4, 46–49]. These pathways play a fundamental role in thyroid carcinogenesis. It is conceivable that an activating ALK mutation could be a driver mutation in a small subset of ATCs, which would offer the possibility of targeted treatment for these patients. An in vivo proof is not available yet.

We also identified *ATRX* mutations in 10 %ATC samples. This corresponds to data from a previous study with an

ATRX mutation prevalence of 6 % and could represent a novel mechanism contributing to tumor cell immortalization [27]. PTPN11 was sequenced, but no mutations were found. PTPN11 therefore appears to play no role in the development of ATC.

In summary, NG panel sequencing data from 30 ATC samples demonstrate the accumulation of genetic alterations in ATC, because in 90 % of samples mutations were already found in the investigated nine genes alone. Mutations were identified in established candidate's genes in ATC, such as *TP53* and *RAS*, but also in genes that may be less often affected, but harbor the potential for targeted treatment in a subset of ATC patients, such as *ALK* or *NF1*. Their oncogenic potential needs to be clarified in future in in vitro and in vivo studies.

Funding This work was supported by the Deutsche Forschungsgemeinschaft DFG (FU356 3-3 to D.F.).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Suppl. Table 1. 371 amplicons were used to sequence exons of 9 different genes.

Gene	amplicons
TP53	exons 1-12
NF1	exons 1-55
ALK	exons 1-29
ATRX	exons 1-35
BRAF	exons 11+15
NRAS	exons 2-3
HRAS	exons 2-3
KRAS	exons 2-3
PTPN11	exons 2-15

Publication 2

A 6-Base Pair in Frame Germline Deletion in Exon 7 Of *RET* Leads to Increased RET Phosphorylation, ERK Activation, and MEN2A

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J Clin Endocrinol Metab. 2016 Mar;101(3):1016-22. doi: 10.1210/jc.2015-2948.

Contribution

Herein I performed and designed all cell culture experiments on my own and analyze and interpretation the results. Furthermore, I performed mutagenesis and cloning of RET variants. Bioinformatics resulted by the Whole Exome Sequencing from our patient were analyzed by myself. I contributed to writing of abstract, introduction, methods, results and discussion of the publication.

ORIGINAL ARTICLE

A 6-Base Pair in Frame Germline Deletion in Exon 7 Of *RET* Leads to Increased RET Phosphorylation, ERK Activation, and MEN2A

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Context: Multiple endocrine neoplasia type 2 (MEN2) is usually caused by missense mutations in the proto-oncogene, *RET*.

Objective: This study aimed to determine the mutation underlying MEN2A in a female patient diagnosed with bilateral pheochromocytoma at age 31 years and with medullary thyroid carcinoma (MTC) 6 years later.

Methods: Leukocyte DNA was used for exome and Sanger sequencing. Wild-type (WT) RET and mutants were expressed in HEK293 cells. Activation of MAPK/ERK and PI3K/AKT was analyzed by Western blotting and luciferase assay. The effect of RET mutants on cell proliferation was tested in a colony forming assay.

Results: Exome sequencing revealed a 6-nucleotide/2-amino acid in-frame deletion in exon 7 of *RET* (c.1512_1517delGGAGGG, p.505_506del). In vitro expression showed that phosphorylation of the crucial tyrosine 905 was much stronger in the p.505_506del RET mutant compared with WT RET, indicating ligand-independent autophosphorylation. Furthermore, the p.505_506del RET mutant induced a strong activation of the MAPK/ERK pathway and the PI3K/AKT pathway. Consequently, the p.505_506del RET mutant cells increased HEK293 colony formation 4-fold compared with WT RET.

Conclusion: The finding of bilateral pheochromocytoma and MTC in our patient was highly suspicious of a *RET* mutation. Exome sequencing revealed a 6-base-pair deletion in exon 7 of *RET*, an exon not yet associated with MEN2. Increased ligand-independent phosphorylation of the p.505_506del RET mutant, increased activation of downstream pathways, and stimulation of cell proliferation demonstrated the pathogenic nature of the mutation. We therefore recommend screening the whole sequence of *RET* in MTC and pheochromocytoma patients with red flags for a genetic cause. (***J Clin Endocrinol Metab*** **101: 1016–1022, 2016)**

Multiple endocrine neoplasia type 2 (MEN2) is an autosomal-dominant genetic syndrome with two variants, MEN2A and MEN2B, depending on the underlying activating *RET* (rearranged during transfection) proto-oncogene mutation. The most common features of MEN2 are medullary thyroid cancer (MTC), which occurs in 70–100% of MEN2 patients, and pheochromocytoma with a frequency of 40–50% (1, 2).

RET is encoded by 21 exons, but *RET* germline mutations are mostly localized in eight exons (exons 5, 8, 10,

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in USA

Received July 22, 2015. Accepted January 11, 2016.
First Published Online January 14, 2016

Abbreviations: MEN2, multiple endocrine neoplasia type 2; JNK, Jun NH2-terminal protein kinase; MTC, medullary thyroid carcinoma; PVDF, polyvinylidene fluoride; SRE, serum response element; WT, wild type.

doi: 10.1210/jc.2015-2948

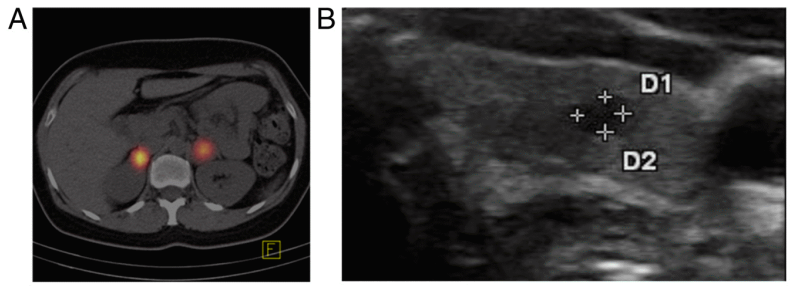

Figure 1. A, I-131 meta-iodobezylguanidine positron emission tomography–computed tomography revealed bilateral adrenal masses with increased tracer accumulation suggesting bilateral pheochromocytoma. B, Thyroid ultrasound showed a 4-mm hypoechogenic nodule in the left thyroid lobe. D, diameter.

11, and 13–16). Gain-of-function mutations of *RET* lead to autophosphorylation within the tyrosine kinase domain (2). RET activates different signaling pathways including the RAS/RAF pathway, which results in activation of ERK1/2 (3, 4), and the PI3K/AKT pathway (3, 5, 6). Furthermore, PLC-γ (7), p38MAPK (8), ERK5 (9), and Jun NH2-terminal protein kinase (JNK) (10, 11) are activated by RET. Constitutive activation of RET and subsequently these pathways increases cell proliferation and survival and ultimately results in cell transformation. Screening for *RET* germline mutations in first-degree relatives of MEN2 patients is recommended to identify affected individuals and allows for prophylactic thyroidectomy prior to onset of MTC (12, 13). Here we report a novel six-nucleotide in-frame deletion in exon 7 of *RET*, identified in a patient with clinical diagnosis of MEN2A, in whom routine genetic testing for a *RET* germline mutation had been negative.

Patients and Methods

Patient

A 31-year-old Caucasian woman presented with severe headache, hypertension (170/100 mm Hg), tachycardia (100 bpm), and deteriorating general condition. Clinically, pheochromocytoma was suspected. The diagnosis was biochemically confirmed by high plasma and urinary metanephrines (plasma: normetanephrine, 432 ng/mL [normal < 200]; metanephrine, 927 ng/mL [normal < 90]; urine: normetanephrine, 2523 μg/d [normal < 600]; metanephrine, 4110 μg/d [normal < 350]). Magnetic resonance imaging revealed bilateral adrenal masses with increased tracer accumulation on I-131 meta-iodobezylguanidine positron emission tomography–computed tomography (Figure 1A). Histopathology after bilateral adrenalectomy confirmed the diagnosis of bilateral pheochromocytoma (Supplemental Figure 1A). In search of a germline mutation as an explanation for the bilateral occurrence of pheochromocytoma, we tested *VHL* (exons 1–3), *SDHB* (exons 1–8), *SDHD* (exons 1–4), or *RET* (exons 10, 11, and 13) with negative results. Still, given the bilateral disease, we suspected a genetic cause and the patient was regularly seen in our outpatient clinic with special attention paid to potential manifestations of syndromic disease. Six years later, at age 37 years, thyroid ultrasound showed a thyroid nodule with a diameter of 5 mm (Figure 1B). Basal calcitonin, normal in all visits before, was now mildly elevated with 7.9 pg/mL (normal < 5.0). After stimulation with pentagastrin, calcitonin increased to 492 pg/mL within 2 minutes and MTC was strongly suspected. The patient underwent thyroidectomy and central lymphadenectomy. Histopathology confirmed a 4-mm MTC with stromal desmoplasia (Supplemental Figure 1B), which was positive for calcitonin and chromogranin A in immunohistochemical staining. Lymph nodes were free of tumor cells and the final TNM classification was pT1a, N0, L0, V0, R0. The patient had plasma metanephrines within normal range (< 88 pg/mL) and nondetectable serum calcitonin (<2.0 pg/mL), and is considered to be in complete remission. PTH and serum calcium were always within the normal range. The patient has no mucosal neuromas of the tongue or lips, no skeletal deformations or joint laxity, and no Marfanoid habitus. Therefore, the clinical diagnosis in this patient is MEN2A. After histological diagnosis of MTC, sequencing of *RET* was repeated and included exons 8–11 and 13–16, again with negative results.

Sequencing

Informed consent was obtained from all individuals before sequencing. DNA was extracted from leukocytes (QIAamp DNA Blood Maxi Kit, QIAGEN) and quantified with NanoDrop 2000c Spectrophotometer (Thermo Scientific). Genomic DNA (500 ng) was fragmented by Covaris shearing following the manufacturer's instructions. Genomic libraries were prepared using NEB Next Ultra kit and appropriate barcodes following the manufacturer's instructions (New England Biolabs) with eight postligation PCR cycles. Exome enrichment was performed on a pool consisting of three adapter-ligated DNA samples (333 ng each) using the Roche Nimblegen EZSeqCap EZ Human Exome Library v2.0 protocol and 18 postcapture PCR amplification cycles. Sequencing of enriched DNA fragments was performed on an Illumina HiSeq2500 platform using the paired-end sequencing protocol with a read length of 101 base pairs (bp) each. The p.505_506del mutation in exon 7 of *RET* was confirmed on gDNA by PCR using 5′taggggtggtcacctca 3′ as forward primer and 5′cagcatgttcctaaagggca 3′ as reverse primer. The PCR product was isolated (QIAquick Gel Extraction Kit, QIAGEN) and sequenced (Seqlab).

Mutagenesis of RET9 plasmids

An expression vector containing the wild-type (WT) short isoform of the human protoRET gene (pBabe-RET9 vector, kindly provided by Massimo Santoro, Pisa, Italy) was used for transient and stable transfections. The p.505_506del RET mutation found in the patient with MEN2A and a C634R mutation were introduced by site-directed mutagenesis (Quick Change Site Directed Mutagenesis KIT, Agilent Technologies) with the following primers: p.505_506del forward 5′-cagctgcttgtaacagtgtcatatgtggccgagg-3′, reverse 5′-cctcggccacatatgacactgttacaagcagctg-3′ and C634R forward 5′-gacgagctgcgccgcacgg-3′, reverse 5′-ccgtgcggcgcagctcgtc-3. After transformation into

XL1-blue competent cells, DNA was isolated from cultured single clones and sequencing confirmed successful mutagenesis (Seqlab).

Cell culture, transfection, and Western blotting

Twenty-four hours before transfection, 0.5×10^6 HEK293 cells (obtained from ATCC) were seeded in a six-well plate and cultured in DMEM (Gibco) supplemented with 10% FCS (Gibco) and 1% ZellShield (Minerva Biolabs). HEK293 cells were transiently transfected with RET9 WT, C634, and p.505_506del plasmids using Lipofectamine 2000 (Life Technologies). Fourty-eight hours after transfection, cells were lysed and PhosSTOP and protease inhibitor (Roche) were added. Protein (10 μg) were separated on a 6 or 10% SDS-polyacrylamide gel, transferred onto polyvinylidene fluoride (PVDF) filters, and blocked with 5% milk powder. Proteins of interest were detected with the following primary antibodies: RET (Cell Signaling, No. 3223 Lot:6), phospho-RET (Tyr905) (Cell Signaling, No. 3221 Lot:3), pAKT (S473) (Cell Signaling, No. 4060 Lot:16), or pERK1/2 (T202,Y204) (Cell Signaling, No. 4370 Lot:12). Stable transfection of HEK293 cells was achieved with Lipofectamine 2000 and subsequent culture in medium supplemented with 500 ng/mL puromycin (Gibco). Nonreducing conditions were achieved by omitting β-mercaptoethanol from Laemmli's buffer.

Dual-luciferase reporter assay

MAPK/ERK pathway activation was measured with a luciferase reporter gene assay (Promega). HEK293 cells (4×10^4) were seeded in a 24-well plate as triplicates and cultured to approximately 75% confluence. Cells were then transiently cotransfected with the RET9 WT, C634, or p.505_506del plasmids (17 ng/well), a reporter construct encoding the firefly luciferase gene (pGL4.33[luc2P/SRE/Hygro] Vector, Promega, 280 ng/well,) as well as renilla luciferase expression vector under the control of the serum response factor (also known as serum response element [SRE]) promoter (pRL Renilla Luciferase Reporter Vector, Promega, 33 ng/well). SRE regulates the transcription of luciferase reporter gene after activation of MAPK/ERK pathway. For transfection, the FuGENE (Promega) reagent was used in a 1:3 (DNA:FuGENE) ratio. Cells were collected 48 hours after transfection and the activities of firefly and renilla luciferases were determined with the Dual-Glo Luciferase Assay System (Promega) in a Sirius luminometer (Berthold Detection Systems). Firefly luciferase luminescence values were divided by renilla luciferase luminescence values from the same transfection sample to control for differences in transfection efficiency.

Colony formation assay

HEK293 cells (1×10^5) were seeded in a six-well plate in triplicate and the RET9 WT, C634, and p.505_506del plasmids were transfected with polyethylenimine (Polysciences Inc.) in a 1:4 (DNA:polyethylenimine) ratio. Medium was changed to 5% FCS in DMEM and 1 μg/mL puromycin 24 hours after transfection and then every 2–3 days. After 14 days, colonies were fixed and stained (0.5% crystal violet [Roth], 1% formaldehyde, and 1% methanol in PBS). This experiment was repeated three times.

Statistical analysis

Statistical analysis was performed using one-way ANOVA (GraphPad Prism 6, GraphPad). Differences were considered significant if *, $P < .05$; **, $P < .01$; ***, $P < .001$; ****, $P < .0001$.

Results

A novel RET mutation was identified in this patient with MEN2A

Exome sequencing from leukocyte DNA revealed a heterozygous in-frame deletion of six nucleotides in exon 7 of *RET* (c.1512_1517delGGAGGG), leading to loss of two amino acids, glutamic acid, and glycine (p. 505_506del) (Figure 2A). Sanger sequencing confirmed the mutation (Figure 2B).

The RET p.505_506 deletion leads to increased MAPK pathway activation

To study the consequences of the p.505_506 deletion in *RET*, we constructed expression vectors for the WT RET9, the most frequent activating *RET* mutation C634R (14, 15), located in the cysteine-rich region and previously found in patients with MEN2A, and the novel p505_506 deletion. After expression in HEK293 cells, the p.505_506del RET9 mutant led to significantly higher MAPK/ERK activation than WT RET9 (Figure 3). MAPK/ERK activation by the positive control C643R was significantly higher than both RET9 WT and RET9 p.505_506del.

The RET9 p.505_506 deletion leads to increased RET, AKT, and ERK phosphorylation

Western blot analysis confirmed that HEK293 cells do not express endogenous RET (Figure 4A). After transfection of the WT, C634R, and p.505_506del mutant plasmids, we detected double bands representing the glycosylated and partially glycosylated RET proteins: the partially glycosylated protein is predominant in WT RET9, the p.505_506del mutant, and RET9 C634R mutant used as a positive control (Figure 4A). Without addition of a ligand, phosphorylation of the crucial amino acid tyrosine 905 (16–18) occurred in RET9 C634R and p.505_506del but not in WT RET9, which suggests ligand-independent autophosphorylation (Figure 4B). Next, the effect on the MAPK/ERK and PI3K/AKT signaling pathways was determined. We found a stronger phosphorylation of ERK1/2 (Figure 4C) and also AKT (Figure 4E) after transfection of RET9 p.505_506del and RET9 C634R compared with WT RET9. Therefore, the p.505_506del mutant activates the MAPK/ERK and PI3/AKT pathways but to a lesser degree than the C634R mutant. Nonreducing conditions revealed dimerization for RET9 C634R but not for the p.505_506 deletion mutant (data not shown).

doi: 10.1210/jc.2015-2948

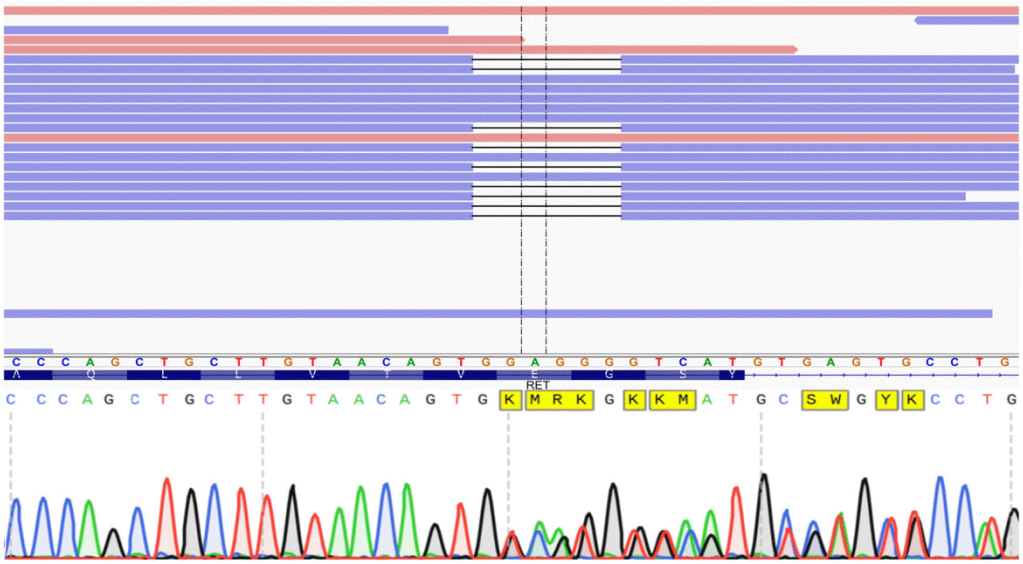

Figure 2. A, Visualization of *RET* sequencing exon 7 (amino acids on blue background) and right flanking intron with the Integrative Genomics Viewer. Reads that match the reference sequence are displayed in blue and red. Deletions are indicated with a black dash (—). Exome sequencing revealed a heterozygous in-frame deletion of 6-bp (c.1512_1517delGGAGGG)/2 amino acids (glutamic acid and glycine). B, Sanger sequencing confirmed the mutation.

The RET9 p.505_506 deletion leads to increased cell proliferation

The influence of RET9 p.505_506del on cell growth was analyzed in a colony-formation assay. HEK293 cells were transfected with the RET plasmids and cultured for 14 days in selection medium. HEK293 cells formed significantly more colonies after transfection with RET9 p.505_506del and RET9 C634R compared with RET9 WT (Figure 5).

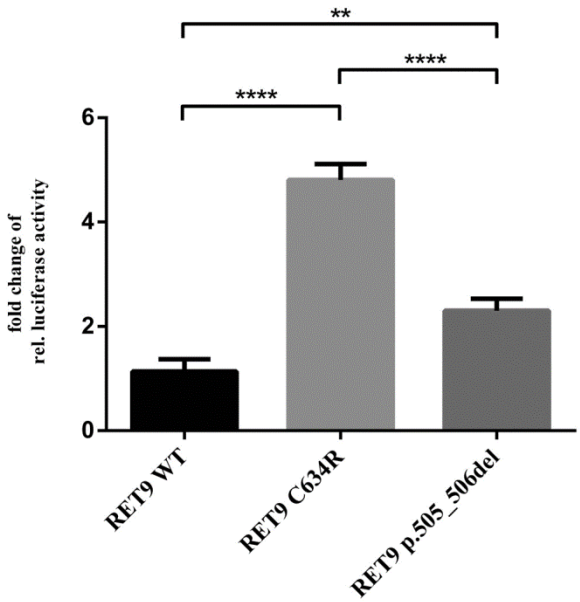

Figure 3. For RET9 p.505_506del, MAPK pathway activation is significantly stronger compared with RET9 WT. HEK293 cells were transfected with the indicated plasmid together with luciferase reporters induced by MAPK activation. Relative luciferase activity is expressed as fold increase of RET9 WT normalized for renilla luciferase. (One-way ANOVA, **, $P < .01$; ****, $P < .0001$; mean ± SEM; n = 3).

Family screening reveals that RET p.505_506del is a de novo mutation

After establishing the pathogenic nature of the 505_506del *RET* mutation, we contacted the family and

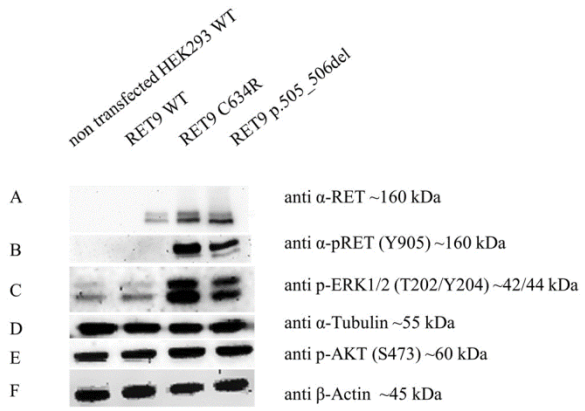

Figure 4. (Auto)phosphorylation and activation of downstream pathways by RET9 p.505_506del after transient transfection of HEK293. 10-μg protein lysate from HEK293-transfected cells with RET9 WT (negative control), C634R (positive control), p505_506del, or nontransfected HEK293 cells were separated on SDS-PAGE and transferred on a PVDF membrane (n = 4). PVDF membranes were stained with antibodies anti-RET (A), anti-pRET (B), anti pERK1/2 (C), anti-α-Tubulin (control for A–C) (D), anti pAKT (E), or anti β-Actin (control for C and D) (F).

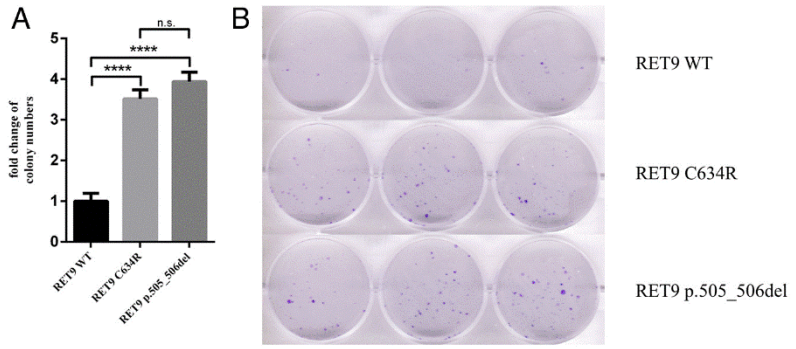

Figure 5. RET9 p.505_506del forms significantly more colonies than RET9 WT. A, HEK293 cells were stably transfected with plasmid encoding for the respective plasmid as indicated. Cells were cultured in a humidified incubator at 37°C and 5% carbon dioxide for 14 days under 5% FCS in DMEM supplemented with 1 μg/mL puromycin. (mean ± SEM; n.s., not significant; ****, $P <$ .0001; one-way ANOVA; n = 3). B, Example of one colony formation assay.

tested the proposita's parents (mother, age 66 y; father, age 71 y) and brother (age 43 y). No one of these relatives had a history of MTC, pheochromocytoma, or primary hyperparathyroidism, and no one harbored the proposita's *RET* mutation, which is therefore a de novo mutation (Figure 6).

Discussion

Our patient first developed bilateral pheochromocytoma, which already suggested an underlying genetic defect, but routine sequencing, including part of *RET*, *VHL*, *SDHB*, and *SDHD*, failed to detect a mutation commonly associated with pheochromocytoma. Considering the possibility of syndromic pheochromocytoma, the patient was followed closely. Six years later, biochemical and ultrasound screening raised the suspicion of a MTC, which was histologically confirmed, establishing the clinical diagnosis of MEN2A. An extended routine genetic screening found no mutations in the eight most commonly affected *RET* exons (8–11, 13–16) associated with MEN2, constituting an "orphan" status of our patient with clinical evidence of MEN2A, but no mutation. Exome sequencing finally detected a deletion of six nucleotides in exon 7 of *RET*. The fact that this *RET* variant has not been described as a polymorphism and was not present in a database of more than 135 healthy probands against which the exome sequencing data were compared, strongly suggests that this deletion is indeed a mutation. This mutation is listed neither in COSMIC nor in the MEN2 database of the department of pathology in Utah (http://arup.utah.edu/database/MEN2/MEN2_display.php) and is considered novel. Mutations in other genes besides *RET* can predispose for pheochromocytoma, but exome sequencing revealed no mutation in any of these genes in the proposita (*NF1*, *VHL*, *SDHD*, *SDHC*, *SDHB*, *EGLN1/PHD2*, *KIF1*, *SDH5/SDHAF2*, *IDH1*, *TMEM127*, *SDHA*, *MAX*, and *HIF2*).

The novel p.505_506del RET germline mutation is located in the extracellular part of RET, at the end of the cadherin-like domain and outside the cysteine-rich region. Pathogenic mutations in *RET* are usually found in the extracellular cysteine-rich domain (exons 10 and 11) or the intracellular tyrosine-kinase domain (16). So far, only one pathogenic mutation is known upstream of the cysteine-rich region. This mutation is located in codon 292 in

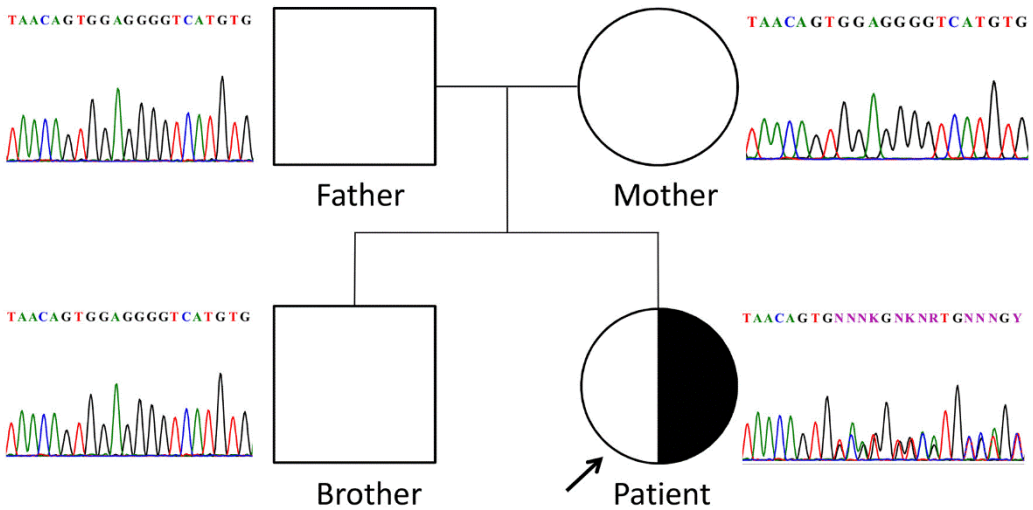

Figure 6. Sanger sequencing of the parents and the brother of the proposita revealed that the mutation occurred de novo.

doi: 10.1210/jc.2015-2948

exon 5 (cadherin like domain 3) and is associated with a mild form of MEN2 (19). For MTC, a genotype-phenotype correlation is well known (20–22). The p.505_506del mutation is a gain-of-function mutation but leads to a weaker activation of MAPK/ERK and PI3/AKT pathway than the most frequent activating C634R mutation, which we used as a positive control for functional characterization of our novel deletion mutation. This may explain the unusual phenotype of our patient with development of bilateral pheochromocytoma 6 years before manifestation of an MTC. The course of disease in our patient and the in vitro characteristics of the 505_506del RET mutant seem to be comparable to the V292M RET mutation in exon 5 (19).

The molecular mechanism underlying the activating effects of the novel 505_506del *RET* mutant are not fully clear. Usually, mutations in the cysteine-rich region affecting a cysteine lead to an aberrant interchain disulphide bonding, which then mimics ligand-induced RET homodimerization (23). We could not observe homodimerization and assume that the p.505_506del mutation affects the conformation of the tyrosine kinase domain, facilitating ligand-independent downstream signaling.

Although *RET* has been described as the susceptibility gene for MEN2 25 years ago, neither this 505_506del mutation nor any other activating mutations in exon 7 of *RET* have been reported. This may be explained by the fact that a tiered approach, starting with analysis of the most commonly affected *RET* exons, already solved the vast majority of cases. If this first step failed to detect a *RET* mutation, further search for novel mutations outside those hotspot exons was not mandatory and certainly not clinical routine, possibly leaving rare exon 7 mutations undiscovered (24). In addition, the phenotype of our patient differs from the common MEN2A patient with development of pheochromocytoma many years before an MTC would have become apparent. Limited sequencing of only hotspot exons, especially in pheochromocytoma patients with no other manifestations suggestive of MEN2, may explain why no other mutations in exon 7 of RET have been reported until now. It is conceivable that among patients with pheochromocytoma and no known mutation some actually harbor an uncommon *RET* mutation and are predisposed for development of MTC, but have not yet been diagnosed as *RET* mutation carriers and MEN2A patients. This may especially be true for patients with de novo mutations, such as our patient, because the negative family history provides no immediate clues for a germline mutation.

The specific 505_506del *RET* mutation reported here is certainly not frequent enough to justify whole-exome sequencing and could have been detected by Sanger sequencing. But this novel mutation in exon 7 of *RET* provides an example that demonstrates the importance of sequencing the entire coding region of *RET* in patients with MEN2 when no mutation is identified in the commonly affected exons (25).

Acknowledgments

We thank Dr Massimo Santoro for providing the RET9 wil-type plasmid and Sebastian Hoenes for excellent support during the experiments.

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This work was supported by Deutsche Forschungsgemeinschaft DFG Grants FU356/8-1 (to D.F.), MO1018/2-1 (to L.C.M), and the IFORES program, Faculty of Medicine, Duisburg-Essen.

Disclosure Summary: The authors have nothing to disclose.

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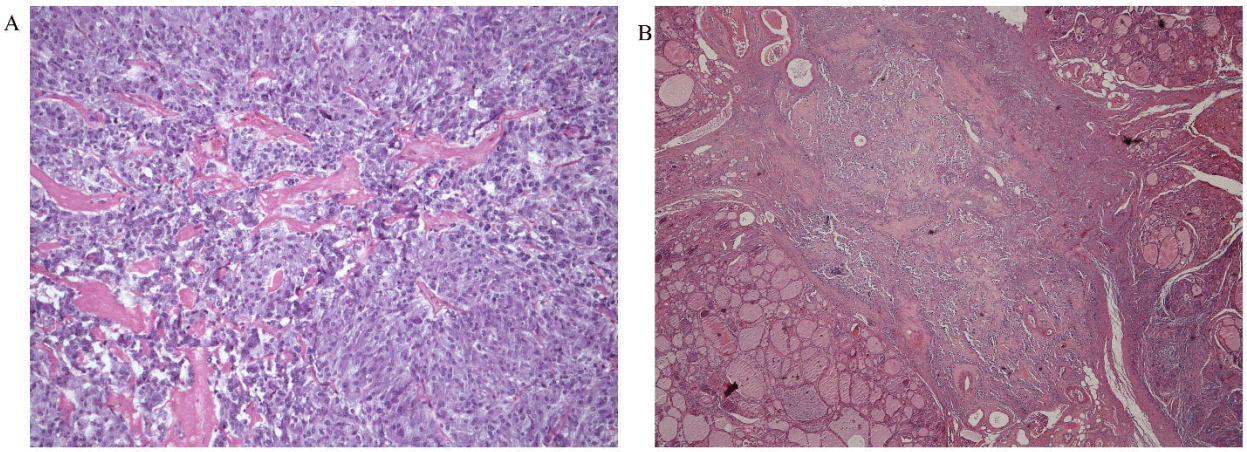
Figure 1S



Supplemental Figure 1. Pathology slides of the proposita's tumors. A) Adrenal pheochromocytoma with typical groups of cells with granular cytoplasm; B) Small medullary thyroid carcinoma with stromal desmoplasia and invasive growth.

Publication 3 (Submitted)

Thyroxine Promotes Tumor Growth And Angiogenesis In An Orthotopic Lung Cancer Mouse Model

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Submitted to *Molecular Cancer*

Contribution

Herein I wrote the animal test application and designed all the experiments. I performed all animal experiments including handling, operations and injections. FACS analysis, in *vivo* imaging and immunohistochemistry was done and analyzed by myself. I wrote abstract, introduction, methods, results and discussion of the publication.

Thyroxine promotes tumor growth in an orthotopic lung cancer mouse model

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Abbreviated Title: T4 promotes lung cancer growth in vivo

Key Terms: integrin $\alpha_v\beta_3$, thyroid hormones, Lewis lung carcinoma, Tetrac, T_4, tumor growth

Word count: 2300

Number of figures and tables: *4 Figures*

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Abstract

Introduction: Thyroid hormones are important for physiology and homeostasis. Besides the nuclear thyroid hormone receptors, a plasma membrane protein, integrin $\alpha_v\beta_3$, has been shown to be a receptor for both T_3 and T_4. Our hypothesis was that thyroid hormone signaling via $\alpha_v\beta_3$ promotes tumor growth.

Material and Methods: To test this hypothesis, murine lung carcinoma cells (3LL), stably transfected with luciferase, were injected into mouse lungs. Mice then remained untreated or were rendered hypothyroid and treated with T_3 or T_4 with or without the $\alpha_v\beta_3$ inhibitor 3,5,3′,5′-tetraiodothyroacetic acid (Tetrac). Tumor progression was determined by serial *in vivo* imaging of bioluminescence emitted from the injected luciferase expressing 3LL cells. Tumor weight was recorded at the end of the experiment. Neoangiogenesis was determined by immunohistochemistry for CD31 and flow cytometry analysis was employed to characterize tumor microenvironment

Results: Tumor growth was reduced in hypothyroid mice and increased in T_4 treated mice. Strikingly, only T_4, but not T_3, treatment promoted tumor growth. This T_4 effect was completely abrogated by co-treatment with the $\alpha_v\beta_3$ inhibitor Tetrac. Tumor weight was significantly higher in the T_4 treatment group and immunohistochemistry showed significantly increased neoangiogenesis only in tumors of T_4 treated mice. Again, the T_4 effect on tumor weight and neoangiogenesis was abolished by Tetrac. No effect was found on stroma cells.

Conclusion: We demonstrate that thyroxine promote tumor growth in an orthotopic lung cancer mouse model. This tumor promoting effect includes increased neoangiogenesis and is preferentially mediated via the plasma membrane integrin $\alpha_v\beta_3$. We suggest that such effects of levothyroxine may need to be considered in cancer patients on T_4 substitution.

Introduction

The thyroid hormones (TH) thyroxine (T_4) and triiodothyronine (T_3) are mostly recognized for their roles in normal growth, development, and metabolism, especially during fetal development and early childhood. TH action is typically mediated by the intracellular thyroid hormone receptors (TRs) α and β. TH are transported into target cells and either activate the canonical pathway where they bind to the TR, which act as a transcription factors, promoting expression of target genes (type 1 TH signaling) [1]. Another potential mechanism is rapid activation of the PI3K-pathway via TRs (THR-dependent signaling of TH without DNA binding, type 3) [2]. In addition to these intracellular TH actions, TH can bind to the plasma membrane protein integrin $\alpha_v\beta_3$, which mediates the signal across the membrane (TR-independent TH signaling, type 4) [1, 3]. $\alpha_v\beta_3$ is expressed in invasive tumors and inflammatory sites, especially in activated macrophages, monocytes, neutrophils, natural killer cells, naive T lymphocytes and endothelial cells [4-7]. This integrin $\alpha_v\beta_3$ has two binding sites, S1 and S2 which activate different downstream pathways [8, 9]: S1 binds exclusively T_3 and activates the PI3K/AKT-pathway whereas S2 binds T_3 and with a higher affinity T_4 and activates PI3K/AKT-pathway and MAPK-pathway [10]. TH action at $\alpha_v\beta_3$ is inhibited by the deaminated T_4 derivative Tetrac (3,3,5′,5′-tetraiodothyroacetic acid) [3].

Besides its role in development and physiology, TH may also play a role in the course of diseases. Thyroid hormone can directly stimulate cancer cell lines *in vitro*, e.g. lung adenocarcinoma [11], breast carcinoma [12] and prostate cancer cells [13]. These TH effects are thought to be mediated by integrin $\alpha_v\beta_3$ and not the canonical TRs α and β, because the stimulatory effect can be inhibited by pretreatment with Tetrac in e.g. lung adenocarcinoma cells [10, 14, 15]. Furthermore, Tetrac suppressed the expression of EGFR, VEGF, multiple cyclins, catenins, and cytokines in human tumor cells [16].

As thyroxine is one the most widely prescribed drugs worldwide and cancer is the second

leading cause of death, a considerable number of cancer patients will be on thyroxine substitution. It is therefore important to study the effects of TH on cancer progress *in vivo*. Here we report the effects of TH in a syngeneic murine Non-Small Cell Lung Cancer (NSCLC) model using *in vivo* imaging and show that TH promotes tumor growth and neoangiogenesis.

Material & Methods

Cell culture

Lewis lung carcinoma cells (3LL) were obtained from ATCC and stably transfected with pCCL-MNDU3-LUC plasmid containing the firefly luciferase gene as previously described [17]. Cells were cultured in RPMI supplemented with 10% fetal bovine serum in 5% CO_2 at 37°C to 65-70% confluence. Cell numbers were adjusted to 500 000 and washed in RPMI w/o phenol and w/o fetal bovine serum twice. Cell viability was measured via trypan blue dye staining and cells were used if viability was >95%. The cell pellet was resuspended in 36 µl HBSS and 4 µl Matrigel "Matrix Growth Factor Reduced" (BD Biosciences, San Jose, USA) and immediately injected into the right lung of mice.

Animals and study design

B6(Cg)-*Tyr*$^{c\text{-}2J}$ (C57BL/6J) were obtained from The Jackson Laboratory (Bar Harbor, Maine, United States). All animal studies were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (LANUV, Germany) and were performed according to the German regulations for Laboratory Animal Science (GVSOLAS) and the European Health Law of the Federation of Laboratory Animal Science Associations (FELASA). Animals were housed under standard laboratory conditions (temperature (22 ±1°C) and 12 h light, 12 h dark light cycle. Food and water was provided *ad libitum.* At the age of seven weeks, male mice were randomized into treatment groups (n=6 per group). The euthyroid group received control diet

In vivo bioluminescence imaging

Mice were anesthetized with 1.5-2% isofluorane during imaging procedures. Imaging was performed with an IVIS Lumina II imaging system (Perkin Elmer, Massachusetts, USA) five minutes after intraperitoneal injection of 60 µg/g D-luciferin (Perkin Elmer, Massachusetts, USA) dissolved in phosphate buffered saline (PBS). Mice were imaged with sequential 30, 60, 90 and 120 seconds exposures. The total flux was calculated in regions of interest (ROI) on each mouse with Living Image 3.2 acquisition and analysis software (Caliper Life Sciences, Hopkinton, USA). Data are presented in photons/second (p/sec).

Flow cytometry

Lung tumor tissue was digested with collagenase type IV (150 U/ml, Worthington Biochemical, Lakewood, USA) and DNase (10 U/ml, Sigma Aldrich, Taufkirchen, Germany) for 1 h at 37°C and minced through cell strainers to dissociate cells. Single cell suspensions of $1^{*}10^{6}$ cells were incubated with anti-CD16/32 antibody (Biolegends, San Diego, USA) for 10 min, washed and then incubated for extracellular receptors (anti-CD3, anti-CD4, anti-CD8, anti-CD11, anti-CD51, anti-CD61, anti-NK1.1, anti-Ly-6G/Ly-6C, anti-F4/80. All antibodies were obtained from Biolegends, San Diego, USA. for 20 min followed by fixation and flow cytometry on a BD FACS Aria III ((BD Biosciences, San Jose, USA). Intranuclear staining was performed for Foxp3 (Biolegends) for 30 min. Data were analyzed with FlowJo V10 (FlowJo, Ashland, USA)

Immunohistochemistry

All tissue sections were deparaffinized and rehydrated through graded alcohol baths (70% - 96% - 100% ethanol, Sigma Aldrich, Taufkirchen, Germany). After pretreatment in citrate buffer (pH 9.0) at 95 °C for 20 min, tissue sections were blocked in an aqueous hydrogen peroxide solution (3% H_2O_2). The primary anti-CD31 antibody (D8V9E, Cell Signaling, Cambridge, UK) was

incubated for 30 min at room temperature. Immunoreactivity was detected with a classical polymer system (Zytomed, Berlin, Germany). Cell nuclei were stained with Haematoxylin (1:8; Roth, Karlsruhe, Germany) for 5 min (Dako Autostainer, Dako, Glostrup, Denmark) and analyzed on an Olympus BX51 Upright microscope with Cell Sens Dimension software.

Serum Measurements

Total T_4 (TT_4) and free T_3 (FT_3) concentrations in serum of mice were measured using commercial ELISA kits according to the manufacturer's instructions (DRG Instruments GmbH, Marburg, Germany; FT_3: EIA-2385; TT_4: EIA-1781) with a detection limit of 0.5 µg/dL for TT_4. TSH was measured as previously described [18].

Statistical Analysis

Statistical analysis was performed using one-way ANOVA (GraphPad Prism 6, GraphPad Software, La Jolla, USA).

Results

Induction of hypothyroidism

Mice were rendered hypothyroid and treated with either T_4, T_3, Tetrac or a combination of TH and Tetrac (Figure 1). Modulation of thyroid state was confirmed by body weight measurements and measurement of serum concentration of thyroid hormones in final serum. Hypothyroidism led to a body weight plateau in hypothyroid mice whereas euthyroid mice gained weight (data not shown) [19]. Induction of hypothyroidism resulted in severe changes of TH serum concentrations compared to euthyroid mice (TSH >8000 vs. 51 mU/l; FT_3 1.4 vs. 2.6 pg/ml and TT_4 0.34 vs. 2,7 µg/dl). Treatment of hypothyroid mice with T_3 or T_4 resulted in increased FT_3 and increased TT_4 concentration respectively.

T_4, but not T_3, treatment promotes tumor growth, which is abolished by Tetrac

Tumor progression was determined as bioluminescence emitted from the luciferase expressing 3LL cells *in vivo*. Induction of hypothyroidism in mice resulted in a reduction of the bioluminescence signal intensity and tumor weight compared to euthyroid mice (Figure 2a, b). Strikingly, tumor weight in mouse lungs increased significantly with T_4 but not T_3 treatment. Moreover, Tetrac abolished this T_4-mediated effect resulting in diminished bioluminescence signal intensity and smaller tumor weight of T_4 and Tetrac treated animals compared to T_4 treated mice. Overall survival, however, was not different between the groups possibly due to predefined termination criteria (supplemented Figure 1).

T_4 promotes tumor neoangiogenesis

As it was shown previously that T_4 influences neoangiogenesis in chick chorioallantoic membrane assays, we assessed the degree of neoangiogenesis in the tumors by CD31 staining (platelet endothelial cell adhesion molecule). Hereby, we observed a significantly increased vascularization in the tumors of T_4 treated mice which again was abrogated by co-treatment with of Tetrac (Figure 3).

Treatment with TH does not alter the composition of infiltrating immune cells

To investigate if the composition of infiltrating immune cells, which also express $\alpha_v\beta_3$, is altered in tumors we characterized the tumor infiltrate by flow cytometry. No significant differences were found between tumors from euthyroid mice or any of the treatment groups for the composition of infiltrating T-regs (CD4+, Foxp3+), cytotoxic T-cells (CD3+, CD8+), tumor associated macrophages (F4/80+, Gr-1+), macrophages (F4/80+), natural killer cells (NK 1.1+), T-helper cells (CD3+, CD4+) or dendritic cells (CD11c+) (Figure 4).

Discussion

TH can bind to the plasma membrane receptor integrin $\alpha_v\beta_3$, which mediates signaling across the membrane [3]. $\alpha_v\beta_3$ is expressed in invasive tumors and inflammatory sites, especially in activated macrophages, monocytes, neutrophils, natural killer cells and naive T lymphocytes [4-6]. In our study, we could show that Lewis lung cell carcinoma grew dependent on thyroid hormone concentration. Thus, we observed a significant increase in tumor growth exclusively in mice treated with T_4 but not T_3.

This observation is in agreement with previous studies in non-orthotopic approaches where 3LL, mammary adenocarcinoma (C3HBA) and fibrosarcoma (T241) cells were injected subcutaneous into mice [11, 20, 21]. Furthermore, we found that tumor promoting effect in T_4 treated mice was abrogated in the presence of Tetrac suggesting that this effect is mainly mediated by $\alpha_v\beta_3$ and not by the thyroid hormone receptors itself.

Since the target cells of T_4 within the tumor are still not exactly known we tested 2 hypotheses. First, we assumed that increased tumor growth might be linked to neovascularization since $\alpha_v\beta_3$ is also expressed on endothelial cells and interacts with proangiogenic agents like FGF-2 [22] and VEGFR2 [23]. Indeed, a significant increase (4-fold) of new vessels was found in T_4, but not T_3, treated mice and this effect was abrogated in the presence of Tetrac.

In a previous study, Carmon-Cortes *et al.* observed in their mouse model with 3LL an increased tumor weight under T_4 treatment which was however not accompanied by changes in the tumor tissue hemoglobin concentration indicating a proportional increase in neovascularization with tumor growth [24]. Neovascularization by e.g. CD31 staining was however not assessed in this model. Mousa *et al.* could show that xenografts of human NSCLC (H1299) in nude mice results in lower hemoglobin concentration when treated with Tetrac indicating that T_4 and Tetrac indeed modulate neovascularization. Our results in an orthotopic and immunocompetent mouse model support this finding.

Another target cell for T_4 within the tumors might be the infiltrating immune cells where subtypes of immune cells also express $\alpha_v\beta_3$. However, in our study, we did not detect any changes in immune cells, expressing $\alpha_v\beta_3$ such as macrophages, natural killer cells, and T lymphocytes. These results do not exclude involvement of other cell subsets in the tumors of our mice. In a recent study involvement of $\alpha_v\beta_3$ mesenchymal stem cell (MSC) was explored by Schmohl *et al.* In their study they $\alpha_v\beta_3$, MSC were injected into the tail vein of mice and a significantly increased recruitment and invasion of MSC into hepatocellular tumor was observed which was abolished in the presence of Tetrac indicating a $\alpha_v\beta_3$ mediated effect. However, no significant effect on tumor growth regarding TH treatment was observed in this model.

In summary, we showed for the first time T_4 dependent tumor progression by *in vivo* imaging under altered TH status in an orthotopic mouse model of non-small cell lung cancer. We suggest that this effect is mediated preferably via integrin $\alpha_v\beta_3$ receptor and involves neoangiogenesis rather than alterations in infiltrating immune cells.

Importantly, the observed effects in our NSCLC mouse model may also have implications in cancer patients under TH substitution. Hypothyroidism may develop under tyrosine kinase inhibitor (TKI) treatment since TKI influence TH uptake, metabolism and presumably also TH transport [26, 27]. Furthermore, hypothyroidism due to autoimmune thyroiditis is a frequent side effect of checkpoint inhibitor treatment in advanced malignancies including lung cancer [26]. In these scenarios, which thyroid hormone should be used for substitution in the future, T_4 or rather T_3? Or should TH treatment installed at all since it seems that hypothyroidism might slow tumor progression at least in some malignancies (e.g. pancreatic and prostate cancer) [26, 28].

Clearly, our study cannot answer these questions, but suggests that further *in vivo* and importantly clinical studies are warranted since thyroid hormone substitution in cancer patients may have more consequences than just normalizing TSH.

Disclosure Statement

The authors have nothing to disclose.

Funding

This work was supported by Deutsche Forschungsgemeinschaft DFG grants MO1018/2-1 to L.C. Moeller and FU356/8-1 to D. Führer and the IFORES program, Faculty of Medicine, University of Duisburg-Essen.

Authors' contributions

SL and LCM designed the studies; SL conducted experiments; SL, DZ, DF, LCM analyzed data; SL and LCM wrote the manuscript.

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Figure legends

Figure 1: Schematic representation of the study design. Induction of hypothyroidism was started 3 weeks before 3LL were inoculated into mice lungs. Treatment started 1 week after inoculation of 3LL and continued until mice were sacrificed.

Figure 2: T_4 treatment increases tumor growth. A) Example illustration of tumor burden of one mouse per treatment group over time measured by *in vivo* imaging. B) A significant increase of tumor weight was observed in the thyroxine treated group. Furthermore, in the presence of Tetrac which blocks the binding of T_4 on the integrin $\alpha_v\beta_3$, T_4 effect on tumor growth was abolished. **$p<0.01$ (T_4 treated mice vs euthyroid mice); a = $p<0.0001$ for T_4 treated mice vs hypothyroid, T_3, Tetrac and T_3 + Tetrac treated mice; b = $p>0.01$ for T_4 treated mice vs euthyroid mice. Mice were sacrificed when predefined criteria were reached.

Figure 3: Increased CD31+ blood vessel upon T_4 treatment. Immunohistochemistry staining of CD31+ from formalin fixed paraffin embedded tumor of A) euthyroid mice, B) T3 treated mice C) T4 treated mice and D) T4 + Tetrac treated mice. E) T4 treated mice showed a significant increase of CD31+ vessels in thyroxine treated mice compared to all other groups indicating neoangiogenesis. This effect was abolished in the presence of Tetrac. Bars represent 50 µM; a = $p<0.0001$ for T_4 treated mice vs all other groups

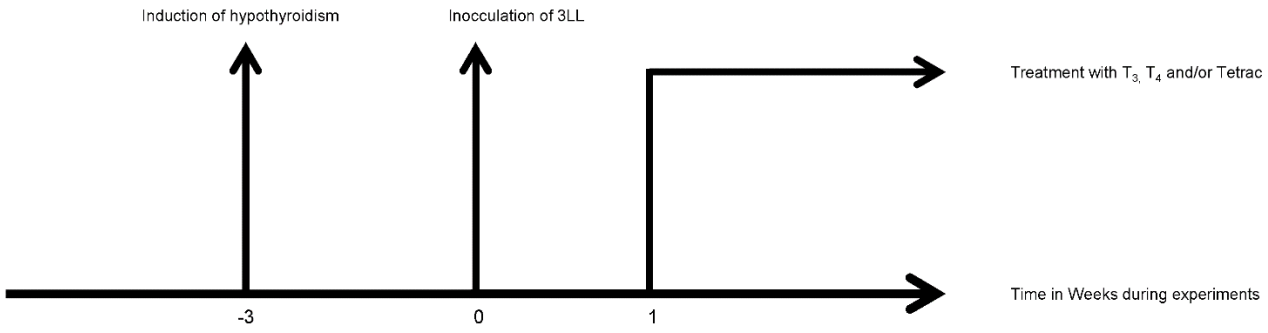
Figure 4: Flow cytometry analysis revealed no change in the subgroups of infiltrating immune cells throughout different treatments.

Supplemented Figures

S. Figure 1:

Effect of euthyroidism, hyperthyroidism and hypothyroidism on survival. Mice were sacrificed when predefined criteria were reached which might lead to a non-significant change in survival.

Figure 1: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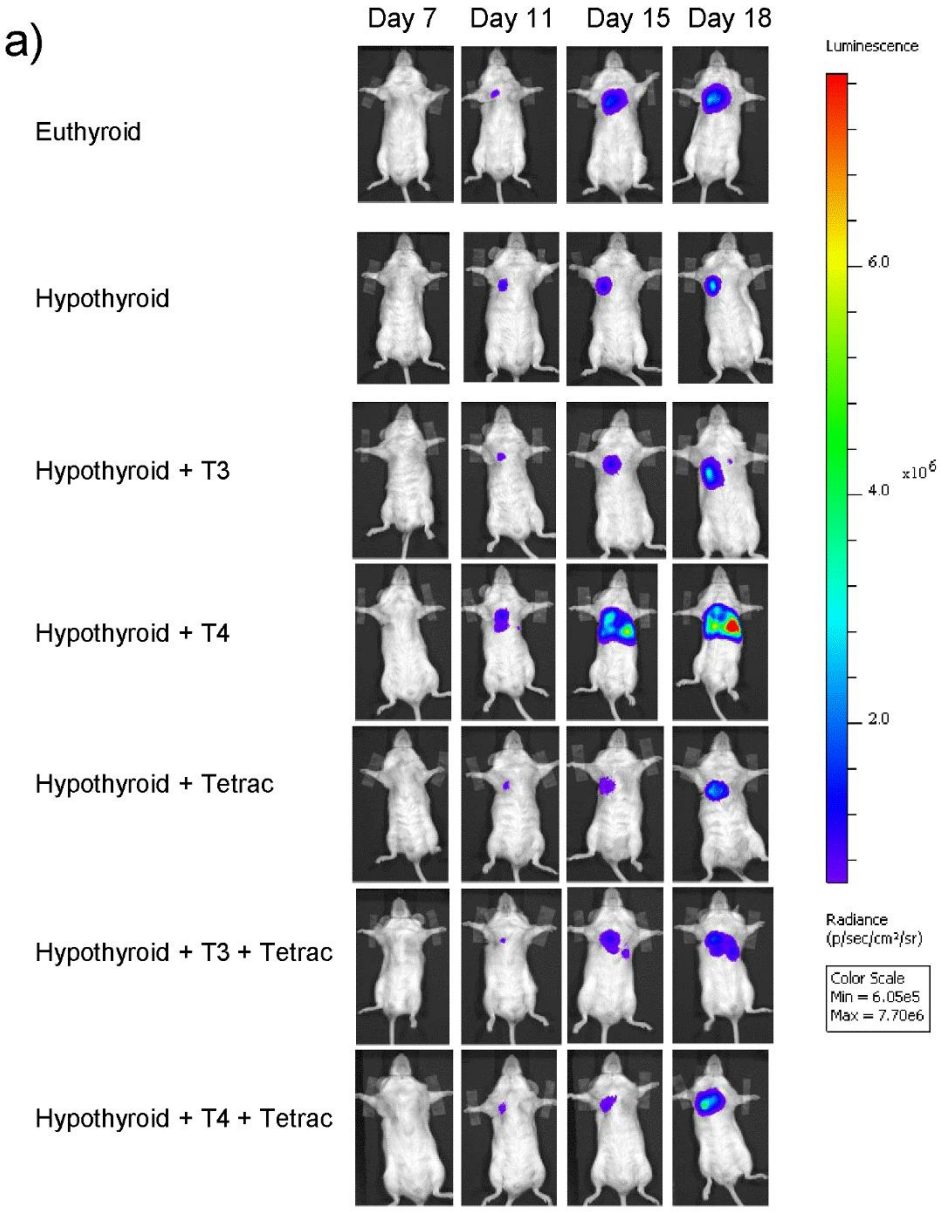
Inocculation of 3LL
Treatment with T_3, T_4 and/or Tetrac
Time in Weeks during experiments
-3
0
1

Figure 2:


a)
Day 7
Day 11
Day 15
Day 18
Euthyroid
Hypothyroid
Hypothyroid + T3
Hypothyroid + T4
Hypothyroid + Tetrac
Hypothyroid + T3 + Tetrac
Hypothyroid + T4 + Tetrac
Luminescence
6.0
4.0
2.0
x10^6
Radiance (p/sec/cm²/sr)
Color Scale
Min = 6.05e5
Max = 7.70e6

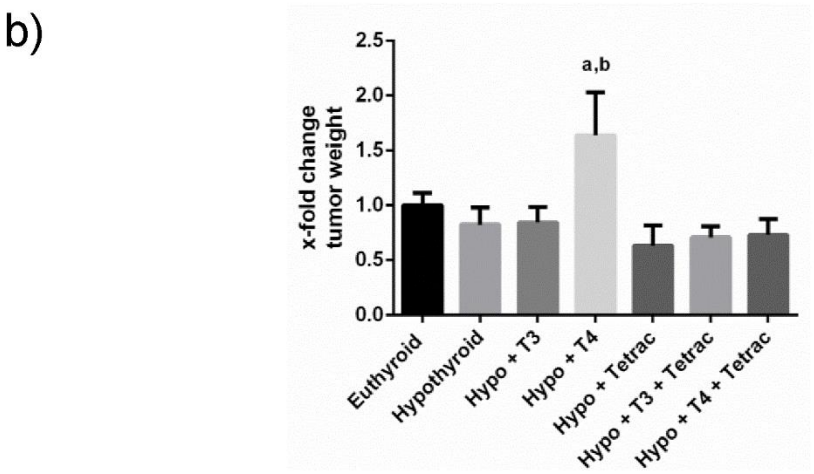

b)
x-fold change tumor weight
2.5
2.0
1.5
1.0
0.5
0.0
a,b
Euthyroid
Hypothyroid
Hypo + T3
Hypo + T4
Hypo + Tetrac
Hypo + T3 + Tetrac
Hypo + T4 + Tetrac

Figure 3:

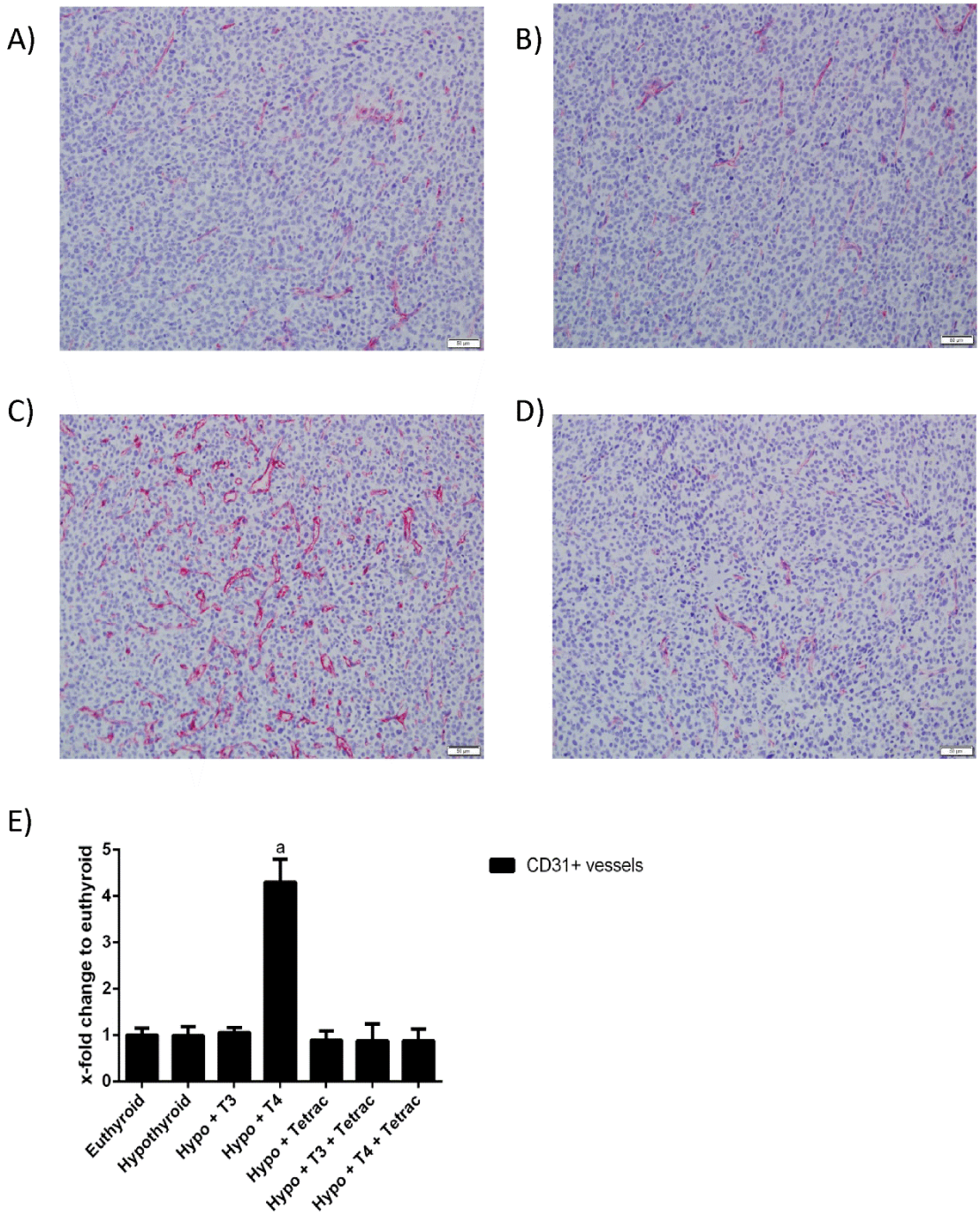
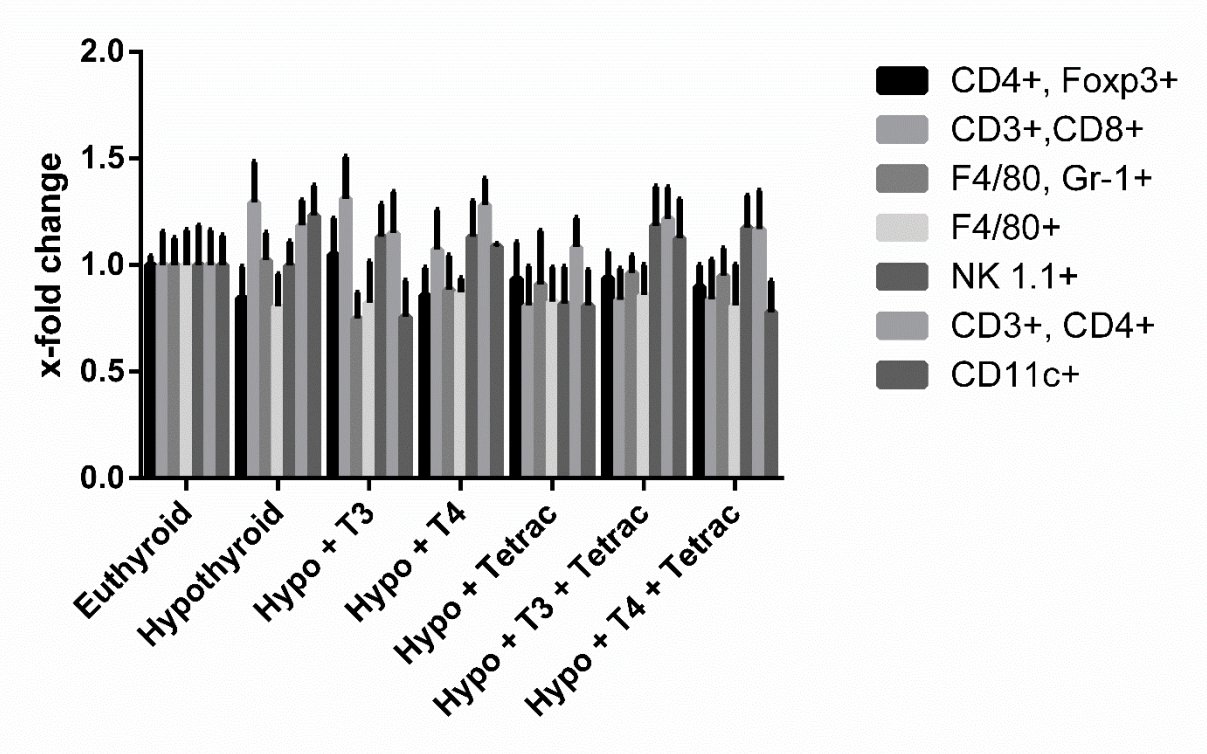
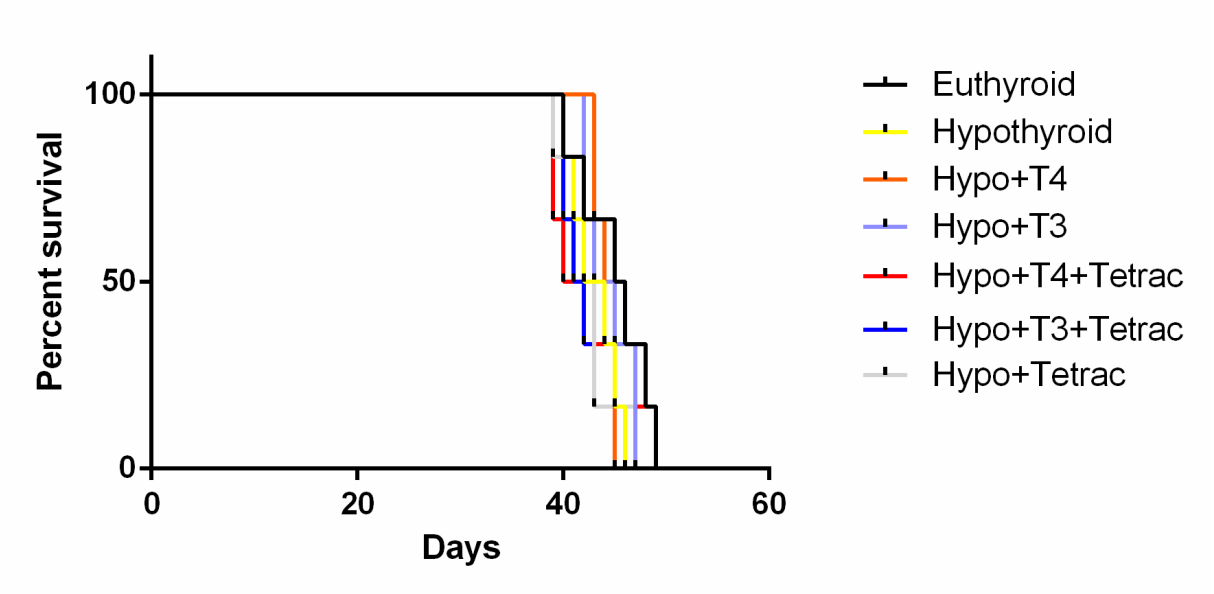

A)
B)
C)
D)
E)
x-fold change to euthyroid
0
1
2
3
4
5
a
CD31+ vessels
Euthyroid
Hypothyroid
Hypo + T3
Hypo + T4
Hypo + Tetrac
Hypo + T3 + Tetrac
Hypo + T4 + Tetrac

Figure 4:


2.0
1.5
1.0
0.5
0.0
x-fold change
Euthyroid
Hypothyroid
Hypo + T3
Hypo + T4
Hypo + Tetrac
Hypo + T3 + Tetrac
Hypo + T4 + Tetrac
CD4+, Foxp3+
CD3+,CD8+
F4/80, Gr-1+
F4/80+
NK 1.1+
CD3+, CD4+
CD11c+

Supplemented Figure 1:



5. Discussion

Thyroid cancer presents with a very different clinical course from curative approach to life threatening disease. They display various different morphologies and are quite heterogeneous regarding genetically landscape. Especially the treatment of ATC patients is challenging due to a complex interaction regarding molecular processes which are not fully understood. The physical and mental burdens of ATC patients are quite high. One possible approach might be targeted therapy. Therefore, characterization of the genetic landscape is necessary and urgently required to identify driver mutations that can be targeted.

In contrast, in patients with hereditary MTC the genomic landscape is well characterized; but, it is essential to identify those patients for adequate treatment and screening of families at an early stage. There are still 5% of all MEN2 patients where no germline mutation can be found. The identification of hereditary MTC at an early stage helps to provide suitable treatment (e.g. prophylactic thyroidectomy) of affected individuals. Not only thyroid cancer presents a problem for patients but also TH seems to influence tumor growth in the periphery.

In this thesis, the molecular landscape of ATC was characterized by targeted Next-Generation Sequencing and novel candidates were revealed. These results will be used to generate first preclinical mouse models that are expected to develop an ATC for which targeted therapy will be possible. Furthermore, in a hereditary MTC patient,h it was possible to identify the underlying mutation were several extended routine diagnosis failed. The case report has significant impact for the clinicians. In cases with red flags for genetic causes where routine diagnose fail to identify the underlying mutation, not only hotspots of genes should be sequenced, but the also theentire gene should also be sequenced.

Depending on the underlying mutation, cancer patients (e.g. lung cancer) are already treated with targeted therapy, especially with tyrosine kinase inhibitors. Tyrosine kinase inhibitors can lead to hypothyroidism in certain patients. Those patients are currently supplemented with T_4. It was possible to show in this thesis that T_4, not T_3, has a tumor promoting effect in an orthotopic immunocompetent male mouse model

by probably stimulating neoangiogenesis within the tumor. These results suggest that tumor patients may be better supplemented with T_3 than with T_4.

5.1. Molecular landscape of anaplastic thyroid carcinoma - Targeted next-generation sequencing for *TP53*, *RAS*, *BRAF*, *ALK* and *NF1* mutations in anaplastic thyroid cancer

Anaplastic thyroid carcinoma is still an orphan disease with a very poor prognosis. Within the last 50 years, the treatment options could not prolong overall survival.

First successful results were achieved with targeted therapy regarding the underlying mutation, e.g. with Vemurafenib [Zelboraf®] ($BRAF^{V600E}$, (Rosove et al., 2013)), Crizotinib [Xalkori®] (ALK-Rearrangment, (Godbert et al., 2015)) or Everolimus [Certican®] (TSC2 Q1178*, (Wagle et al., 2014)). But no large cohorts of ATC patients were sequenced to study if targeted therapy might be an option for ATC patients at this time. To answer this question, the largest cohort at this time of 39 ATC samples was investigated.

The aim was not only to identify mutations in genes where targeted therapy might be possible but also to reveal novel genes in the context of ATC. In the recent years, high throughput sequencing studies were performed on ATC samples as it was performed in this thesis. Moreover, a multiplex PCR was performed in the course of this thesis followed by parallel targeted next-generation sequencing on 39 ATC samples from the archives of the Pathology in Essen. 30% of all ATC samples co-exist with DTC. Hence, the majority of the ATC samples seem to develop *de novo* in this cohort.

From 9 samples, the DNA quality was insufficient resulting in heterogeneous readouts and were therefore excluded from further analysis of target genes: (*TP53* (60%), *NF1* (37%), *ALK* (20%), *NRAS* (14%), *ATRX* (10%), *BRAF* (7%), *HRAS* (7%), and *KRAS* (3%)). In 28 samples, at least one mutated gene was found with an overall of 45 distinct genetic variants.

Before high throughput sequencing methods were available, classic identification of mutations in cancer samples had usually been performed by Sanger sequencing to characterize the genetic landscape of ATC. In the early 1990`s, the first molecular

alterations in ATC were identified in the tumor suppressor gene *TP53.* At codon 273, an arginine was changed to a histidine with a high prevalence of 71%-83% (Donghi et al., 1993; Fagin et al., 1993). Step by step, further genes were identified in the context of ATC like phosphatase and tensin homolog (*PTEN*, 10-20%), *PI3KCA* (25-40%) (Garcia-Rostan et al., 2005; Karakas et al., 2006), *RAS* (20%-30) (Basolo et al., 2000; Costa et al., 2008; Fukahori et al., 2012; Garcia-Rostan et al., 2003; Hou et al., 2007; Liu et al., 2008; Ricarte-Filho et al., 2009; Volante et al., 2009), *BRAF,* (Begum et al., 2004; Fugazzola et al., 2004; Fukushima et al., 2003; Namba et al., 2003; Nikiforova et al., 2003; Nikiforova et al., 2013; Puxeddu et al., 2004) and *β-Catenin (42%)* (Garcia-Rostan et al., 1999).

As expected, *TP53* mutations were themost frequent mutational event in these ATC series with 60% (Donghi et al., 1993; Fagin et al., 1993; Xing, 2013). It seems that mutation in *TP53* play a central role in the tumorigenesis of ATC. *TP53* is a tumor suppressor gene whose main function is the maintenance of the genomic stability. Alteration of *TP53* in the context of ATC usually leads to a loss of function resulting in genomic instability. Targeted therapy, which restores the function of *TP53,* is not yet available.

5.1.1. Alterations in the MAPK-pathway and PI3K-pathway are characteristic features in ATC

Neurofibromin 1 (NF1) is a tumor suppressor gene which inactivates RAS. It was possible to identify mutations in *NF1* in 37% of ATC samples. Interestingly, NF1 activation mainly regulates the MAPK-pathway. Loss of function by NF1 results in constitutive activation of the MAPK-pathway. Inhibitors of the crucial protein´s dual specificity mitogen-activated protein kinase 1/2 showed promising results in combination with other drugs and might be a treatment options for ATC patients with inactivating NF1 mutation (Infante et al., 2017; LoRusso et al., 2017; Tai et al., 2017).

However, since NF1 was not specifically analyzed before, NF1 mutations were only identified in whole exome sequencing and massive parallel sequencing by Kunstman *et al.*, Landa *et al.* and Sykorova *et al.* (2/22, 3/33 and 2/5 respectively) in lower frequency (Kunstman et al., 2015; Landa et al., 2016; Sykorova et al., 2015). *NF1* is a driver mutation for neurofibromatosis. Patients suffering from neurofibromatosis

only have a slightly increased risk for thyroid cancer indicating that NF1 is definitely no driver mutation in the context of anaplastic thyroid carcinoma.

The Cancer Genome Atlas Research Network study of PTC samples showed that 0,5% of all samples harbored *NF1* mutation. In the present cohort, 43% of all ATC derived from PTC showed mutations in NF1 indicating that NF1 contributes to progression from a PTC to a ATC.

Mutations in the proto-oncogenes *RAS* (comprising H-Ras, N-Ras and K-Ras) can be found in 20-30% of all human cancer with the highest incidences in adenocarcinomas of the pancreas (90%) (Bos, 1989). RAS is a binary molecular switch cross talking between different pathways regulating cell proliferation, cell differentiation, apoptosis, and cell metabolism. Although RAS is a key player in activation of the PI3K-pathway and MAPK/ERK-pathway, the frequency of RAS mutations is surprisingly quite low in ATC (20-30%) and even lower in the present study (*NRAS* (14%), *HRAS* (7%), and *KRAS* (3%)). Interestingly, in less aggressive FTC (30-45%), PTC (12%) and PDTC (20-40%), mutation frequent seems to be higher and can also be found in benign follicular adenoma (Basolo et al., 2000; Challeton et al., 1995; Costa et al., 2008; Fukahori et al., 2012; Garcia-Rostan et al., 2003; Hou et al., 2007; Liu et al., 2008; Ricarte-Filho et al., 2009; Volante et al., 2009).

One of the first interaction partners of RAS within the MAPK/ERK-pathway is the serine/threonine-specific protein kinase RAF (BRAF) (Van Aelst et al., 1993). The codon 600 and its close proximity in *BRAF* is of great interest. In this codon, a single point mutation T1799A accounts for 80% of all *BRAF* mutations and results in an amino acid change from valine to glutamic acid (Davies et al., 2002). This single nucleotide exchange results in a substitution of an amino acid with hydrophobic side chain (Valine) to an amino acid with negatively charged side chain (Glutamic Acid). No phosphorylation from the extracellular matrix is necessary to activate the MAPK/ERK-pathway (Xing, 2005). In this cohort, no $BRAF^{V600E}$ was identified. However, $BRAF^{V600E}$ mutations in ATC are controversially discussed and must be viewed with caution since several studies report that $BRAF^{V600E}$ mutations were absent (Fugazzola et al., 2004; Fukushima et al., 2003; Puxeddu et al., 2004) or present (20-50%) (Begum et al., 2004; Namba et al., 2003; Nikiforova et al., 2003; Nikiforova et al., 2013). In The Cancer Genome Atlas Research Network study, 60% of all PTC harbor a $BRAF^{V600E}$ mutation. If ATC dedifferentiate from PTC the

percentage of $BRAF^{V600E}$ mutations should be higher if the $BRAF^{V600E}$ mutation is required for dedifferentiation. In this study, 7 ATC coexisted with a PTC and only 1 ATC showed a BRAF mutation supporting the idea that BRAF mutations are not required for dedifferentiation. ATC derived from DTC display a higher mutation load than without incidence for other DTC (1,85 vs 1,57 mutations per sample).

ALK mutations had been already described in cancers like neuroblastoma (Berry et al., 2012), non-small cell lung cancer (NSCLC) (Kwak et al., 2010) or anaplastic thyroid carcinoma (Murugan and Xing, 2011) and usually result in rearrangements with other proteins like echinoderm microtubule-associated protein-like 4 (EML4) (Pyo et al., 2016). In this cohort, it was possible to identify mutations in ALK in 20% of all ATC patients which is similar to the initially reported frequent (11%) (Murugan and Xing, 2011). Rearrangements were not analyzed. Six ALK variants were found including two nonsense mutations resulting in an early termination of translation. The other four ALK variants include mutations at amino acid 1424 which is in close proximity to the tyrosine kinase domain. These mutations might result in constitutive activation of downstream signaling. Interestingly, a mutation at this position was observed in two different patients although resulting a different amino acid. An *in vivo* proof is not yet available. In principle, treatment of ALK-positive ATC with ALK inhibitors appears possible. The first ALK inhibitors (e.g .Crizotinib [Xalkori®], Ceritinib, [Zykadia®]) have been approved by the Food and Drug Administration (FDA) for the metastatic ALK-positive NSCLC and are now regarded as standard therapy for this NSCLC subgroup (Khozin et al., 2015; Malik et al., 2014; Scagliotti et al., 2012; Soda et al., 2007). A case report of a targeted inhibition of the ALK/EML4 fusion protein in an ATC patient showed a reduction of metastases by up to 90% (Godbert, et al., 2015). The restored quality of life (ECOG 0) over more than 2 years after initial diagnosis suggests that ALK inhibitors might be effective for a subpopulation of ALK-positive ATC. The authors emphasize that the role of ALK mutations in the formation of ATC still needs to be investigated (Godbert et al., 2015). ALK screening should therefore be included in all molecular routine diagnostic of ATC patients.

5.1.2. Next-generation sequencing reveals hallmark mutations in ATC

Targeted next-generation sequencing is limited regarding pathway analysis. Whole exome sequencing like Kunstman *et al.*, Landa *et al.* or Sykorova *et al.* have performed can reveal cluster of mutations in certain pathways. They observed higher mutation load (23.7 mutations per samples (Kunstman et al., 2015)) and also found novel mutations like *EIF1AX* and *USH2A* in 3/22 ATC and 4/22 ATC samples. They proposed that, in ATC a cluster of mutations occured in the MAPK/ERK pathway (*BRAF, HRAS, KRAS, NRAS, RAF1, AKT2,* and *PIK3CA*) and the ErbB pathway (*MAPK10, ERBB2, ERBB3, RAF1, RAC1,* and *NF2*) rather than random single independent mutations.

Another hallmark study was published by Landa *et al*. They investigated 341 tumor specific genes in 84 PDTC and 33 ATC. The results were compared with the Cancer Genome Atlas Research Network study. In the recent years, one additional gene seems to play a crucial role in the context of ATC, the *telomerase reverse transcriptase* (*TERT*) which was found to be most frequently mutated in the study performed by Landa *et al.* (73%).

TERT is the catalytic subunit of the enzyme telomerase, which leads to the extension of the telomeres during the S phase of proliferation (Kirkpatrick and Mokbel, 2001; Weinrich et al., 1997) and might result in immortalization of tumor cells. It was first identified to be mutated in thyroid cancer in the year 2013 (Liu et al., 2013). Landa *et al.* showed that *TERT* promotor mutations increase during dedifferentiation (9% PTC, 40% PDTC, 73% ATC) and are significantly associated with *BRAF* and *RAS* mutations (Cancer Genome Atlas Research, 2014; Landa et al., 2016). Compared to PTC it seems that the frequencies of *BRAF*-like mutations decrease and *RAS*-like mutations increase. Rearrangements found in DTC (RET/PTC, PAx8-PPARG, and ALK fusions) (Kelly et al., 2014; Kroll et al., 2000; Pierotti et al., 1992) were still present in PDTCs but gone in ATCs.

A larger study of 144 ATC samples, 50 genes were recently sequenced and analyzed by Bonhomme *et al.*. Only 62.5% of the samples could be analyzed due to poor DNA quality. Again, *TP53* mutations were most frequent with 54.4% followed by *TERT* with 54%, 33% (*HRAS, NRAS, RAS*) *RAS* genes, 13.8% *BRAF* mutation, and 17% PI3K-

AKT pathway mutation which presents similar results to the previously described study (Bonhomme et al., 2017).

In a follow up study, we again sequenced a large cohort of 118 ATC samples with a broader next-generation sequencing panel this time including *TERT*. We found mutations in *TERT* (73%), *TP53* (55%), *RAS* (*HRAS, KRAS,* and *NRAS* (19.5%)), *CDKN2A* (16.9%), *PI3KCA* (11.8%), *BRAF* (11 %), and *RET* (7.6%). In *ALK*, *KIT* and *MET,* no mutations were found. In *EGFR*, *ERBB2*, *PDGFRA*, *RB1* and *TSC2* mutations were found in less than 2% of all ATC (Tiedje et al., 2017). This time, no *ALK* mutations were found. In this study, only the hotspots were sequenced. In the presented study included in this thesis, the whole sequence was analyzed and might explain the differences.

In the second cohort, the same amount of *TERT* mutations were found compared to Landa *et al.* (73%) and furthermore also $BRAF^{V600E}$ mutations (11%) were detected. The difference in *BRAF* mutations in our cohort (7% and 11%) compared to Kunstman *et al.* and Landa *et al.* (27% and 45%) might be due to the finding of coexisting of DTC in the ATC samples (30% and 29,6% in our cohorts compared to 40-45% by Kunstman *et al.* and Landa *et al.*). The majority of the ATC observed in our study may have arisen *de novo.*

However, the sequencing results in the present study, as well as the other studies, cannot answer the open question if an ATC develops *de novo* or as a consequence of dedifferentiation from a DTC. Taken together, the findings in the literature support both hypotheses.

In recent decades, and especially in recent years, the genetic landscape of ATC has become clearer, although it is very heterogeneous. The current literature suggests that three major events take place during dedifferentiation in thyrocytes. i) Constitutive activation of the MAPK-pathway and/or PI3K-pathway (e.g. *BRAF, RAS* or *PIK3CA*) is one of the first events in the genome since mutations are often found in PTC. ii) Immortalization of the tumor cells is required by mutations in the *TERT* promoter region as well as to a low-frequency in ATRX (Landa et al., 2016; Latteyer et al., 2016b). iii) Genomic instability through mutations in tumor suppressor genes such as *TP53*, *NF1* and *PTEN* results in heterogeneous genetically background.

5.2. Identification and characterization of a *RET* germline mutation which escaped the routine sequencing - A 6-base pair in frame germline deletion in exon 7 of *RET* leads to increased RET phosphorylation, ERK activation, and MEN2A

In contrast to ATC the genetic landscape in MTC is well characterized and is mainly limited to mutations in the proto-oncogene *RET.* However, in routine sequencing, the underlying mutation in MEN2 patients is usually identified. Here, it was possible to identify a 6-base pair in frame germline deletion of *RET* in a female Caucasian patient although even extended routine sequencing could not identify any mutation. This deletion was cloned and transfected into HEK293 cells in which it was possible to show independent (auto)-phosphorylation of RET in the absence of its ligand, significant and constitutive activation of MAPK/ERK-pathway by luciferase-assay as well as significant growth by colony formation assay.

The female Caucasian patient initially presented with severe headache, hypertension, tachycardia and deteriorating general condition at the age 31 years. Magnetic resonance imaging revealed bilateral pheochromocytoma which was biochemically confirmed. The finding of bilateral pheochromocytoma was highly suspicious for a germline mutation but recommended routine sequencing from the ATA guidelines of certain hotspots in the context of pheochromocytoma from *RET*, *VHL*, *SDHB* and *SDHD* failed to identify any mutation. Six years later, the patient developed a 4 mm small MTC which was histologically confirmed and the clinical diagnosis was MEN2A. Again, routine sequencing of extended exons recommended by the ATA and commonly associated with MEN2 by *RET* (8-11, 13-16) failed to detect a mutation.

Whole exome sequencing was performed and a 6 nucleotide in frame heterozygous deletion was identified resulting in a loss of two amino acids (Glutamic Acid and Glycine, p505_506del RET). To exclude the possibility that we identified a polymorphism, sequencing results were compared to 135 healthy probands suggesting indeed a pathogenic property. Furthermore, at this time, the deletion was not listed in the Catalogue of Somatic Mutations in Cancer (COSMIC) as well as the MEN2 database of the department of pathology in Utah (http://arup.utah.edu/database/MEN2/MEN2_display.php). Today, the deletion is

officially listed in the MEN2 database of the department of pathology in Utah and described as pathogenic.

All genes known to be involved in pheochromocytoma were examined to exclude an additional mutation (*NF1, VHL, SDHD, SDHC, SDHB, EGLN1/PHD2, KIF2, SDH5/SDHAF2, IDH1, TMEM127, SDHA, MAX,* and *HIF2*).

According to the MEN2 database of the department of pathology in Utah, the novel p.505_506del RET deletion is still the only known pathogenic mutation in exon 7 (August 2017). The altered protein part resulting from the deletion is located in the extracellular part of RET at the end of the cadherin-like domain and outside the cysteine-rich region. The most common mutation in *RET* especially in the extracellular compartment appear in the five cysteine codons 609, 611, 618, and 620 of exon 10 and codon 634 of exon 11 (Eng et al., 1996; Lallier et al., 1998).

5.2.1. Molecular consequences for mutations in *RET*

The cysteine residues in the cysteine-rich region are involved in the formation of intramolecular disulfide bonds with other cysteines (Airaksinen and Saarma, 2002; Anders et al., 2001; Santoro and Carlomagno, 2013). Mutations of one cysteine to any other amino acid lead to an unbound cysteine in the monomers of RET. The unbound disulfide bond of the cysteine of the monomer of RET binds to the unbound disulfide bond of another monomer of RET resulting in dimerization. Then dimerization leads to in constitutive activation.

However, RET9 p505_506del results in constitutive activation without dimerization in the absence of its ligand. Although the mutation is in the extracellular region, one would expect a primary alteration in the extracellular region which either mimics ligand binding or ligand independently induced dimerization. Since no dimerization is observed, the mutation seems to affect the tyrosine kinase domain somehow.

Upstream of the cysteine-rich region, only one pathogenic mutation is known (RET V292M) in exon 5 of RET and this seems to be comparable to the novel RET p505_506del mutation (Castellone et al., 2010).

Mutations in *RET* have been known since more than 25 years. RET p505_506del was not observed before maybe due to the fact that routine diagnosis usually sequences only the recommended hotspots by the ATA of *RET*. Although we used whole exome sequencing in this patient, the frequency is not high enough to justify the expensive search by whole exome sequencing. In addition, the RET p505_506del mutation causes a mild clinical phenotype which does not necessarily require an exact genetic analysis and thus perhaps remains undiscovered to this day.

However, it was possible to show that, in the remaining 5% of MEN2 patients without genetic diagnose, a *RET* mutation can be found when the whole gene is sequenced. The updated ATA guidelines now include the recommendation to sequence the whole sequence of *RET* if sequencing of hotspots fails to detect any mutation referring to the results of this thesis (Latteyer et al., 2016a; Wells et al., 2015)

The *in vitro* data confirm the pathogenic nature of the mutation, which then allowed screening of the family and prophylactic thyroidectomy of affected children might be possible. Especially for the descendants of MEN2 patients in whom no mutation was found, it is important to know the mutation to act at an early stage in the case of an inherited mutation.

5.2.2. *RET* mutations in other cancer entities

Mutations in *RET* are frequently observed in pheochromocytoma, sporadic and hereditary MTC. However, *RET* mutations in other cancer types are only reported in low numbers. Large cohorts of 4871 (Kato et al., 2017) and 6011 (Cerami et al., 2012; Gao et al., 2013) tumor patients with diverse cancer types were sequenced regarding *RET* mutations.

RET mutations were only found in 88/4.871 (1.8%) and 181/6.011 (3%) tumor samples. Most frequently, *RET* mutations were observed in ovarian epithelial carcinoma (4/54 – 7.3%), lung adenocarcinoma (23/412 – 5.6%) non-small cell lung carcinoma (4/125 – 3.2%), and melanoma (2/136 – 1.6%). *RET* alterations were mainly mutations (34/88 - 38.6%) followed by fusions (27/88 - 30.7%) and amplifications (22/88 - 25.0%). Even one loss of function was detectible.

The limitation of these studies is that there is no correlation between clinical outcome and underlying mutations since no data was available. In addition, the mutations that were found were not investigated for the existence of germline mutations.

Taken together, *RET* mutations are highly frequent and driver mutations in the context of MTC and pheochromocytoma but less frequent in other cancer types. Nevertheless, targeted therapy of RET by Vandetanib, Cabozantinib and Lenvatinib might be a treatment option of certain cancer subtypes. None of the inhibitors are currently approved by the FDA or EMA for targeting *RET* mutations in other cancer entities than MTC (Mulligan, 2014; Shaw et al., 2013).

5.3. Tumor promoting effects of TH - Thyroxine Promotes Tumor Growth And Angiogenesis In An Orthotopic Lung Cancer Mouse Model

TH seem to be involved somehow in tumorigenesis (Kinoshita et al., 1991; Kumar et al., 1979; Shoemaker et al., 1976), but the exact mechanisms are still not fully understood. To investigate the effects of T_3, T_4 and/or Tetrac on tumor growth, infiltrating immune and angiogenesis, an immunocompetent orthotopic cancer mouse model was used.

The effects of T_3, T_4 and Tetrac on tumor growth were analyzed *in vivo* and *ex vivo.* It was possible to detect a significant increase of tumor weight and bioluminescence signal intensity. Compared to control treated euthyroid mice, a non-significant decrease of the bioluminescence signal intensity and tumor weight in hypothyroid mice were measured over time.

Interestingly, the treatment of immunocompetent orthotopic hypothyroid mice with biologically active T_3 and T_4 resulted in an increase of the bioluminescence signal intensity and a significant increase of tumor weight only in the T_4, not T_3, treated group. This observation is in agreement to previous studies although the T_4 concentration used was much lower compared to Kumar *et al.* (50% lower) and Kinoshita *et al.* (80% lower). The previous studies were performed in non-immunocompetent mice (Kinoshita et al., 1991; Kumar et al., 1979; Shoemaker et al., 1976). Nevertheless, T_4 is still referred as a prohormone in textbooks and only T_3 is considered as the only biological active form (Fleisher, 2012; Kansagra M., 2010).

All mice groups, except the euthyroid group, were rendered hypothyroid throughout the whole entire experiments. Measurements of TSH, TT4 and FT4 confirmed the successful induction of hypothyroidism and therefore the effects of endogenous released TH by the thyroid gland can be neglected.

Beside the significantly increased tumor weight and increased bioluminescence signal, also a significant increase of $CD31^+$ vessels was observed in T_4 treated mice. The results obtained from T_4 treated mice regarding $CD31^+$ vessel indicate that the increased tumor growth might be favored by higher neovascularization. However, the exact molecular cascade after T_4 treatment is unclear. At this time, still a couple of questions need to be answered:

Do the tumor cells first proliferate faster and, as a consequence, does this results in higher vascularization? Or do the vessels proliferate faster and lead to a better supply of nutrients? Another question is if the observed effect is a primary or a secondary impact. The tumor cells might release signal proteins (e.g. VEGF, bFGF) that promote neovascularization upon T_4 treatment. The remaining questions need to be addressed in future experiments.

The observed effects are not restricted to 3LL cells that were used in this thesis. Several cell lines also showed a significant alteration in cell proliferation and angiogenesis upon TH-stimulation in a dose-dependent manner like human non-small cell lung cancer cells (Mousa et al., 2012), pituitary cancer cells (Barrera-Hernandez et al., 1999), breast cancer cells (Hall et al., 2008), and prostatic cancer cell lines (Tsui et al., 2008). In the human non-small cell lung cancer cells, the effects were also reversible in the presence of Tetrac (Mousa et al., 2012).

5.3.1. Tetrac counteracts T_4 promoted tumor growth

Tetrac, the deaminated T_4 analogue, was shown to bind at the binding site S1 and S2 within the integrin $\alpha_v\beta_3$. When the binding sites are blocked, stimulation by the TH is blocked and signaling by the integrin $\alpha_v\beta_3$ is inhibited (Bergh et al., 2005; Davis et al., 2006). The observed significant increase of tumor weight and $CD31^+$ vessels was gone in the presence of Tetrac although mice were treated with T_4. This observation suggests that the integrin $\alpha_v\beta_3$ might be one of the “driver” proteins. Mice were

treated 3 times per week with Tetrac and 5 times per week with T_4. Still Tetrac was able to inhibit T_4-promoted tumor growth. The reason might be the affinity towards $\alpha_v\beta_3$ which is higher for Tetrac than for T_4 (~100 fold). Tetrac is consequently able to displace T_4 from $\alpha_v\beta_3$ (Bergh et al., 2005).

It was also shown that the integrin $\alpha_v\beta_3$ interacts with pro-angiogenic agents like FGF-2 (Sahni and Francis, 2004) and VEGFR2 (Masson-Gadais et al., 2003) and that this effect is again gone in the presence of Tetrac (Mousa et al., 2008). In Tetrac-treated xenografted nude mice, not only significant reduced tumor volume was observed but also a diminished hemoglobin concentrations indicating lower tumor vascularity (Mousa et al., 2012).

Similar results were obtained regarding increased neovascularization within T_4 treated mice and significant lower CD31+ vessels when mice were treated with Tetrac and T_4 compared to T_4 alone. Furthermore, Mousa et al. showed that Tetrac blocks angiogenesis induced by VEGF in the chick chorioallantoic membrane (CAM) model (Mousa et al., 2008).

In contrast, Carmona-Cortés *et al.* inoculated 3LL subcutaneously into the dorsum of male mice and also observed a significant tumor weight increase upon T_4 treatment but no alteration of Hb concentration. These results indicate that no increased neovascularization took place in this model (Carmona-Cortes et al., 2014).

T_4 seems to promote tumor growth via the integrin $\alpha_v\beta_3$. However, the biological function of the TRs in the context of tumorigenesis was recently described as tumor suppressive (Aranda et al., 2009). The reason for this contradictory dual function of the THs remains unknown.

Overall, Tetrac seems not only to regulate angiogenesis and hence tumor growth but to be also involved in the expression of checkpoint proteins like PD-1 (programmed death-1)/PD-L1 (PD-ligand 1). The PD-1/PD-L1 interaction is a crucial event in the tumorigenesis and is one way how cancer cells can evade T-cell activation. Recently, it was shown that Tetrac treatment reduces PD-L1 mRNA and protein amount in a colorectal carcinoma cell line even though cells were treated with T_4 (Lin et al., 2016).The therapeutic function of Tetrac needs to be elevated in clinical trials.

5.3.2. TH do not modulate infiltrating immune cells in an immune-competent orthotopic male mouse model

The integrin $\alpha_v\beta_3$ is not only expressed on vessels and tumor cells but also on immune cells. The question rose what effect is mediated by the integrin $\alpha_v\beta_3$ within immune cells. First hints showed that TH somehow might modulate immune cells. Kinoshita *et al.* isolated NK cells from spleens of T_3, T_4 and methimazole treated mice and observed an immunosuppressive mode of action mediated by T_4 but not by T_3. Similar results regarding T_4 were obtained by Gupta *et al* (Gupta et al., 1983). In contrast, alveolar macrophages from those mice showed higher mediated cytotoxicity when treated with T_3 and T_4 but not when treated with methimazole alone (Kinoshita et al., 1991). Binding of T_4 to integrin $\alpha_v\beta_3$ results in activation of the MAPK-pathway, but the effect on immune cells is not understood.s

To analyze the alteration of infiltrating immune within the tumor upon T_4, T_3 and/or Tetrac stimulation, certain subtypes of immune cells which express $\alpha_v\beta_3$ were analyzed. No difference in the composition of infiltrating immune cells was observed throughout the differently treated groups. In this model, TH do not have any influence on infiltrating immune cells.

In sum, T_4 promotes tumor growth via S2 of integrin avß3 rather by favoring angiogenesis than suppressing the immune system. Such effects need to be taken into account regarding cancer patients developing hypothyroidism upon anti-cancer treatment.

5.3.3. Clinical relevance of T_4 treatment in cancer patients

The observation made in this study and in literature have indeed clinical relevance in the context of tumor therapy. Several targeted therapies are currently available for the treatment of various carcinomas. But special attention should be paid to their side effects. For example, Sunitinib [Sutent®] and Pazopanib [Votrient®]. Both drugs are tyrosine kinase inhibitors (e.g., VEGFR, PDGFR, KIT) approved by the FDA in 2010 for tumor therapy e.g. pancreatic neuroendocrine tumors, kidney cancer and, soft tissue cancer. They yielded promising results in clinical trials. One of the possible side effects of both drugs is hypothyroidism, which is particularly common in the

treatment of renal carcinoma with Sunitinib and Pazopanib (Vanderpump, 2011a; Vanderpump, 2011b).

Tyrosine kinase inhibitors are of course also able to block tyrosine kinase receptors within the hypothalamus-pituitary-thyroid axis resulting in a drop of TH concentration. This observation is also reported for Sunitinib-treated patients in lung carcinomas (Socinski et al., 2008), gastrointestinal stromal tumors (Uno et al., 2013) or pancreatic carcinomas (2012). Interestingly, the development of hypothyroidism seems to be associated with the success of the therapy.

This “side effect” is treated by administration of T_4. The results obtained in this study and the literature suggest that the treatment of hypothyroidism with T_4 appears to be counterproductive since T_4 could promote tumor growth. Furthermore, hypothyroidism seems to slow down tumor growth.

Ultimately, therapeutic consequences need to be addressed urgently now in the clinics: Should hypothyroidism in tumor patients be compensated by T_4 administration when hypothyroidism is caused by tyrosine kinase inhibitor treatment? Should patients be treated with T_3 instead of T_4?

6. Outlook

In summary, this thesis could answer several open questions of thyroidology, but still many questions need to be addressed.

The sequencing data generated in this thesis could indeed partially answer the question which mutations for targeted therapy can be found in ATC samples. These results will now help to provide novel mouse models for targeted therapy. Especially mutation found in *ALK* and *NF1* might be potential targets for a subgroup of ATC patients. A mouse model developing an ATC requires a thyrocyte specific expression of target genes like ALK^{F1174L}.

Cross breeding with a mouse expressing thyrocytes specific $CreER^{T2}$-Recombinase is necessary to achieve an organ specific expression of ALK^{F1174L}. To achieve the thyrocyte specific expression, currently two mouse models are available like the Tg-Cre^{ERT2} (Kusakabe et al., 2004) driven by the thyroglobulin promoter and the Tpo-Cre^{ERT2} driven by the thyreoperoxidase promoter (Kero et al., 2007). The expression of the ALK^{F1174L} would be prevented by a stop codon and poly-A-tail (hGH-pA) flanked by loxP sequences which can remove the $CreER^{T2}$-Recombinase after Tamoxifen injection.

Since three major events take place during tumorigenesis of ATC, it might be possible that constitutive activation of the ALK^{F1174L} is insufficient and a second genetic event might be necessary like the inactivation of the endogenous tumor suppressor gene *Trp53* or *Nf1.* A mouse that develops an ATC after overexpression of ALK^{F1174L} would be a preclinical *in vivo* model to test ALK inhibitors in a large cohort.

Furthermore, it would also be possible to generate a thyrocyte specific double knock-out of the endogenous tumor suppressor genes *Trp53* and *Nf1* to achieve a preclinical mouse model developing an ATC. The relevant exons of the endogenous Trp53 and Nf1 will be flanked with loxP sequences that can be inducible removed by the Cre-Recombinase-System.

The case report of the proposita´s, who first developed a pheochromocytoma and then 6 years later a MTC where routine diagnosis failed to identify a *RET* mutations, showed that the ATA guidelines were inadequate. This thesis provides a guideline

how the remaining MEN2 patients with no detectable mutation by hotspot sequencing should be analyzed. Physician should have a close look on the molecular level when routine sequencing fails to detect any mutations in the hotspot genes of *RET*. A multiplex PCR panel including primer for the whole sequence of *RET* should be designed.

In this thesis, it was possible to answer the question if TH modulate tumor growth *in vivo*. Furthermore, it was shown that T_4 and not T_3 promotes tumor growth via integrin $\alpha_v\beta_3$ activation. The observed influence of TH on tumor growth needs to be analyzed more precisely in future experiments. First, the question should be answered which target cells responded to T_4 stimulation. *In vitro* analysis of tumor cells might answer the question if tumor cells release proteins that favor neovascularization upon T_4 treatment (e.g. VEGF). Second, the experiments should be carried out with additional tumor cell lines to check if the observation is not only limited to 3LL. Also, cell lines should be included that do not express $\alpha_v\beta_3$ as negative control. Third, tumor cells should be inoculated in orthotopic organs in which a specific knock-out of $\alpha_v\beta_3$ is possible. This might answer the question if $\alpha_v\beta_3$ is required on blood vessels. Fourth, clinical trials are required to translate the findings in rodents and epidemiological studies. The clinical trials should be designed in a way that they can answer the question whether pharmacological doses of T_4 promote tumor growth or if hypothyroidism is able to slow down tumor growth.

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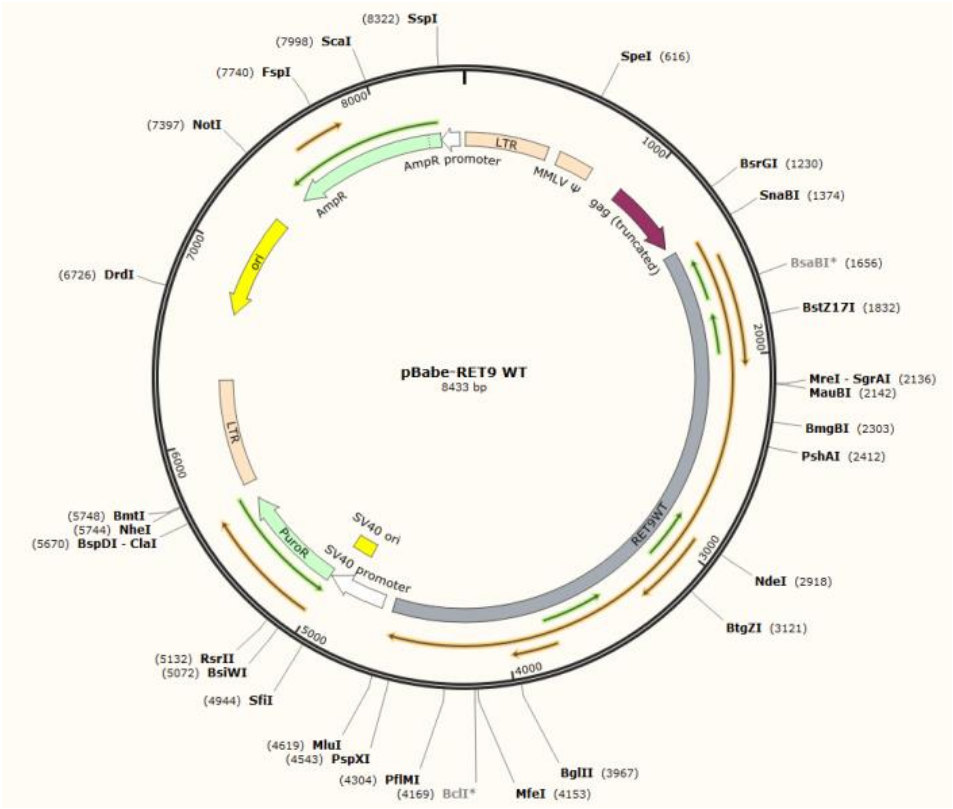
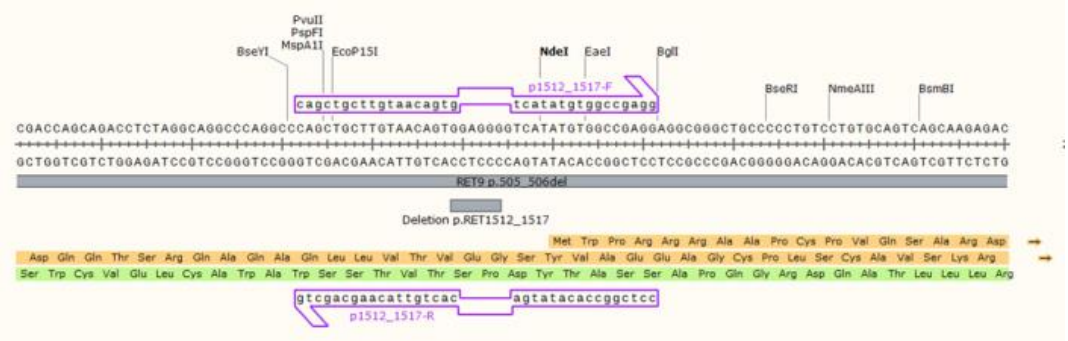
8. Appendix

8.1 Supplementary Figures



Supplementary Figure 1: Plasmid vector map for RET9 WT. This vector was used to create RET9 C634R and RET9 p505_506del by mutagenesis.



Supplementary Figure 2: Primer design for the mutagenesis to delete glutamic acid and glycine at amino acid position 505 and 506 in RET9 WT.

8.2 Abbreviations

%	Percent
3LL	Lewis Lung Cell Carcinoma
AA	Amino acid
AKT	Protein kinase B
ALK	Anaplastic lymphoma kinase
ATA	American Thyroid Association
ATC	Anaplastic thyroid cancer
ATRX	Alpha thalassemia/mental retardation Xlinked
BFGF	Basic fibroblast growth factor
BRAF	V-Raf murine sarcoma viral oncogene homolog B
CD	Cluster of differentiation
COSMIC	Catalogue of Somatic Mutations in Cancer
CTNNB1	β-catenin
DIT	Diiodo-tyrosyl-residue
DBD	DNA binding domain
EGLN1/PHD2	Prolyl hydroxylase domain-containing protein 2
FFPF	Formalin-fixed paraffin embedded tissue
EIF1AX	Eukaryotic translation initiation factor 1A, X-chromosomal
ERK	Extracellular signal-regulated kinases
Fig.	Figure
FTC	Follicular thyroid cancer
G	Grams
GDNF	Gliant cell line-derived neurotrophic factor
GFL	GDNF-family ligands
GFRα	Gliant cell line-derived neurotrophic factor receptor-α
HIF	Hypoxia-inducible factor
H&E	Hematoxylin and eosin staining
JNK	Jun NH2-terminal protein kinase
KIF1	Kinesin-like protein
LBD	Ligand binding domain
MAPK	Mitogen-activated protein kinase
MEN	Multiple endocrine Neoplasia

MIT	Monoiodo-tyrosyl residue
MTC	Medullary thyroid cancer
MAX	Myc-associated factor X
NF1	Neurofibromin 1
NGS	Next-generation sequencing
NIS	Sodium iodine symporter
PAX8-PPARγ1	Paired-Box-Protein 8 - Peroxisome proliferator-activated receptor gamma 1
PI3K	Phosphatidylinositol-3 kinase
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PD-1	Programme death-1
PD-L1	Programmed death ligand-1
PDTC	Poorly differentiated thyroid cancer
PTC	Papillary thyroid cancer
PTEN	Phosphatase and Tensin homolog
PTPN11	Protein tyrosine phosphatase non-receptor type 11
PVDF	Polyvinylidene fluoride
RAS	Rat sarcoma
RET	Rearranged during transfection
SDHAF2	Succinate dehydrogenase complex assembly factor 2
T_3	3,3’,5-triiodo-L-thyronine
T_4	3,3´,5,5’-tetraiodo-L-thyronine
TMEM127	Transmembrane protein 127
Tetrac	3,3′,5,5′-Tetraiodothyroacetic acid
TERT	Telomerase reverse transcriptase
TG	Thyroglobulin
TH	Thyroid hormone
TP53	Tumor suppressor p53
TPO	Thyroid peroxidase
TR	Thyroid hormone receptor
TRH	Thyrotropin-releasing hormone
TSH	Thyrotropin stimulating hormone
TSHR	Thyrotropin stimulating hormone receptor

SDHB	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit
SDHD	Succinate dehydrogenase [ubiquinone] cytochrome b small subunit
SRE	Serum response element
STRN	Striatin
VHL	Von Hippel–Lindau tumor suppressor
WT	Wild type

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2. Introduction

Publication 1:

9. Eidesstaatliche Erklärungen

Bestätigung:

Hiermit bestätige ich die Darstellung zu den Anteilen von Herrn Sören Latteyer an Konzeption, Durchführung und Abfassung jeder Publikation (Publication 1-3) gem. der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat.

Essen, den _____

PD Dr. Lars Möller

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, g der Promotionsordnung der Fakultät für Biologie zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „**Genetics of thyroid cancer and effects of thyroid hormone on tumor progress *in vivo***“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von (***Sören Latteyer***) befürworte.

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PD. Dr. Lars Möller

Erklärung:

Hiermit erkläre ich, gem. § 7 Abs. 2, d und f der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

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Sören Latteyer

Erklärung:

Hiermit erkläre ich, gem. § 7 Abs. 2, e und g der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe, dass diese Arbeit von keiner anderen Fakultät abgelehnt worden ist, und dass ich die Dissertation nur in diesem Verfahren einreiche.

Essen, den _____

Sören Latteyer

10. Curriculum Vitae

The Curriculum Vitae is not included in the online version for privacy reasons.

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

11. Acknowledgment

Zuallererst möchte ich meinen beiden Betreuern Frau Prof. Dr. Dr. Dagmar Führer-Sakel und Herrn PD. Dr. Lars Möller meinen tiefen Dank aussprechen. Sie haben mir beide die Möglichkeit eröffnet an äußerst interessanten und vielseitigen Themen zu arbeiten. Dabei konnte ich von ihren Erfahrungen und deren außerordentlichen Wissen in allen Belangen profitieren. Ihre Motivation und kontinuierliche Unterstützung waren mir in der gesamten Zeit stets eine Stütze. Ich bedanke mich auch für das entgegengebrachte Vertrauen.

Ein besonderer Dank geht an jene Helfer die mir bei der täglichen Arbeit unterstützend geholfen haben. Julius gilt mein Dank für die Unterstützung bei tierexperimentellen Arbeiten und Pflege der Tiere. Ein großer Dank gilt auch dem gesamten Team des Zentralentierlaboratoriums für ihre Hilfe rund um das Thema Mäuse. Dankeschön an die Pathologinnen Saskia Ting und Sarah Synoracki für ihre Hilfe bei der Auswertung der Immunhistochemie. Ebenfalls gilt mein Dank Vera Tiedje für ihre Hilfe bei der Beantwortung klinischer Fragen. Mein Dank gilt auch Sandra Christoph für das Erlernen der OP-Techniken. Ebenfalls möchte ich mich bei Steffi für ihre clevere Hilfe bei täglich anfallenden technischen Problemen bedanken. Ein ganz besonders großer Dank geht an Alexandra Brenzel für ihren unermüdlichen Einsatz und die Hilfe am Durchflusszytometer. Vielen Dank an Massimo Santoro (Mailand, Italien) für das Bereitstellen des RET-Plasmids. Ein Dank an all jene die diese Arbeit Korrekturlesen mussten.

Ich möchte auch die Gelegenheit nutzen mich bei allen Mitgliedern des Labors zu bedanken: Andrea, Denise, Daniela, Helena, Irina, Janina, Judith, Kathrin E., Kathrin S., Mathias, Sabrina und Sebastian. Vielen Dank an jeden einzelnen für diese tolle Zeit.

Ein großer Dank geht an meine Familie und Freunde für ihren unermesslichen Einsatz und Unterstützung in allen Lebenslagen. Ohne euch wäre das alles nicht möglich gewesen. Ihr wart und seid die Besten!

Zuletzt möchte ich mich bei jenen bedanken, die mich auf meinem Weg begleitet haben und stets für mich da waren, aber leider das Ende der Dissertation nicht miterleben durften. Danke!