Genetic Characterization and Identification of Novel Treatment Targets in Anaplastic Thyroid Carcinoma

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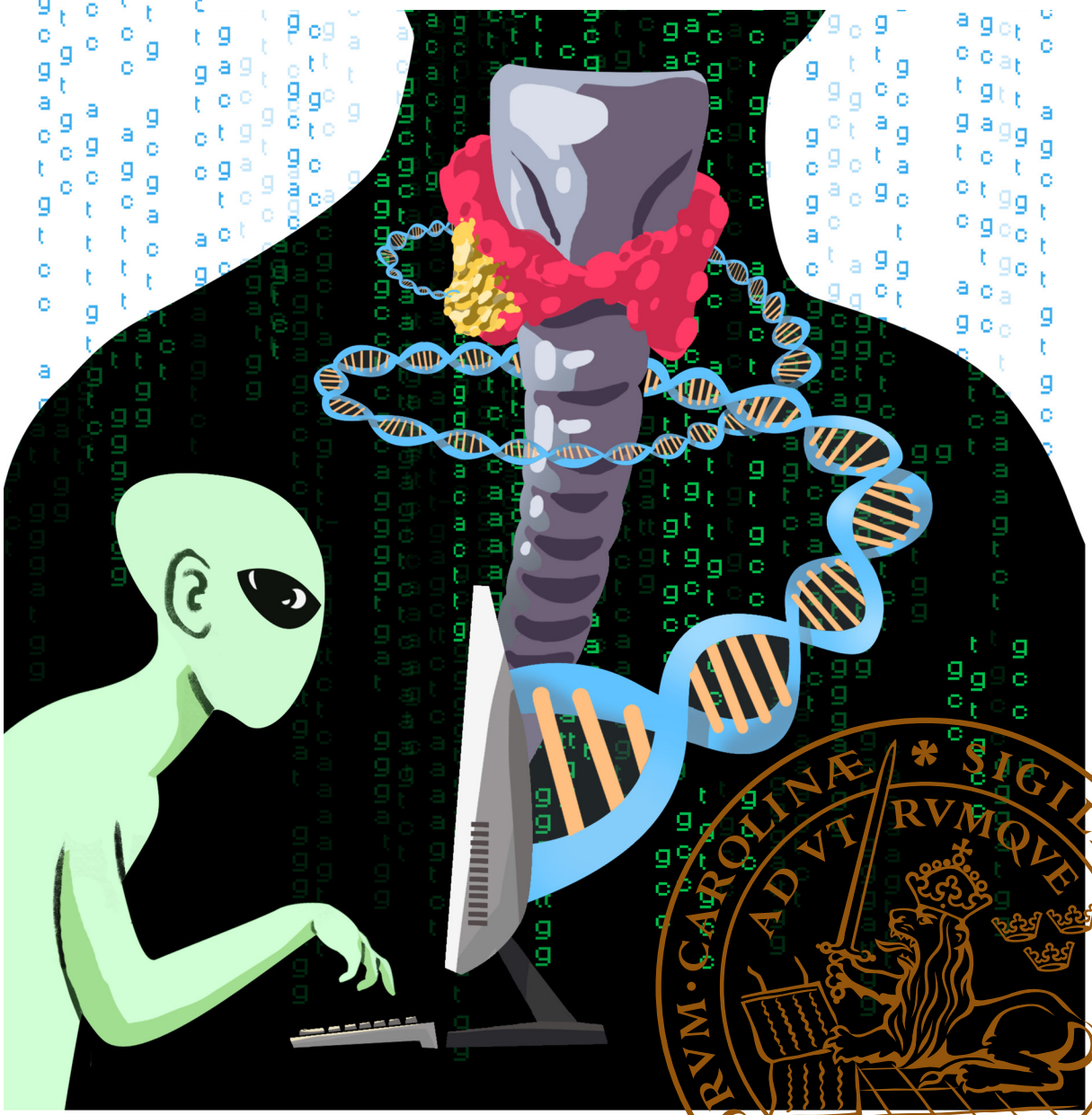

Naveen Ravi started his adventure in science with Master's in Molecular Biology at Lund University, Sweden. His adventure continued as a doctoral student in Kajsa Paulsson's group at the division of Clinical Genetics. This thesis was undertaken with an objective to understand the underlying molecular genetics in Anaplastic Thyroid Cancer.

Apart from research, he has closely supervised the works of Elon Musk & Iron Man to combat crises in both real and virtual world.

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Naveen Ravi

LUND
UNIVERSITY

DOCTORAL DISSERTATION

by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended on Thursday 28th of November 2019, at 13.00
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Faculty opponent
Dr Khalil Helou
University of Gothenburg

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Abstract

Anaplastic thyroid cancer (ATC) is a rare and highly aggressive thyroid malignancy, usually resistant to conventional therapeutic strategies. The prognosis of ATC is extremely poor with a median survival rate of 6 months. Current treatment therapy includes surgical resection in combination with external radiotherapy and chemotherapy. The underlying mechanisms involved in ATC tumorigenesis is understudied. In this thesis, we have focused on genomic characterization of ATC that could possibly lead to identification of novel treatment strategies in ATC.

Article I & III highlights the genomic heterogeneity in ATC cell lines and primary cases. ATC displayed massive aneuploidy with frequent variations in copy number. Additionally, we found frequent mutations in *TP53*, *TERT*, *BRAF* and *RAS* family genes. Furthermore, the most frequent mutational signature in ATC cell lines and primary cases was increased activity of the cytidine deaminase apolipoprotein B editing complex (APOBEC). Moreover, we found interstitial deletions in *NEGR1,* resulting in aberrant splicing, which could possibly be a driver event in ATC. Furthermore, we detected amplifications in *CCNE1*, *CDK6* and *TWIST1*; these patients could be treated with targeted therapy. In Article II, we investigated the tumor initiation, progression and clonal evolution of papillary thyroid cancer in a conditional mouse model. Stochastic activation of mutant *Braf* leads to development of multifocal microtumors that are oligoclonal in nature. Furthermore, we identified additional mutations at low frequencies, highlighting the presence of subclones that might be associated with tumor progression. In article IV, we studied the genome-wide methylation profile in primary ATC cases. Global hypomethylation was common in ATC, while hypermethylation was noticed in promoters and CpG islands. Furthermore, aberrant DNA methylation in *MTOR* and *NOTCH1* genes was associated with increased expression. Alternatively, hypomethylation in thyroid related genes including *TSHR* and *SLC26A7* was associated with decreased expression in gene body regions. Moreover, we found that processes related to the cell cycle were upregulated, while TP53-regulated genes and thyroid-related pathways were downregulated in ATC.

Taken together, this thesis provides a better understanding of the complex processes involved in ATC tumorigenesis. Furthermore, a substantial proportion of ATC patients could be suitable for personalized treatment, including CDK and TWIST1 inhibition therapy.

Key words: Anaplastic thyroid cancer, copy number alterations, somatic mutations, mutational signatures, DNA methylation analysis and gene expression analysis

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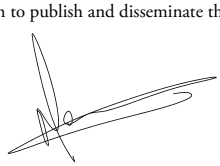
Signature  Date 2019-10-22

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To my family

Table of Contents

Original Articles

This thesis is based on the following articles.

Article I

Woodward EL, Biloglav A, Ravi N, Yang M, Ekblad L, Wennerberg J, Paulsson K: **Genomic complexity and targeted genes in anaplastic thyroid cancer cell lines.** *Endocr Relat Cancer* 2017, **24**(5):209-220.

Article II

Schoultz E*, Johansson E*, Moccia C, Jakubikova I, Ravi N, Liang S, Carlsson T, Montelius M, Patyra K, Kero J, Paulsson K, Fagman H, Bergö M, Nilsson M. **Modeling sporadic thyroid cancer in vivo reveals spatiotemporal control of tumor initiation and clonal growth.** (Manuscript)

Article III

Ravi N, Yang M, Gretarsson S, Jansson C, Mylona N, Sydow SR, Woodward EL, Ekblad L, Wennerberg J, Paulsson K. **Identification of targetable lesions in anaplastic thyroid cancer by genome profiling**. *Cancers* 2019, **11**(3):402.

Article IV

Ravi N, Yang M, Mylona N, Wennerberg J, Paulsson K. **Global RNA expression and DNA methylation patterns in primary anaplastic thyroid cancer.** (Submitted)

Articles not included in the thesis:

Forslund O, Sugiyama N, Wu C, Ravi N, Jin Y, Swoboda S, Andersson F, Bzhalava D, Hultin E, Paulsson K, Dillner J, Schwartz S, Wennerberg J, Ekblad L. **A novel human *in vitro* papillomavirus type 16 positive tonsil cancer cell line with high sensitivity to radiation and cisplatin.** *BMC Cancer* 2019 **19**(1):265.

Yang M, Vesterlund M, Siavelis I, Moura-Castro LH, Castor A, Fioretos T, Jafari R, Lilljebjörn H, Odom DT, Olsson L, Ravi N, Woodward EL, Harewood L, Lehtiö J, Paulsson K. **Proteogenomics and Hi-C reveal transcriptional dysregulation in high hyperdiploid childhood acute lymphoblastic leukemia.** *Nat Commun* 2019 **10**(1):1519.

Ku A, Ravi N, Yang M, Evander M, Laurell T, Lilja H, Ceder Y. **A urinary extracellular vesicle microRNA biomarker discovery pipeline; from automated extracellular vesicle enrichment by acoustic trapping to microRNA sequencing.** *PLoS One* 2019 **14**(5):e0217507.

Abbreviations

ATC	Anaplastic thyroid cancer
FA	Follicular adenoma
FFPE	Formalin fixed paraffin embedded
FTC	Follicular thyroid cancer
LOH	Loss of heterozygosity
MTC	Medullary thyroid cancer
NGS	Next generation sequencing
PDTC	Poorly differentiated thyroid cancer
PTC	Papillary thyroid cancer
RNA-seq	RNA sequencing
SNP	Single nucleotide polymorphism
WDTC	Well differentiated thyroid cancer
WES	Whole exome sequencing

Introduction

Tumorigenesis

In general, it is said that cancer is as old as human race. However, the first documented case of cancer in humans was from Egypt five thousand years ago, a case report on breast cancer which was deemed as untreatable [1]. In 2018, cancer was responsible for death of over 9 million people worldwide, thus making it the second-most leading cause of death [2].

Cell is a basic structural and functional unit of human body. The continuity of life is based on the never-ending process of cell proliferation; a highly complex, well-coordinated and tightly regulated process called cell cycle. DNA replication is the process of duplicating the genome of the parent cell prior to mitosis. Various complex regulatory mechanisms ensure that DNA replication is performed with high efficiency and accuracy [3]. Nevertheless, errors might occur during this process. Besides, various environmental factors can also damage the DNA. Despite multiple proofreading and DNA repairing mechanisms a small number of errors will be passed on to the daughter cell during cell division. These nucleotide variants can be further classified into single nucleotide variants (point mutations), insertions, deletions, inversions and translocations that lead to cells gaining a proliferative advantage over normal cells, ultimately leading to the transformation of normal cells to cancer cells.

Even before the discovery of the structure of DNA, Boveri in 1914, postulated that genetic abnormalities lead to progression of normal cells to neoplastic cells – this is now famously known as somatic theory of cancer (SMT) [4]. This theory gained attention when Powell in 1960 identified the genetic abnormality named Philadelphia chromosome in chronic myeloid leukemia [5]. Furthermore, SMT gained popularity with, for example, the identification of point mutation that underlies progression of a normal cell to bladder carcinoma [6]. Since then, genetic content of most tumor types have been studied to identify the genetic events that lead to tumor progression and to understand the effects these events have on the cell's phenotype. Numerous studies have validated that a tumor is the product of changes in the genetic material.

Tumors typically develop through sequential accumulation of genetic and epigenetic alterations over time [7]. Mutations that present preferential growth and survival advantage are termed as driver gene mutations, while mutations that do not affect

tumor progression but only happen to occur in the same cell as a driver mutation are termed as passenger mutations. Driver gene mutations can be further classified as oncogenes or tumor suppressor genes, since they regulate cell growth positively and negatively, respectively [7]. Epigenetic alterations such as histone modifications, DNA methylation and mircoRNAs alter the expression of genes; thereby aiding in initiation and progression of tumors [8]. Numerical and structural chromosomal instability (CIN) is widely observed in various tumors, these alterations drive the intratumoral heterogeneity and thereby increased resistance to therapy [9, 10]. Numerical CIN leads to loss or gain of whole chromosomes, whereas structural CIN encompasses small genomic alterations and gross chromosomal aberrations [9, 10] that might lead to creation of fusion gene, aiding in deregulation of genes and formation of a novel fusion protein or truncated proteins.

In 2000, Hanahan and Weinberg published a landmark paper titled "The hallmarks of cancer" that summarized tumorigenesis as a multistep process and stated that six essential changes in cell physiology are required for the malignant growth of tumors. Tumor cells should proliferate by attaining self-sufficiency in growth signals (a), thereby decreasing dependency on external growth stimulus, develop resistance against growth inhibitory signals (b) and develop mechanisms to evade apoptosis (c). Furthermore, these cells should attain limitless replication potential by regulating telomerase activity (d). Moreover, tumors should induce and sustain angiogenesis for gaining access to essential nutrients required for growth and survival (e). Additionally, malignant tumors need to develop the ability to infiltrate surrounding tissues by invading the blood or lymphatic vessels (f) [11].

An updated version of the article was published a decade later, that added two new emerging hallmarks; reprogramming cellular metabolism (g) and evading immune destruction (h) [12]. Furthermore, they also highlighted that genome instability (i) enable subclones of cells that can become dominant and outgrow other cells in the tumor microenvironment; and tumor promoting inflammation (j), which enhances tumor proliferation by delivering essential molecules to the tumor environment, as the two enabling characteristics required for acquisition of any hallmark competences (Figure 1) [12]. It is thus safe to conclude that tumors are not just an insular mass of rapidly dividing cancer cells, but are complex tissue harboring different cell types that are capable of interacting among themselves, creating a favorable microenvironment that enable cells in expression of hallmark features [11, 12].

It is highly unlikely that the tumor characteristics are generally gained by single somatic mutation, although Philadelphia chromosome appears to be sufficient for generation of chronic myeloid leukemia. Instead, accumulation of genetic and epigenetic changes over time generally drives tumor progression. Moreover, the number of mutations across different tumor types is an extensive spectrum ranging from a few in leukemia's to thousands in lung cancer. Exposure to mutagens and defective DNA repair

mechanisms are reflected in highly mutated tumors [7]. In fact, advanced tumors harbor highly complex genomes with larger variation in structural variants, multiple mutations and encompassing various epigenetic modifications. This thesis mainly focuses on identifying genetic and epigenetic changes that contribute to anaplastic thyroid cancer tumorigenesis.

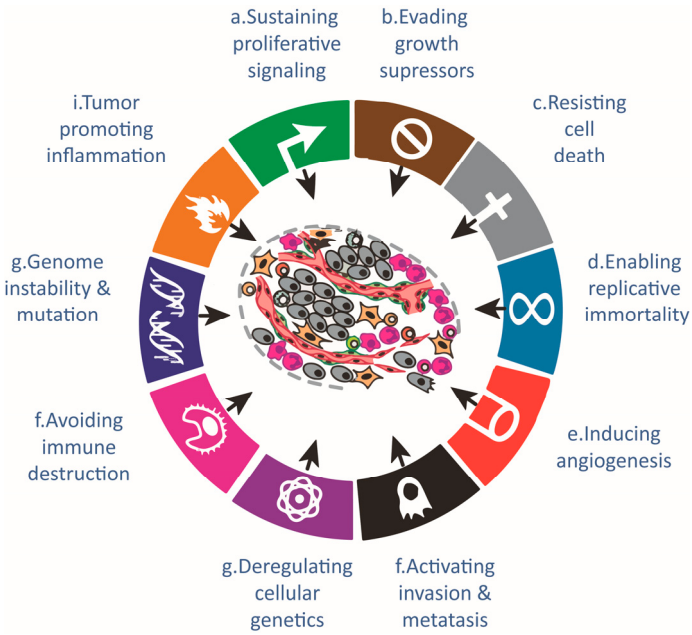

Figure 1: The hallmarks of cancer. Adapted from Hannahan and Weinberg 2011 [12].

Thyroid Gland

Physiology and function

The thyroid gland resembles a butterfly shape and is one of the largest endocrine organs, consisting of two lobes located on either side of the trachea that are connected by an isthmus. It weighs around 15-20 grams and consists of two different cell types: thyroid follicular and parafollicular cells.

The essential functional components of the thyroid gland are follicles. These are highly organized spherical structures with a central cavity filled by colloid, which serves as a reservoir for thyroglobulin. Colloids are densely packed together by connective tissue

to form lobule. Parafollicular cells are in proximity of the colloids, regulating the calcium homeostasis by the secretion of calcitonin hormone [13, 14].

Iodine is essential for synthesis of triiodothyronine (T3) and thyroxine (T4) hormones that are produced by the thyroid gland. Thyrotropin-releasing hormone (TRH) produced by hypothalamus stimulates pituitary gland to release thyroid-stimulating hormone (TSH) which regulates follicular function. Thyroid hormones regulate TSH by negative feedback mechanisms, with increased levels of T3 and T4 leading to decreased levels of TSH produced by the pituitary gland. TSH stimulates sodium-iodine symporter (NIS) to uptake iodide from the basolateral side of the follicle. On the apical side, pendrin transports iodide from the follicular cytosol to the lumen. Thyroid peroxidase (TPO) catalyzes oxidation of iodide to iodine and covalently links it to thyroglobulin, thereby synthesizing thyroid hormones, which are concentrated in the colloid. These hormones are endocytosed and severed in the follicular cell, releasing T3 and T4 into the bloodstream (Figure 2) [13, 14].

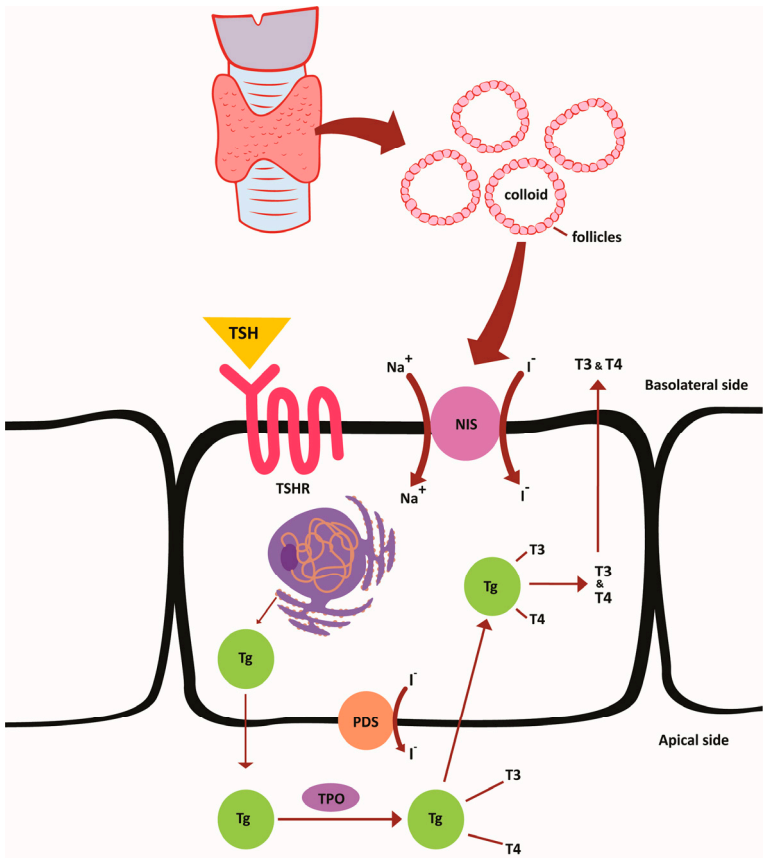

Figure 2. Thyroid hormone synthesis pathway. (TSH) thyroid stimulating hormone, (TSHR) thyroid stimulating hormone receptor, (NIS) sodium-iodide symporter, (PDS) pendrin, (TPO) thyroid peroxidase, (TG) thyroglobulin (T3) triiodothyronine and (T4) thyroxine.

The thyroid gland predominantly produces T4 in comparison to T3, even though T3 is more biologically active than T4. Deiodination of T4 to T3 takes place in the peripheral tissues or thyroid gland [15]. The function of thyroid hormones is tissue specific upon binding of thyroid hormones to thyroid hormone receptors, which are expressed by most of the cell types, initiating expression of target genes. Of the many functions of the thyroid, some of the key ones worth mentioning are - development of fetal nervous system, bone growth and regulation of cardiac output, basal metabolic rate and thermogenesis [16].

Thyroid cancer

Thyroid cancer is the most common endocrine malignancy, with more than half a million cases reported globally in 2018 [2]. It is the fifth most common type of cancer detected in women, of which more than 50% of patients diagnosed were below 50 years and they have higher tendency to develop thyroid malignancies than men with 3:1 ratio [2].

Classification of thyroid tumors is based on histological and cellular origin characteristics. Benign tumors comprise of follicular ademonas, while malignant tumors consist of well differentiated (papillary and follicular), poorly differentiated and anaplastic variants that arise from follicular cells. Tumors originating from parafollicular cells are called medullary thyroid cancer (MTC) [17].

Incidences of thyroid cancer has increased gradually with around 3.6% annually over the last four decades in USA [18]. Similarly, the prevalence of thyroid cancer in Sweden has increased from 3/100,000 to 8/100,000 individuals during 1998-2017 [19]. These trends could be attributed to the rapid development of diagnostic tools such as computerized tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography (PET), that has increased the diagnosis of small tumors at an early stage [20]. However, the underlying reason for increased frequencies cannot be solely attributed to better diagnosis, as the incidence of patients with larger and aggressive tumors has increased steadily over the years [20].

Exposure to ionizing radiation is major risk factor for developing thyroid cancer [21]. Atomic bombing of Hiroshima and Nagasaki along with Chernobyl nuclear power plant disaster caused release of radioactive substances into the atmosphere, including isotopes of iodine. Exposure to this ionizing radiation resulted in increased frequencies of differentiated thyroid cancer in the pediatric population [22, 23]. Additionally smoking, diet, diabetes mellitus and obesity are risk factors for developing thyroid malignancies [24-27].

Follicular Adenoma

Follicular adenomas (FA) are benign tumors of the thyroid gland frequently detected in middle aged women; most of them are euthyroid [28]. These tumors are characterized by the presence of round or oval nodule surrounded by fibrous capsules. Tumor size varies from 1-3 cm, but can be larger and are palpable by physical examination or identified using ultrasound scan [29]. Exposure to radiation and iodine deficiency can increase the risk for developing adenomas [30, 31].

Follicular adenomas are classified based on histological features. Diagnosis of follicular adenomas by fine needle aspiration (FNA) can be problematic, as these benign tumors share similar morphological features as follicular thyroid cancer (FTC). It can be distinguished based by the absence of capsular invasion, vascular invasion, lymph node metastases and distant metastases in follicular adenoma; however, cases with indeterminate FNA cytology do exist [29, 32]. Moreover, several mutations including *RAS* family genes (20–40% of cases) and *PAX8-PPARG* chromosomal rearrangements (11%) have been found in FA [32-35]. Furthermore, such FA have similar mutations as FTC [33, 34]. This shared genetic and cytological features between FA and FTA further complicates the diagnosis of indeterminate cases. However, recent studies have focused on using RNA and miRNA profiling to identify new biomarkers that can complement FNA cytology to classify indeterminate cases [32, 36]. The argument of benign adenomas progression to malignant tumors is debated [32].

Papillary thyroid cancer

Papillary thyroid cancer (PTC), the most common endocrine malignancy, accounts for 80-85% of all the thyroid malignancies. Incidences of PTC has progressively increased worldwide in both sexes, in contrast to a steady decline in mortality over last 30 years. These tumors occur three times more often in women than in men. Patients diagnosed with PTC have an excellent 5-year survival rate of over 90% [18, 37-39].

Increased incidences of PTC cannot be solely attributed to better diagnosis of small tumors <1 cm, as the incidences of advanced PTC have also increased over the years [18]. The diagnosis of PTC is based on cytology, fine needle biopsies, ultrasound and other imaging modalities of all thyroid nodules. According to WHO classification, there are 14 subtypes of PTC based on histological features [31]. Exposure to ionizing radiation is a major risk factor associated with PTC [40].

The genomic landscape of PTC is well characterised across different populations [41-43]. Typically, the mitogen-activated protein kinase (MAPK) pathway is upregulated by somatic activating mutations in *BRAF* (59-74%) and *RAS* family genes (*NRAS* (2-7%), *HRAS* (0.6-3.5%) and *KRAS* (0.3-2%)). These mutations in *BRAF* and *RAS* are mutually exclusive. Mutations in *PTEN* (0.3-2%), *PIK3CA* (0.3-6%) and *AKT* (0.3-

1%) are also detected at lower frequencies; these genetic alterations activate phosphoinositide 3-kinase AKT (PI3K–AKT) pathway, playing an important role in thyroid tumorigenesis [41, 43, 44]. Similarly, mutations in tumor suppressor genes (*TP53* (0.3-10%), *MEN1* (0.3-3%), *NF1* (0.5-2%) and *NF2* (2%)) and DNA double-strand break repair genes (*ATM* (0.3-4%), *PPM1D* (1.2%), *CHEK2* (1.2%), *BRCA2* (1%) and *BRCA1* (1%)) occur at low percentages in *PTC* [43-45]. Additionally, low frequency mutations are observed in *EIF1AX* (1.5-2%), *EZH1* (0.5-2.2%) and *TERT* (2-9.4%) [42, 44, 45].

Among structural rearrangements observed in PTC, the most common is *RET-PTC* (6.8-8.5%) [43, 45]. *RET* codes for a receptor tyrosine kinase that recombines with a strong promoter, leading to ligand-independent dimerization of the fusion protein. Although 10 different types of functional *RET* chimeric transcripts exist, the most common ones are *RET-PTC1* (53%) usually found in sporadic PTCs and *RET-PTC3* (62.3%) which is frequent in post-Chernobyl PTC patients [17, 46-48]. Additionally, less frequent fusions involving genes such as *PPARG* (0.8%), *BRAF* (2.3%), *ALK* (0.8%), *NTRK3* (1.3%), and *LTK* (0.83%) have been reported in PTC [41]. These fusion genes are mutually exclusive to driver mutations. The incidence of fusion genes as oncogenic drivers is higher in radiation induced thyroid cancer compared to sporadic PTC [49].

PTCs are primarily driven by genetic alterations in *BRAF* and *RAS*, these genetic events lead to distinctive expression profiles. Principal component analysis (PCA) revealed that PTCs cluster into 3 different molecular subtypes: *BRAF*–like, *RAS*–like, and non–*BRAF*–non–*RAS* (NBNR) tumors [42]. Additionally, the BRAF-RAF score (BRS) classifier consisting of the expression of 71 genes, also segregates PTC's tumors into two groups (*BRAF*–like and *RAS*–like tumors) based on the gene expression profile. Similarly, the expression of thyroid specific genes quantified using thyroid differentiation score (TDS) varies between *BRAF*–like and *RAS*-like tumors, with the former having relatively lower TDS score and the latter maintaining more normal expression levels [42, 45].

The treatment of patients diagnosed with PTC is dependent on the type and stage of the tumor. Usually, total thyroidectomy is performed if the tumor size is >1 cm, followed by radioactive iodine therapy. Post-surgery, TSH suppressor hormone therapy might be recommended to suppress the proliferation of follicular cells. Radioactive iodine therapy is used to completely ablate residual follicular cells, eradicate distant metastases and residual tumor cells. However, *BRAF* mutant PTCs have lower expression of thyroid specific genes, thereby attaining resistance to radioactive iodine therapy [50, 51]. FDA-approved multikinase inhibitors (MKI), such as lenvatinib and sorafenib, are frequently used to treat patients with radioiodine refractory PTC, thereby increasing the duration of disease-free survival [51]. Vemurafenib, which specifically targets mutant BRAF, has been approved for treatment of patients diagnosed with

melanoma. Phase II clinical trials of treatment in refractory radioiodine PTC patients with vemurafenib showed that it could be a viable treatment option for *BRAF* mutant PTC [52].

Follicular thyroid cancer

Follicular thyroid cancer (FTC) is the second most common type of endocrine tumor, accounting for 10-15% of all thyroid malignancies. Higher frequencies of FTC are observed in women as compared to men. Increased incidences of FTC were detected in iodine deficient regions. Patients diagnosed with FTC are usually over 45 years with tumor size >1cm and presence of distal metastases was observed in 15–27% of patients at the time of diagnosis. Iodine deficiency is a major risk factor for FTC [53-56].

Based on WHO classification, FTC is a malignant epithelial tumor that lacks diagnostic features of papillary thyroid cancer and shows signs of follicular differentiation [31]. The diagnosis of FTC is based on the presence of capsular and vascular invasion. These tumors are classified into minimally invasive and widely invasive tumors, based on the extent of capsular and vascular invasion infiltration [55]. Widely invasive FTC are aggressive tumors, as they metastasize into distal sites through vascular invasion [57]. Widely invasive tumors are associated with poor prognosis due to presence of extrathyroidal extension, distant metastases and tumor size in excess of 4cm, usually considerably higher than seen in minimally invasive tumors [31].

Genes in the *RAS* family (*NRAS*, *KRAS* and *HRAS*) are frequently mutated in FTC, usually in the 61st codon. In particular, mutations in *NRAS* are common in FTC (15% to 40%) [55]. RAS is a dual activator of MAPK and PI3K-AKT pathways. Recent studies using next generation sequencing have identified mutations in *FLT3* (51.4%), *TP53* (28.6%), *IGF2BP3* (23%), *TSHR* (10.3%), *KMT2C* (7.6%), *DICER1* (5.1%), *PTEN* (5.1%), *NF1* (5.1%), *IDH1* (2.9%), *JAK3* (2.9%), *KIT* (2.9%), *PIK3CA* (2.9%), *BRAF* (2.6%) and *EIF1AX* (2.2%) in FTC [33, 34, 42, 55]. The *PAX8-PPARG* gene fusion is found in 12% to 56% of FTCs [55]. The fusion protein inhibits the normal function of the tumor suppressor PPARG and also stimulate certain PAX8 responsive genes [17]. Although FTC and FA share similar somatic mutations, FTC harbors more mutations than FA [34].

FTC harbor frequent DNA copy number changes with loss of entire chromosome 22 or 22q, 1p21 and13q21; conversely, recurrent gains are seen in 1q and 17q [58, 59]. Minimally invasive tumors have fewer copy number events as compared to widely invasive tumors [59]. Recurrent copy number gains involving the genes *IQGAP1* and *PIK3CA* have been reported to be involved in tumor progression. *IQGAP1* amplification is associated with invasiveness in FTC [60]. Amplification of *PIK3CA* is associated with aberrant activation of the PI3K-AKT pathway, thereby promoting tumorigenesis [61]. Mutations in *PIK3CA* and copy number gain in *PIK3CA* are

mutually exclusive events, indicating that either one of these genetic events is sufficient for the tumor progression via PI3K-AKT pathway [17].

Almost all patients diagnosed with FTC are treated with surgery. Based on tumor size, presence of distant metastases and age of the patient, thyroid lobectomy or total thyroidectomy is performed followed by radioiodine treatment for effective management of FTC [56]. Thyroid-stimulating hormone (TSH) suppressive therapy is also recommended as part of post-surgery management. With the recent advances in targeted therapy, protein kinase inhibitors such as lenvatinib and sorafenib have increased the disease-free survival duration among patients with radioiodine refractory FTC [51, 56].

Medullary thyroid cancer

Medullary thyroid cancer (MTC) is the third most common endocrine malignancy and has gradually become more common over the last three decades [62]. It accounts for 2-5% of thyroid cancers, yet it is responsible for 13% of mortality related to thyroid malignancies. These tumors originate from parafollicular cells or C cells that synthesize calcitonin. The mean age at diagnosis is 52 years and there is no difference between the sexes in terms of number of cases reported [62-64].

Diagnosis of MTC is based on fine needle aspirate, measurement of calcitonin levels and ultrasound scan. In patients diagnosed with MTC, roughly 35% are found to have lymph node metastases, while about 20% are found to have distant metastases. The 10 year disease free survival in patients with localized tumor exceeds 90%, which is reduced to 78% and 40% in patients with regional and distant metastases, respectively [65]. Age, tumor size, presence of distant metastasis, calcitonin levels and *RET* mutations are the common prognostic markers in MTC [66].

Almost 75% of MTC cases are sporadically developed tumors whereas approximately 25% of cases have an inherited form of the disease; as part of multiple endocrine neoplasia syndrome type 2 (MEN2) caused by germline mutations in *RET* proto-oncogenes leading to gain of function, which have an autosomal dominant mode of inheritance [31]. MEN2A and MEN2B are the two clinical variants of MEN2, while familial is a subtype of MEN2A. Although several *RET* mutations are associated with MEN2A, most of the patients diagnosed with MEN2A harbor germline mutations found in exon 10 and 11. Similarly in MEN2B, frequent mutations were observed in either exons 15 or 16 but they are more common in exon 16 [65].

Majority of sporadic MTCs (85%) have acquired mutations in *RET* (65%) or *RAS* (*KRAS* (15%) and *HRAS* (5%)). Mutations in *RET* and *RAS* genes are mutually exclusive, suggesting that either of these driver mutations are sufficient for tumor progression [67, 68]. Additionally, copy number loss in cell cycle regulator genes such

as *CDKN2C* (19%) was associated with worst prognosis [69]. Furthermore, mutations observed in *BRAF*, *PIK3CA* and *TP53* are not common in MTC, thus making their diagnostic utility uncertain [67, 69].

MTC harbor recurrent loss of chromosomal regions in 1p, 13q, 3q26.3-q27 and 22q [70]. The target of 1p32 deletion is believed to be *CDKN2C*; loss of *CDKN2C* leads to haploinsufficiency, thereby deregulating cell cycle and aiding in tumor progression. Loss of *CDKN2C* frequently co-occurs with somatic mutations in *RET* and is associated with a poor prognosis [69].

Based on risk stratification, thyroidectomy is performed in majority of MTC cases, followed up by constant monitoring of calcitonin levels and glycoprotein carcinoembryonic antigen (CEA) to check for local reoccurrence of the primary tumor. Chemotherapy is administered to patients with local and distant metastases [65]. Multikinase inhibitors such as vandetanib and cabozantinib are used to target the action of RET, VEGF and EFGR are frequently used in treatment of patients with advanced MTC [66].

Poorly differentiated thyroid cancer

Poorly differentiated thyroid cancer (PDTC) is a rare type of thyroid malignancy, accounting for 2-15% of all cases [71, 72]. PDTC originates from follicular cells and can arise either from well differentiated tumors (WDTC) such as papillary or follicular variants or *de novo* [71]. The mean age at diagnosis is 59 and these tumors have a female predominance with a ratio of 6:1 in women versus men [71].

More than 50% of patients diagnosed with PDTC harbor lymph node metastases, whereas 50-85% of cases metastasize into surrounding regions, commonly into lung and bone. Tumor size, age, extra thyroidal extension and presence of distant metastases are the predictive factors that influence the overall survival in PDTC cases. Patients diagnosed with PDTC have an overall 5-year survival rate of 62-85% [71, 73, 74].

Although WDTC and PDTC share the same oncogenic mutually exclusive driver mutations in *BRAF* (33%) and *RAS* family genes (29%) [75-77], PDTC have higher mutation burden than WDTC [75]. Tumor suppressor genes that are frequently mutated, include *TP53* (10%), *ATM* (10%), *PTEN* (5%), *LATS2* (6%), *CTNNA2* (6%), *RB1* (1%) and *MEN1* (1%); however, the frequency of these mutations are lower than in anaplastic thyroid cancer (ATC) [44, 75-77]. Mutations are infrequent in genes that regulate PI3K-AKT pathway (11%) [75-77]. Mutation in *EIF1AX* (11%) occurs at low frequency and is predictive of worse survival in PDTC. Similarly, infrequent mutations in PDTC were observed in genes involved in histone methyltransferases (7%), SWI/SNF chromatin remodeling complex (6%) and DNA mismatch repair pathway (2%) [75-80]. Mutations in *TERT* (40-46%) promoter is a common

occurrence in PDTC [75, 76]. These mutations are associated with *BRAF* or *RAS* mutations, and usually exhibit an aggressive phenotype [75, 81].

PDTC harbor frequent changes in copy number involving gains in 1q and 20q and losses in 1p, 8p, 13q, 15q, 17p and 22q. Additionally, gain in 1q is associated with a poor prognosis, while loss in 22q is common feature among *RAS*-mutant PDTC [71, 75, 82]. Fusion genes are infrequent events in PTDC involving *RET*/*PTC* (6%), *PAX8*/*PPARG* (4%) and fusions involving different partners of *ALK* (4%) [75].

Clinical trials on PTDC have been restricted mainly by the unavailability of samples. Clinical management of patients diagnosed with PDTC is similar to WDTC. Total thyroidectomy is performed for resectable tumors when feasible, followed by adjuvant radioiodine therapy [73]. Adjuvant external beam radiation therapy (EBRT) can be employed to reduce risk of relapse [83]. Additionally, very limited data is available on the effect of adjuvant chemotherapy on PDTC. As these tumors are rare, development of new drugs and subsequent conducting of clinical trials is challenging [71, 83, 84].

Anaplastic thyroid cancer

Anaplastic thyroid cancer (ATC) is a rare and malignant endocrine tumor, accounting for 1-2% of all thyroid cancer, yet it is liable for more than 50% of all the fatalities related to thyroid cancer [85-87]. These tumors occur more frequently in women as compared to men. The mean age at diagnosis is over 65 years with a median survival of less than 6 months and only 10-15% survive 2 years after diagnosis [88, 89].

In USA, age adjusted frequency rates of ATC have gradually increased over the last 3 decades from 0.2 cases per million in 1973 to 1.2 cases per million in 2014, although the overall incidences of ATC still remains very low at <2% compared to all thyroid cancer [90].

Classification of ATC is based on their histological and clinical features at diagnosis. The American Joint Committee on Cancer (AJCC) classifies ATC as stage IV, regardless of the tumor size, spreading to the lymph nodes or distant metastases, with intrathyroidal disease as stage IVA, gross extrathyroidal extension or cervical lymph node metastases as stage IVB, and distant metastases as stage IVC. Most ATC tumors are more than 3cm in size. Some of the most common symptoms include dysphagia, dyspnea, hoarseness, neck pain, vocal cord paralysis and weight loss [85, 91].

Histologically, anomalous patterns of growth are seen in ATC. These patterns mainly constitute the presence of numerous atypical mitotic cells and multinucleated cells with large peculiar nuclei. ATCs are highly invasive on detection and are characterized by the presence of spindle, epithelioid, squamoid, giant or osteoclast-like cells with either patches of differentiated tissue or no trait of differentiated thyroid tissue [91]. Exposure to ionizing radiation, history of longstanding goiter and age are the main risk factors

associated with ATC, whereas the presence of distant metastasis, size of tumor, sex and age are indicative of a poor prognosis in ATC [85, 88].

Clonal evolution of ATC

Although ATC may arise through *de novo* pathways, it is believed that approximately 50% of ATC develops from pre-existing well-differentiated tumor [91, 92]. This is based on the presence of well-differentiated areas in ATCs, suggesting post-malignant dedifferentiation leading to aggressive subtypes [91].

Recently a couple of studies have focused on deciphering the post-malignant dedifferentiation of ATC from WDTC by genomic analysis of ATC cases with coexisting WDTCs [93, 94]. The first study consisted of a small cohort of 3 ATCs with coexisting PTC that were analysed using whole exome sequencing. Very few mutations were shared between the ATCs and PTCs. Additionally, in one case immunohistochemistry (IHC) staining confirmed the lack of cells expressing both BRAF V600E and TP53. These results suggest that ATC and PTC are two separate molecular subtypes that evolve independently [93].

On the contrary, a small cohort consisting of 5 ATCs with coexisting WDTC analysed using whole exome sequencing found higher percentage of trunk mutations shared between these tumors. Moreover, in 2 cases a subclone of cells harbored genetic features like ATC. These results suggest that these tumors share a common ancestor and a linear transition occurs through subsequent accumulation of mutations that lead to progression of tumors from WDTC to ATC [94]. Taken together, our understanding of the clonal evolution in ATC is very limited, further investigations are required to delineate the evolutionary process involved in ATC.

Genetic alterations in ATC

With the advent of next generation sequencing, various studies have focused on identifying genetic events responsible for the aggressive phenotype observed in ATC. However, most of these studies were performed using targeted gene panels [44, 75, 95-101]. Only a handful of published studies have employed whole exome sequencing and whole genome sequencing to completely decipher the mutational landscape of ATC [76, 86, 93, 94, 102]. Nonetheless, the genomic landscape is yet to be elucidated.

ATC have higher mutation burden compared to PTC with a mean number of nonsynonymous variants of 13 vs 23 in PTC and ATC [45, 86]. *BRAF* and *RAS* mutations are the primary oncogenic events that drive the tumor progression in WDTC. These mutations in *BRAF* (25-91%) and *RAS* family genes (9-43%) are frequently detected in ATC (Table 1); while these mutations are mutually exclusive event [44, 75, 76, 103]. These genetic alterations lead to deregulation of MAPK pathway and PI3K-AKT pathway. PI3K-AKT pathway can also be deregulated by acquired mutations in *PTEN* (15%) and *PI3KCA* (6-18%) (Table1)[100].

Mutations in *TP53* (27-73%) occur at a higher frequency in ATC compared to PDTC (10%) or PTC (0.3-10%) [44, 45, 75-77]. The process of de-differentiation from WDTC to ATC might be accelerated by acquiring mutations in *TP53* [75, 86]. This hypothesis has been validated by a mice study, with loss of *TP53* in a *BRAF* mutant mice accelerates the progression from PTC to ATC [104]. Similarly, the de-differentiation can be triggered by acquiring mutations in *PI3KCA* (Table1) [105].

Frequent *TERT* promoter mutations are observed in ATC, usually at two hotspot locations C228T and C250T [75, 86]. *TERT* mutations are a hallmark of advanced thyroid tumors, as incidences of *TERT* promoter mutations are higher in advanced PTC (61%), PDTC (40-46%) and ATC (27-73%), compared to PTC (9%) (Table1) [41, 44, 75]. Constitutive expression of *TERT* leads to further activation of MAPK signaling [75].

Table 1: Overview of common alterations found in ATC

Alterations	Genes	Frequency	References
RAS-MAPK-pathway	*BRAF*	25-91%	[44, 75, 76, 80, 82, 86, 93, 96, 97, 99, 106]
	NRAS	7.6-29%	[75, 76, 86, 93, 96, 99]
	KRAS	3-9%	[86, 96, 99]
	HRAS	4-14%	[76, 93, 96, 99]
PI3K-AKT pathway	*PTEN*	7-15%	[44, 75, 76, 93, 97]
	PIK3CA	6-18%	[44, 75, 86, 97, 99, 100]
	MTOR	9%	[75]
Cell cycle	*CDKN2A*	22%	[44, 76]
	CDKN2B	13%	[44]
	CCNE1	4%	[44, 98]
	RB1	1-9%	[44, 75, 98-100]
Tumor-suppressor genes	*TP53*	27-73%	[44, 75, 76, 80, 86, 93, 96, 97, 99]
	USH2A	18%	[86]
	NF1	10-37%	[44, 75, 86, 96, 100, 101]
	NF2	5-27%	[44, 75, 86, 97, 98]
	ATM	8-57%	[44, 75, 100, 101]
Additional genes	*EIF1AX*	9-14%	[75, 86]
	TERT	15-75%	[44, 75, 76, 82, 86, 93, 98-100]

Cell cycle deregulation is frequently observed in ATC by acquired copy number loss of *CDKN2A* (22%) and *CDKN2B* (13%), mutations in *RB1* (1-9%) and amplifications of *CCNE1* (4%) [44, 75, 76]. Additionally, genetic alterations were observed in DNA

mismatch repair genes (MMR) (4-27%), SWI/SNF chromatin remodeling complex (9-36%) and histone methyl transferases (HMT) (18-24%) [44, 75, 76, 86]. Mutations were also reported in *USH2A* (18%), *ATM* (8-57%), *NF1* (10-37%), *MTOR* (9%) and *EIF1AX* (9-14%) (Table1) [44, 75, 76, 86]. Particularly, *EIF1AX* mutations commonly co-occur with RAS mutations [75].

ATC are heterogenous tumors with complex karyotypes and larger variation in copy number. Regarding cytogenetic data, only a handful of cases have been published on primary ATC, showing that they display massive aneuploidy with chromosome numbers ranging between 65 and 120 [107-112]. Frequent losses in 8p (20-36%), 1q (40%), 9p (58%), 11p (33%), 11q (33%), 13q (18-42%), 17p (6-44%), 17q (43%), 19p (36%), and 22q (38%) while gains in 1q (18%), 10p (20%), 19p (33%), 19q (40%), 20q (30-47%) and Xp (20%) have been reported [75, 111, 113]. Loss of 13q and gain in 20q has been linked to shorter survival [75].

Gene fusions in ATC

Chromosomal rearrangements resulting in fusion genes are a common feature in WDTC; however, there are very few fusion genes reported in ATC [75, 76]. Although *RET* fusions are frequent in PTC, they are rare in ATC [114]. Similarly, *ALK* fusions were reported to be associated with two different genes, including *EML4* and *STRN* in single cases [115, 116]. Additionally, fusion involving *BRAF* with *MKRN1* was detected in one case [117]. Lastly, *FGFR2*/*OGDH*, *NUTM1*/*BRD4* and *SS18*/*SLC5A11* were the fusions identified in single cases [75, 117].

Epigenetic modifications in ATC

Epigenetic modification is a tightly regulated process essential for growth, development and regulation of tissue, and cell specific functions. Disruption of epigenetic processes can possibly have a major impact on disease progression, including cancer. Epigenetic modifications are mediated by changes in DNA methylation, histone modifications, chromatin remodeling and non-coding RNAs [118, 119]. DNA methylation mostly occurs at CpG dinucleotides, affecting the regulation of gene expression. Furthermore, post transcriptional modification of histone tails also regulate gene expression. Similarly, noncoding RNAs regulate gene expression by post-transcriptional mechanisms. Moreover, chromatin remodeling leads to changes in gene expression as it regulates chromatin accessibility.

Genome wide methylation investigations performed in cohorts of two and three cases revealed that ATC harbors a distinctive methylation pattern when compared to differentiated thyroid cancers and normal thyroids. [120, 121]. Global hypomethylation is common in ATC. In addition, a higher proportion of hypomethylated probes are located in CpG islands, whereas most hypermethylated probes were in intergenic and gene bodies. Furthermore, several pathways were deregulated in ATC as identified from pathway enrichment analysis. Of the several, G-

protein alpha-i (*Gαi*) signaling pathway was activated, that might stimulate the kinase activity of cSRC protein [120, 121].

In ATCs, tumor suppressor genes such as *PTEN* (69%), *RASAL1* (33%), *RAP1GAP* (33%), *RASSF1A* (77%) and *RASSF2* (83%) have been reported to be frequently inactivated by DNA hypermethylation [121-126]. *PTEN* negatively regulates PI3K-AKT pathway; downregulation of *PTEN* leads to aberrant activation of PI3K-AKT pathway. Hypermethylation of *PTEN* is also frequently found in FTC [125]. Similarly, hypermethylation of RASAL1, which inactivates RAS, thereby modulating RAS signaling pathway, is reported in ATC [124]. Additionally, *TSHR* (61.8%) and *NKX2-1* (60%) genes involved in the regulation of thyroid function are hypermethylated [127, 128]. On the contrary, only a handful of genes such as *MAP17* (33%), *TCL1B* (64%) and *NOTCH4* (45%) have been reported to be hypomethylated [120].

Transcriptome profile of ATC

Only a handful of studies have focused on finding the transcriptomic profile in ATC [76, 102]. From unsupervised principal component analysis, it is evident that ATC clusters separately from PTC [75, 76, 129]. Gene set enrichment analysis has identified upregulation of genes involved in MAPK signaling, cell cycle, TGF-beta signaling, epithelial to mesenchymal transition and glycolysis metabolism in ATC compared with DTC and/or normal thyroid tissue, while genes involved in fatty acid metabolism, glycolipid metabolism and calcium signaling pathway have been reported to be downregulated in ATC [76, 129-132].

Genes involved in thyroid synthesis have been reported to be significantly downregulated in ATC. Iodide is essential for the production of thyroid hormone, it is transported into the follicles via sodium iodide symporter (NIS). As NIS is frequently downregulated in ATC, this leads to loss in ability to concentrate iodide, causing tumors refractory to radioiodine therapy [75, 76].

Programmed death ligand-1 (PD-L1), is an immune inhibitor molecule that constrains T cell-mediated immune responses. Expression of PD-L1 is observed in ATC and is associated with poor prognosis [76, 133]. However, these studies related to expression of PD-L1 in ATC were conducted on small cohorts of samples [133, 134].

Tumor microenvironment

The tumor microenvironment of ATC is extensively infiltrated by inflammatory cells, mainly consisting of tumor assisted macrophages (TAM) that might constitute around 50% of the tumor mass in some cases [135]. Additionally, expression data reported overexpression of genes associated with M2 macrophages signatures [75]. Higher density of infiltration of TAMs is observed in ATC as compared to PDTC. Increased incidences of TAMs correlate with invasiveness of the tumor and is associated with worst prognosis [136].

Treatments

As ATC is a highly aggressive tumor, an intense and multimodal therapy consisting of surgical resection in combination with external radiotherapy and adjuvant chemotherapy is applied [103, 137]. At diagnosis, most patients have extra thyroidal extension, spreading to surrounding regions such as the larynx, trachea and esophagus [88, 138]. However, complete surgical resection is possible in patients diagnosed with stage IVA and a small subgroup of patients with IVB. Multimode intensive therapy with combination of surgical intervention and chemoradiation has improved the median overall survival time to 8 months [139]. Additionally, a single institute retrospective study concluded that multimode intensive therapy improved the median overall survival to 22 months compared to 4 months in palliative intent therapy [140]. For patients with stage IVB ATCs that are unresectable, neoadjuvant chemotherapy was effective in reducing tumor size in 33% of the patients with one patient displaying complete response and two patients had partial responses. Subsequently, enabling gross resection of the tumor and followed up with adjuvant therapy. Furthermore, patients who underwent neoadjuvant chemotherapy following surgery displayed no signs of distant metastasis during 11-32 months follow up [141].

Identification of molecular targets in cancer has led to increased number of preclinical studies, both in cellular and mouse models. Inhibitors of multikinases, BRAF, PI3K/mTOR, EGFR and VEGR are in phase II clinical trials [85, 103, 142-146]. Sorafenib, a multikinase inhibitor, has been tested in different phase II single arm clinical trials in patients with advanced thyroid cancer, consisting of smaller groups of patients with ATC [143-146]. A multi-institutional phase II clinical trial, consisting of 20 patients with ATC were treated with sorafenib. The overall median survival was 3.9 months, 10% of the patients had partial response and 25% of the patients had stable disease [144]. A second single arm phase II clinical trial was tested in a subgroup of four ATC patients with one patient (25%) displayed stable disease [143]. A third single arm phase II clinical trial included 10ATC patients, (40%) of the patients experienced stable disease with a median overall survival of 5 months [145]. Similarly, lenvatinib a multikinase inhibitor, was tested in a single arm phase II clinical trial including a subgroup of 17 ATC patients out of 51 enrolled for the study. The overall survival was 10.6 months, 25% of the patients had partial response and 71% of the patients had stable disease [142]. Everolimus, mTOR inhibition therapy has been explored in several phase II clinical trials in smaller groups of patients with ATC [101, 147, 148]. Of the seven ATC patients included in the study, one patient demonstrated partial response and remained progression-free for 17.9 months, while two (28%) patients had stable disease [101]. The other two studies included a minor group of six and seven ATC patients, none of the patients displayed any response to treatment with everolimus [147,148]. Vermurafenib, a BRAF inhibitor, was investigated in non-melanoma patients [149]. Of the seven ATC patients included in the study, one patient had complete response, another

patient had partial response, while 57% of the patients had progressive disease [149]. Recently, FDA approved targeted therapy for *BRAF*-mutant ATC, consisting of a combination of BRAF and MEK1/2 inhibitors (dabrafenib and trametinib). Of the 16 ATC patients included in phase II clinical trial using dabrafenib and trametinib inhibition therapy, overall response rate was 69% with one patient displaying complete response and 10 patients demonstrating partial responses [140, 150].

The present study

Aims

As ATC has a very poor prognosis, the aim of this thesis was to achieve a better understanding of the complex genetic mechanisms involved in ATC. Additionally, genetic characterization of ATC could lead to development of potential new treatments that could improve the overall survival time for patients diagnosed with ATC and to investigate the tumor initiation, progression and clonal evolution in PTC mouse model.

Article I To investigate the genomic landscape of ATC by SNP array analysis, RNA-seq and WES in ATC cell lines

Article II To investigate additional mutations that contribute to tumor progression in a sporadic mice model of PTC

Article III To investigate the genomic landscape of ATC using RNA-seq and WES in primary ATC cases

Article IV To investigate the global methylation patterns in correlation to expression patterns in primary ATC

Material and Methods

The following section is a concise outline of the materials and methods employed during the study. For more elaborate explanations please refer to the original articles.

Patients and tumor samples

In article I, tumor biopsies from patients diagnosed with ATC and PTC were used to establish ATC and PTC cell lines at the Departments of Oncology, ENT/H&N Surgery at Skåne University Hospital in Lund, Sweden. For Article II, mouse tumor samples with matched normal kidneys were obtained through collaboration at Gothenburg University, Sweden. In articles III & IV, primary tumor biopsies and fresh frozen paraffin embedded tumor blocks were obtained from patients diagnosed with ATC prior to treatment with chemotherapy or radiotherapy, from the Pathology Department, Laboratory Medicine, Skåne, Sweden.

All these studies were approved by the local ethics committees.

SNP array analysis

Naturally occurring single nucleotide variants with alleles displaying an incidence of >1% in the population is commonly defined as single nucleotide polymorphisms (SNPs). In SNP array analysis, these naturally occurring variants can be used to identify a wide variety of genomic changes such as amplifications, deletions and loss of heterozygosity (LOH).

Both Illumina (San Diego, CA, USA) and Affymetrix arrays (Santa Clara, CA, USA) were used in article I, each containing 5 million and 1.8 million markers, respectively. In brief, SNP arrays comprise millions of oligonucleotide probes. Fragmented DNA is hybridized to the oligonucleotide probes. The signal emitted by the bound DNA fragment by fluorescent attached tags is scanned and measured. For each locus two values are generated. One is the log2 ratio, which indicates the copy number, obtained by normalized signal intensity The other is the B-allele frequency which indicates allele

distribution at the locus. The resolution of SNP array is highly dependent on the number of markers utilized in the array. The inability of SNP arrays to detect balanced chromosomal rearrangements is a major disadvantage of using SNP arrays.

For data analysis, raw intensity files were analysed by using Genotyping Module from Illumina GenomeStudio software and Nexus Copy Number software.

In article I, ATC cell lines were investigated using SNP array analysis to identify acquired copy number changes and LOH.

Sporadic papillary thyroid cancer mouse model

The Cre/loxP-System is a highly versatile genetic engineering tool that can produce conditional knock-out or knock-in mice models. Cre is a recombinase enzyme, derived from P1 bacteriophage that recognizes specific target sequence LoxP and catalyse recombination around the LoxP regions. LoxP consists of a 34 bp sequence with a 13 bp palindromic sequence flanking either side of the central 8 bp sequence. The orientation of floxed loxP sites surrounding the gene of interest determines the type of recombination [151].

In tamoxifen-inducible Cre recombinase system (Cre-ER), Cre is bound to a mutated estrogen receptor, which only gets activated upon binding to tamoxifen. Transgenic mice with tissue-specific tamoxifen-inducible expression of Cre recombinase under the control of thyroglobulin promoter ($TgCreER^{T2}$) produces Cre recombinase enzyme specifically in the thyroid gland and it is restricted only to the cytoplasm. $TgCreER^{T2}$ mice was crossed with floxed $Braf^{V600E}$ mice to generate $TgCreER^{T2};Braf^{CA/+}$. Following tamoxifen induction, Cre recombinase is activated in $TgCreER^{T2};Braf^{CA/+}$ mice, resulting in knock-in mouse model, expressing mutated $Braf^{V\!E}$ protein (Figure 3). Since mutated $Braf^{V\!E}$ acts as an oncogene and initiates tumor development all over the thyroid gland, this model of tumor development does not completely mimic the scenario in humans where the entire thyroid gland is not involved in tumorigenesis.

We took advantage of the spontaneous activation of Cre recombinase enzyme even in the absence of tamoxifen (inducible agent) leading to development of multifocal microtumors. Such microtumor development led by leakiness of the system is more comparable to the scenario in human tumorigenesis.

In article II, tumor initiation and tumorigenesis were studied using the conditional tamoxifen-inducible $TgCreER^{T2};Braf^{CA/+}$ mice model.

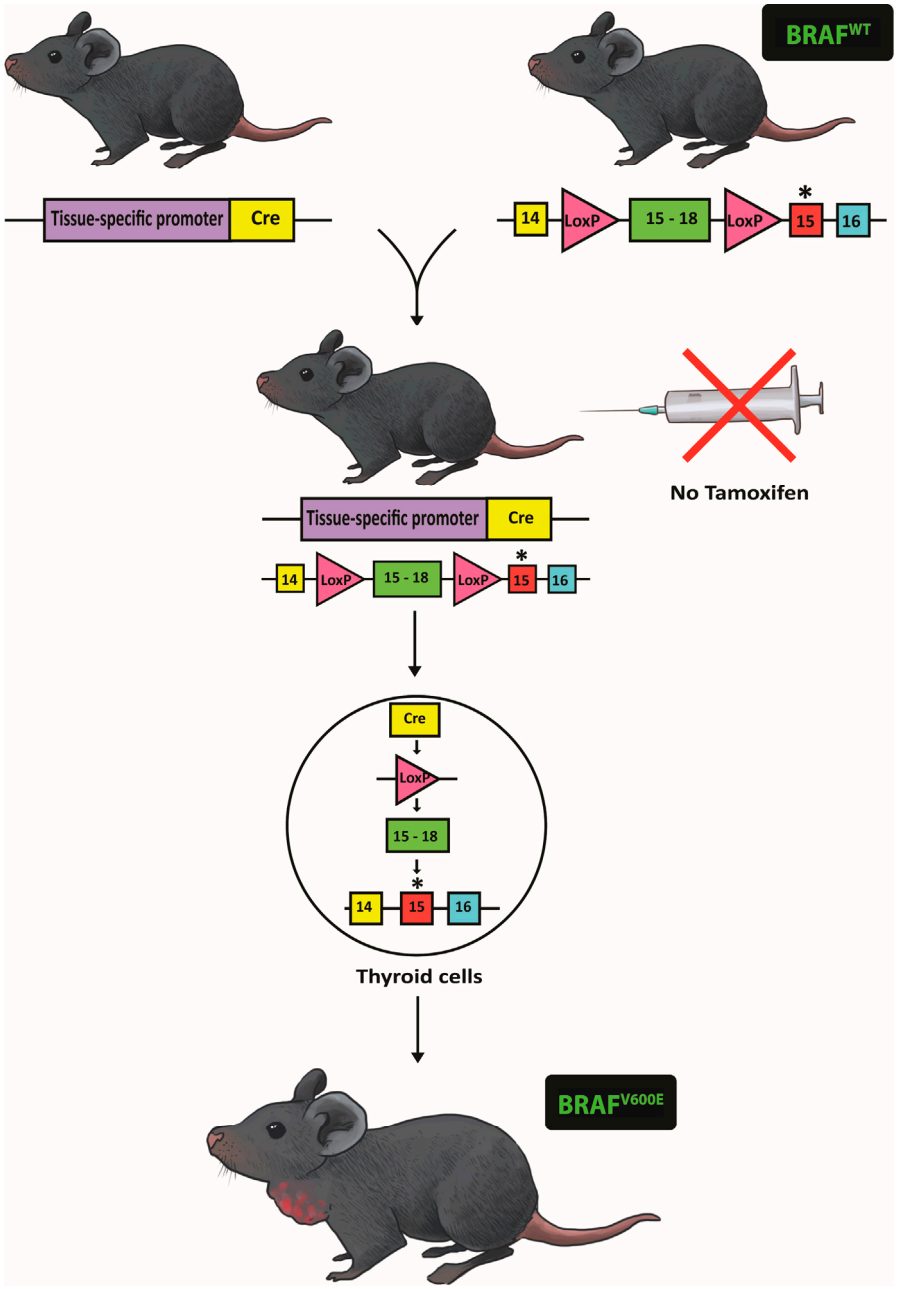

Figure 3. Conditional knock-in mouse model using Cre/loxP system. A transgenic mouse expressing tissue-specific Cre recombinase is bred with another transgenic mouse with a floxed *Braf* gene, expression wildtype BRAF protein (mice on the top). Spontaneous activation of Cre by leakage results in activation of mutated *Braf*^{V600E} gene (mouse at the bottom).

Next generation sequencing

Next generation sequencing (NGS) or massively parallel sequencing is a paradigm-shifting technology that has enabled scientists to address diverse biological problems. NGS offers greater sensitivity, specificity and scalability, aiding in the advancement of cancer genetics by large scale sequencing of billions of DNA and RNA fragments. NGS technologies have been extensively used for the investigation of whole genomes, whole exomes, epigenomes, targeted regions and transcriptomes. Additionally, NGS has aided in *de novo* assembly of genomes of various organisms without any reference genomes [152].

Several commercially available sequencing platforms are available, manufactured by Complete Genomics (Mountain View, CA, USA), Thermo Fisher Scientific (Waltham, MA, USA), Illumina (San Diego, CA, USA), Roche (Basel, Switzerland), 10x Genomics (Pleasanton, MA, USA) and Pacific Biosciences (Menlo Park, CA, USA), employ different sequencing technologies to achieve high throughput sequencing. The global market for sequencing instruments is dominated by Illumina [153]. Illumina (NextSeq500) and Complete Genomics platforms were primarily used for genomic and transcriptomic studies in this thesis.

Illumina employs sequencing by synthesis technology to achieve high throughput sequencing. Constructed libraries are loaded on to a flow cell that consists of oligos that are complimentary sequences to the end fragments of the DNA libraries. Upon hybridization, polymerase generates a complementary strand to the bound fragment. This double stranded structure is denatured, and the hybridized strand is washed away. Each fragment is clonally amplified by bridge amplification generating clonal clusters. Additionally, the reverse strands are cleaved off with the 3' ends primed to prevent undesirable priming. This is followed by sequencing of the forward strands, consisting of a three-step cycle starting with incorporation of a 3' blocked fluorescently labelled nucleotide, which ensures only one nucleotide is added during each cycle. After addition of the nucleotide, a fluorescent signal of specific wavelength is emitted by the cluster by activation via light source. The captured fluorescent signal together with its intensity aids in accurate determination of base call. Finally, the fluorophore dye is cleaved off and 3′-OH is regenerated by chemical agents to reinitiate the process of addition of 3' blocked fluorescently labelled nucleotide in the next cycle. This cycle is repeated several times and the number of cycles determine the read length. After completion of the forward strand, the reverse strand is sequenced as per the same process mentioned above [154].

Whole exome sequencing

Exome is defined as the protein coding regions throughout the genome, encompassing roughly 1% of the entire human DNA; however, many disease-associated variants are within these regions. Whole exome sequencing (WES) is usually performed at a higher depth (>100x), as approximately 1% of the genome is under focus, this reduces the overall cost and time needed for analysing the data compared to whole genome sequencing (60x). Usually, tumor samples are sequenced together with paired normal samples to exclude germline variants.

In this thesis, following sequencing, raw data from Illumina sequencing platform were converted to fastq file format. The fastq file consists of sequence identifier, base calls and base quality scores information for all sequenced reads. After processing of low-quality reads and soft clipping of adapter sequences, reads were aligned to reference genome using BWA-MEM [155]. GATK best practices pipeline was employed to remove redundant reads and local realignment of reads around the indel regions [156]. MuTect2 was used for somatic variant calling and variants were annotated by ANNOVAR [157, 158]. CNVkit was used for calling copy number events from exome data [159].

In articles I & III, WES was performed on ATC cell lines and primary ATC cases to investigate somatic mutations, copy number events and mutational signatures in ATC; while, in article II, WES was performed on the mouse model of PTC.

RNA sequencing

RNA sequencing (RNA-seq) is commonly used for characterization and quantification of whole transcriptomic profiles. Additionally, RNA-seq has broader dynamic range for detecting differently expressed genes when compared to microarrays [160]. Thus, gaining a big advantage over hybridization-based techniques. RNA-seq has enabled the detection of novel isoforms and fusion transcripts.

In this thesis, subsequent to sequencing tumor samples, reads were aligned to the reference genome using STAR [161]. Raw read counts for each gene were calculated using HT-seq. These read counts were normalized by the fragments per kilobase million (FPKM) method. FPKM accounts for the sequencing variability between samples, as it is calculated based on gene length, total number of reads spanning the gene and the total reads mapped for a given library. Analysis of differentially expressed genes was performed using DEseq2 and EdgeR [162, 163]. DAVID and gene set enrichment analysis (GSEA) was used for functional enrichment analysis [164, 165].

FusionCatcher, SOAPFuse, ChimeraScan and InFusion were used for detection of chimeric transcripts [166-169]. As these software are based on different algorithms, their sensitivity and specificity for detecting fusion transcripts varies drastically,

resulting in lower frequencies of similarity between the chimeric transcripts detected by various fusion detection software. Detected fusion genes were validated by Sanger sequencing.

Limitations of RNA-seq include errors incorporated during reverse transcription of RNA to cDNA that introduces artefacts as a product of template switching, resulting in higher incidences of false-positive gene fusions. Additionally, the sequencing quality scores and sequencing depth is highly dependent on the purity of RNA material [170].

In articles I, III & IV, RNA sequencing was performed for detection of fusion genes and to investigate the global gene expression patterns in ATC cell lines and primary ATC cases.

Methylation array

For characterization of methylation profiles in ATC, Illumina Infinium EPIC methylation array targeting more than 850,000 CpG sites across the genome was employed on bisulfite-converted DNA samples. Methylation levels across the genome was quantified based on the genotype of the bisulfite-converted DNA sample.

The EPIC array consists of 866,836 CpG sites, of which around 36% are located in proximity to gene promoter regions, such as 5' untranslated regions (UTR) and transcription start sites, 36% are located in gene bodies and 27% in intergenic regions. Based on the context of CpG location, 56.5 % of CpG sites are located in open sea, followed by CpG islands (17.8%), CpG shores (16.9%) and CpG shelves (8.8%) [171].

For differential methylation analysis, raw data was imported into ChAMP software [172]. After filtering for SNP sites, correcting for batch effect and dye correction, normalised β values were segregated into three groups. Fisher's two-sided t-test was performed to identify differentially methylated probes and these CpG sites were annotated based on Illumina's manifest file.

In article IV, global methylation profile of primary ATC cases was analysed using Illumina EPIC methylation array.

Results

Article I

Genomic complexity and targeted genes in anaplastic thyroid cancer cell lines

In paper I, we investigated genetic aberrations in 10 ATC and 3 papillary thyroid cancer early-passage cell lines by SNP array analysis, RNA-seq and WES.

SNP array analysis of 10 ATC cell lines identified multiple breakpoints with a median of 49.5 breakpoints, ranging from 11 to 167 per case. Breakpoints were frequent in pericentromeric regions, indicating mitotic spindle defects and genomic instability. Larger variations in copy number with frequent deletions in fragile sites were observed in ATC, suggesting replicative stress. Loss of heterozygosity (LOH) involving whole chromosomes were common in chromosomes 13, 18 and 17.

Recurrent small targeted deletions were observed involving genes such as *PTPRD* (six cell lines; 60%), followed by *AUTS2* and *FHIT* (four cell lines; 40%), *CDKN2A* and *MACROD2* (three cell lines; 30%). Furthermore, targeted deletions in *NEGR1* was seen in 4 ATC cell lines and an acquired mutation in one cell line, leading to atypical splice variants, which could be a possible driver event in ATC.

Regarding mutations, *TP53* and *TERT* promoter hotspot mutations were most frequent in ATC cell lines (60%). The second most prevalent genetic alteration was mutations in *BRAF* (40%), while mutations in *NRAS* were seen in 20% of the cell lines.

From RNA-seq data, we identified 21 non-recurrent fusion genes among six ATC cell lines. Functional pathway analysis in ATC and PTC cell lines revealed that TP53 signaling, axon guidance, cell adhesion molecules (CAMs) and autoimmune thyroid disease pathways were downregulated in ATC.

In summary, we presented the genomic landscape of ten newly established cell lines, which closely resembled primary ATC. We noticed complex copy number patterns with massive aneuploidy and signs of replicative stress as well as genomic instability. Furthermore, we detected mutations that are commonly observed in primary ATC

including *TP53*, *TERT*, *BRAF* and *NRAS*. Moreover, we found frequent deletions in *NEGR1* and *PTPRD* that could contribute to tumorigenesis in ATC.

Article II

Modeling sporadic thyroid cancer in vivo reveals spatiotemporal control of tumor initiation and clonal growth

This study is a collaborative work with Professor Mikael Nilsson, University of Gothenburg. All the mice work was done by Mikael Nilsson's group; my main contributions included identification and validation of somatic alterations that could contribute to the sporadic development of tumors in $TgCreER^{T2};Braf^{CA/+}$ mice by WES and targeted sequencing.

Exome sequencing was performed on five thyroid tumors using matched kidney tissue as constitutional controls. Apart from the $Braf^{V600E}$, no additional mutations were detected in mice tumors that stemmed from tamoxifen induction (n=1) and non-induced sporadic tumor (n=1) at 4 months of age. However, in three non-induced sporadic tumors in mice 6-12 months of age, we detected a handful of additional somatic alterations with a median of 2 per case (range 1-3). None of the identified mutations were recurrent. Of all the mutations identified, only *Pclaf* has been reported to be implicated in thyroid cancer. These mutations were validated by targeted resequencing except *Pclaf*. Furthermore, the mutations occurred at a very low mutant allele frequency (range 0.05-0.14), indicating that they were subclonal events. Our findings suggest that acquired somatic mutations are infrequent in the *Braf* mutant mice model of PTC.

Article III

Identification of targetable lesions in anaplastic thyroid cancer by genome profiling

In paper III, we investigated acquired somatic alterations, copy number events, fusion genes and mutational signatures in 14 primary ATC cases using WES and RNA-seq.

Copy number analysis from WES data of 10 ATC cases revealed that these tumors were polyploid with frequent breaks in the centromeric regions. We detected a median of 16

breakpoints per case ranging from 5 to 43 per case. A striking feature of loss of chromosome 8p and subsequent gain of 8q with frequent breaks in the centromeric regions was observed in ATC (6 cases; 60%). Targeted genomic amplifications involving genes such as *CCNE1* (29%), *CDK6* (9%) and *TWIST1* (9%) displayed higher gene expression of these genes.

For the eight matched tumor/normal paired cases subjected to WES, we detected a total of 7478 somatic mutations; the number of somatic alterations detected per case varied from 28 to 6863, with a median of 60 mutations per tumor sample. In the three cases that lacked a matched normal sample for analysis, the list of variants was filtered against several criteria reducing the list to a total of 245 somatic mutations with a range of 58 to 99 per case.

Mutations in *TP53* (6/11, 55%) was the most common genetic alteration observed in primary ATC, followed by *TERT* (36%) promoter mutations detected in four cases. Mutations that were found in three cases (27%) were *ATM* and *ARID2. BRAF* was mutated in two cases (27%), whereas *NRAS* and *HRAS* mutations were observed in one case (9%). Mutations in tumor suppressor genes such as *RB1*, *PIK3CA* and *NF2* were found in two cases (27%).

Regarding mutational signatures, mutational patterns detected in at least 3 cases (27%) were associated with ageing, activity of APOBEC family cytidine deaminases, defective DNA mismatch repair, malfunction of DNA double strand break repair, exposure to tobacco and ultraviolet light.

From RNA-seq data, chimeric transcripts were identified using two fusion detection callers namely InFusion and FusionCatcher. We detected a total of 21 candidate chimeric transcripts in 5 out of 12 cases; none of them were found to be recurrent. Interestingly, we found in-frame reciprocal fusions in case 5 and case 14 involving *MLXIP*/*PTEN* and *NCOR2*/*EP400* genes, respectively. Additionally, we also detected the *FN1* gene to be involved in two different fusions; however, both were out-of-frame fusions.

Our study provides better understanding of the complex mechanisms involved in ATC tumorigenesis. In our cohort of 14 ATC cases, 36% harbored genetic alterations that are appropriate for targeted treatments such as CDK6 and TWIST1 inhibition therapy.

Article IV

Global RNA expression and DNA methylation patterns in primary anaplastic thyroid cancer

In paper IV, we investigated the epigenetic and transcriptomic events in 13 primary ATC cases by genome wide methylation arrays and RNA-sequencing.

Integrative analysis of expression and methylation profiles between primary ATC cases and normal thyroid tissue was performed. Lower expression of thyroid related genes was observed in ATC, indicating dedifferentiation. Cell cycle-related processes, cytokine interactions, extracellular matrix and G-protein-associated signaling were found to be upregulated in ATC, whereas pathways related to thyroid hormone generation, thyroid hormone metabolic processes, translation, transcription, metabolic processes and mitochondria were found to be downregulated.

In ATC, differentially methylated CpG sites were detected across all genomic regions with a higher fraction of hypermethylated CpG probes detected in promoters and gene bodies, whereas hypomethylated CpG probes were frequently found in intergenic regions. Of 222 differentially methylated sites associated with significant differences in gene expression, we found a higher proportion of hypomethylated probes (86%) associated with increased gene expression in ATC, comprising several oncogenes such as *NOTCH1* and *MTOR*. A smaller fraction of hypermethylated probes (14%) were linked to lower gene expression, consisting of tumor suppressor gene *MAGI1*. Furthermore, we observed 32 hypermethylated sites in gene bodies linked to increased gene expression in ATC, consisting of genes such as *MTOR*. Similarly, we found 226 hypomethylated probes in gene bodies exhibiting significantly decreased expression, including genes such as *TSHR* and *SLC26A7* that are involved in the regulation of thyroid hormone synthesis.

To conclude, our study is the first to report genome-wide methylation in correlation to gene expression in ATC. Global hypomethylation in ATC was a common feature observed in our cohort, indicating that epigenetic modifications could play a vital role in thyroid tumorigenesis. Moreover, aberrant methylation was observed in *MTOR*, associated with increased expression. Patients with elevated expression levels of *MTOR* could directly benefit from targeted therapy.

General discussion and future perspectives

ATC is one of the most malignant cancers occurring in humans; these tumors are highly aggressive and are associated with a very dismal prognosis [85, 87, 91]. No effective treatment has been found yet. The genetic events leading to this aggressive tumour phenotype is understudied. In this thesis, we have investigated genomic, epigenomic, and transcriptomic profile of ATC to identify novel treatment targets that could be used to develop new therapeutic strategies in ATC.

It is evident from the copy number analysis of ATC cell lines and primary tumors (articles I & III) that these tumors are generally polyploid, with most of the cases investigated displaying massive aneuploidy. Frequent deletions in fragile sites were observed, signifying replicative stress [173]. These tumors acquire multiple breakpoints across the genome with frequent breaks near the centromeric regions. This could be either attributed to mitotic spindle deficiencies or due to telomeric shortening causing initiation of breakage-fusion bridge cycles that facilitate centromeric breaks [174, 175].

Complex copy number patterns, with frequent deletions and gains of regions, were observed in ATC. In article I, we identified recurrent deletions in tumor suppressor genes such as *PTPRD* (60%), *FHIT* (40%) and *CDKN2A* (30%). Additionally, we found genomic alterations in *NEGR1* (50%), comprising interstitial deletions in four cell lines and an additional cell line harbored a somatic mutation. Moreover, cell lines with deletions had multiple splice variants. *NEGR1* encodes for a glycosylphosphatidylinositol (GPI)-anchored protein that mainly functions in cell adhesion. *NEGR1* is frequently downregulated in several cancers and knockdown of *NEGR1* in an ovarian adenocarcinoma cell line caused increased cellular migration and invasive characteristics, indicating that *NEGR1* is a putative tumor suppressor gene [176]. As *NEGR1* is frequently targeted, alterations in this gene may increase invasiveness and thereby promote metastasis in ATC. However, we could not ascertain targeted deletions of *NEGR1* in primary ATC cases due to the poor resolution of exome sequencing.

Although recurrent focal amplifications of chromosomal regions involving genes were not common in cell lines (article I), we identified three primary cases (article III) with high copy number gains 19q12 involving *CCNE1.* CCNE1 interacts with cyclin dependent kinases (CDKs) causing formation of cyclin E1/CDK2 complex that drives the cell progression into the S phase [177]. Our results are consistent with previous findings of *CCNE1* amplifications in ATC [44] and these amplifications have also been

reported in several tumors such as breast, ovarian and gastric cancer [177-179]. Here, we observed that 29% of our primary cases displayed *CCNE1* amplifications and cases with amplification exhibited high expression of *CCNE1*. Additionally, we found amplifications involving *CDK6* and *TWIST1* in one case. The former's functions are similar to *CCNE1,* as *CDK6* is a downstream target molecule of *CCNE1.* The latter is frequently upregulated in multiple malignancies including prostate, ovarian and pancreatic cancer [180]. *TWIST1* is a transcription factor that enables invasion and metastasis by upregulating the epithelial-to-mesenchymal transition (EMT) pathway [181]. Although currently there are no inhibitors available targeting TWIST1 and CCNE1, several FDA approved drugs targeting CDK4/6 such as ribociclib, palbociclib and abemaciclib have been employed in clinical setting for management of patient with advanced or metastatic ER-positive breast cancer [182]. CDK inhibitors could be beneficial for patients with *CDK6* and *CCNE1* amplifications.

Regarding mutations detected in article I & III, majority of cell lines and primary cases harbored mutations in *TP53* and *TERT* promoter regions. Additionally, we detected mutations in *BRAF* and *RAS* family genes; these were mutually exclusive events. Furthermore, we found mutations in tumor suppressor genes *RB1*, *PTEN* and *NF2* at varying mutant allele frequencies (MAF). The somatic mutations profiles of ATC observed in article I & III are in agreement with previous studies [44, 76, 86]. Moreover, most of the cases had several mutations below <5% MAF, these could be subclonal mutations, highlighting the mutational burden in ATC.

The most common mutational signature in our cohort of cell lines and primary cases was related to increased activation of AID (activation induced cytidine deaminase)/APOBEC (apolipoprotein B mRNA editing enzyme complex) activity that deaminates cytosine leading to C>T transition mutations [183]. Additionally, in our primary tumors, we detected mutational signatures associated with ageing, exposure to tobacco, defective DNA mismatch repair and defective homologous recombination signature. Our results are in line with recent investigations, stating that ATC harbor mutational signatures in ageing, defective DNA mismatch repair and in AID/APOBEC activation [44, 76, 94]. Taken together, ATC harbor multiple mutational processes that varies between cases, indicating diverse mechanisms underlying ATC tumorigenesis.

Recently, Dong *et al*. and Capdevila *et al*. have focused on elucidating the clonal evolution in ATC by investigating ATC cases with presence of co-existing WDTC [93, 94]. The former reported that ATC and WDTC are derived from a common ancestor, as these tumors share a substantial amount of mutations and accumulate unique additional mutations after divergence, while the latter stated that ATC and WDTC are two different entities that evolve independently, as they share very few mutations; however, both groups included relatively few cases in their analyses. In the same way, in article II we investigated the tumor initiation, progression and clonal evolution in an

inducible *Braf* mutant mice model in PTC. Mice developed tumors sporadically due to spontaneous activation of cre recombinase by leakage. These tumors are oligoclonal in nature originating from single follicles. Moreover, these tumors encompass different phenotypes as seen in humans. Furthermore, exome sequencing revealed presence of additional mutations, providing further evidence for the presence of subclones. Additionally, the tumor development in mice exhibits characteristics similar to that seen in humans diagnosed with multifocal PTC. Our results suggest that these tumors diverge at an early stage and evolve independently.

Results from the expression analyses in article I & IV revealed that several pathways were deregulated in ATC. Pathways upregulated in ATC were related to cell cycle, extracellular matrix and G-protein-associated signalling. Our findings are consistent with the recently published study [76]. As these tumors are highly proliferative, this likely underlies the enrichment of cell cycle related pathways. Downregulated pathways were related to thyroid hormone synthesis, TP53 signaling pathways and cell adhesion molecules. As ATC lacked the expression of genes related to thyroid function in our study, this could be accountable for the down regulation of thyroid related pathways, while frequent somatic mutations in *TP53* resulting in loss of function could be responsible for TP53 signaling downregulation.

In articles I & III, RNA-seq data was investigated for identifying candidate chimeric transcripts in ATC. In total, forty-two fusion genes were found in both cell lines and primary tumors, all being non-recurrent. The number of fusion genes detected per case varied and a large fraction were out-of-frame fusions. However, some were in-frame fusions and a fraction of these fusions could aid in tumor progression in ATC. In our cohort of ATC cell lines and primary tumors, none of the previously described low frequency *RET* rearrangements and *ALK* fusions were detected [114, 115]. Additionally, we detected in-frame reciprocal fusion genes involving the *MLXIP*|*PTEN* and *NCOR2*|*EP400* genes. *PTEN* is known to be involved in both in-frame and out-of-frame fusions in various cancers, this subsequently results in impaired functioning of *PTEN* gene [184-186].

In article IV, we investigated genome-wide methylation profile and its impact on expression in 13 primary ATC cases. In both methylation and expression data, ATCs formed distinctive clusters from normal thyroids. A large fraction of these differentially methylated probes were hypomethylated CpG's located in intergenic regions, while a small proportion of differentially methylated probes were hypermethylated in promoter regions and gene body regions. Moreover, genome wide hypomethylation is frequently observed in cancer and is found to induce genomic instability [187], in line with our findings in article III, relating to frequent genomic instability in ATC.

We noticed aberrant DNA hypomethylation resulting in increased expression of genes that are frequently deregulated in cancer such as *HIF1A*. Interestingly, we also found oncogenes such as *MTOR* and *NOTCH1* to be hypomethylated. As *MTOR* is major

regulator of cell growth, metabolism and survival through PI3K/mTOR/AKT pathway, a few MTOR inhibitors are in clinical trials for ATC showing promising results [103]. Our findings should be explored in a larger cohort of patient samples.

Dedifferentiation is one of the salient features of ATC. We found aberrant DNA methylation in gene bodies accompanied by reduced expression of genes involved in thyroid hormone synthesis namely *TSHR* (thyroid stimulating hormone receptor) and *SLC26A7* (sodium/iodide receptor). Our observations agree with previous investigations that reported the downregulation of genes involved in thyroid function and aberrant methylation associated with *TSHR* [128, 131]. In conclusion, our results suggest that aberrant DNA methylation results in loss of thyroid function could play a vital role in the dedifferentiation process in ATC.

Taken together, our findings provide a better understanding of the complex genomic, epigenomic and transcriptomic mechanisms involved in ATC tumorigenesis. The genomic characterization of ATC cell lines and primary tumors has unraveled the presence of massive aneuploidy with frequent changes in copy number. We found a higher proportion of ATC harboring recurrent amplifications in *CCNE1* (29%) in our cohort and identified cases with amplifications in *CDK6* and *TWIST1.* In total, 36% of our patients are candidates for therapeutic interventions with CDK6 and TWIST1 targeted therapy. Clinical trials should be extended to patients with CCNE1/CDK6 amplifications using CDK4/6 inhibitors to explore the benefits of treating ATC patients with CDK4/6 inhibition therapy. Moreover, only a handful of investigations have focused on identifying intratumor heterogeneity as well as clonal evolution in ATC. Further research is required to identify the underlying processes involved in tumor evolution that could have obvious implications in clinical management of ATC.

Conclusions

The main findings from the work presented in this thesis can be summarised as follows.

- ATC are polyploid tumors displaying massive aneuploidy, with complex copy number aberrations
- ATC display genomic instability and replicative stress, possibly stemming from mitotic spindle defects
- Mutations in *TP53* and *TERT* are common in ATC followed by mutations in *BRAF* and *RAS* family genes
- Increased activation of AID/APOBEC was the common mutational signature among cell lines and primary ATC
- Downregulation of TP53-regulated genes and thyroid-related pathways in ATC could explain the metastatic potential and dedifferentiation process.
- Aberrant DNA methylation observed in primary ATC could drive tumor progression in ATC
- Aberrant methylation in *MTOR* associated with increased expression could be targeted by therapeutic interventions
- In ATC cell lines, *NEGR1* is frequently targeted and modifications in this gene may increase invasiveness and thereby promote metastasis
- Primary ATC exhibit amplifications in *CCNE1*, *CDK6* and *TWIST1* that could be suitable for targeted therapy

Populärvetenskaplig sammanfattning

Celler är den grundläggande byggstenen i människokroppen. Vi består av biljoner celler som delar sig genom en tätt reglerad process som kallas cellcykeln. Modercellens genetiska material dupliceras vanligtvis så att varje dottercell har en komplett uppsättning genetiskt material. Även om flera mekanismer arbetar för att säkerställa att DNA-replikationen utförs med hög effektivitet och noggrannhet kan ibland fel införas under denna process. Dessa förändringar i det genetiska materialet kan ge en tillväxtfördel jämfört med de omgivande cellerna, vilket i slutändan leder till omvandling av normala celler till cancerceller.

Cancer klassificeras baserat på ursprungsvävnaden. Maligna tumörer som uppstår i sköldkörteln kallas sköldkörtelcancer. Det finns flera olika typer av sköldkörtelcancer inklusive papillär sköldkörtelcancer (PTC), follikulär sköldkörtelcancer (FTC), medullär sköldkörtelcancer (MTC), lågdifferentierad sköldkörtelcancer (PDTC) och anaplastisk sköldkörtelcancer (ATC).

ATC är en sällsynt och dödlig endokrin malignitet och patienter som diagnostiserats med ATC har vanligtvis en mycket kort överlevnadstid på mindre än 6 månader. Det finns för närvarande ingen effektiv behandling för ATC. Vid diagnosen har de flesta patienter spridning av cancern till andra delar av kroppen, såsom hjärna, skelett och lungor. De komplexa underliggande mekanismerna i ATC-tumörutveckling är dåligt kända. Den här avhandlingen undersöker de genetiska förändringar som är involverade i utvecklingen av ATC. Bättre förståelse av dessa komplicerade processer kan leda till identifiering av nya behandlingsstrategier som kan förbättra överlevnaden hos patienter som diagnostiserats med ATC.

I artikel I & III undersökte vi förvärvade genetiska förändringar i ATC. Vi noterade att ATC-tumörer hade fel antal kromosomer. Dessutom hade dessa tumörer återkommande mutationer i generna *TP53*, *TERT*, *BRAF* och *RAS* familj generna. Dessutom hittade vi förändringar i *NEGR1*-genensom möjligtvis kan göra cancercellerna mer invasiva och kan påverka tumörutvecklingen. Vidare fann vi genamplifieringar i *CCNE1*, *CDK6* och *TWIST1*, som sannolikt bidrar till tumörprogression. En möjlighet är att använda sig av läkemedel som riktar sig mot dessa gener, så kallad målinriktad behandling, vilket skulle kunna hjälpa patienter med ATC.

I artikel II studerade vi tumörinitiering, progression och utveckling av PTC i en musmodell. Spontan aktivering av *Braf* leder till utveckling av olika subtyper av PTC-tumörer hos möss. Vidare identifierade vi mutationer genom DNA-sekvensering, som påvisade närvaro av mindre kloner som kan vara förknippade med tumörprogression.

I artikel IV studerade vi epigenetiska förändringar i ATC. Vi observerade låga nivåer av metylering i genomet, medan relativt högre nivåer av metylering observerades i genpromotorer och CpG-öar. Dessutom fann vi låga nivåer av DNA-metylering i *MTOR* och *NOTCH1* onkogener, som var förknippade med ökat genuttryck. Vi såg också avvikande metylering av gener relaterade till sköldkörtelfunktion, inklusive *TSHR* och *SLC26A7*, associerat med minskat uttryck. Från genuttrycksanalys fann vi att cellcykelprocesser var uppreglerade, medan signalvägar relaterade till sköldkörtelfunktion och TP53-associerade gener nedreglerades i ATC.

Avslutningsvis har resultaten av denna avhandling hjälpt oss att dechiffrera de komplexa processer som är involverade i ATC-tumorigenes. En betydande del av ATC-patienter kan vara lämpliga för målinriktad behandling, inklusive terapi med CDK- och TWIST1-hämmare.

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Article I

Genomic complexity and targeted genes in anaplastic thyroid cancer cell lines

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Abstract

Anaplastic thyroid cancer (ATC) is a highly malignant disease with a very short median survival time. Few studies have addressed the underlying somatic mutations, and the genomic landscape of ATC thus remains largely unknown. In the present study, we have ascertained copy number aberrations, gene fusions, gene expression patterns, and mutations in early-passage cells from ten newly established ATC cell lines using single nucleotide polymorphism (SNP) array analysis, RNA sequencing and whole exome sequencing. The ATC cell line genomes were highly complex and displayed signs of replicative stress and genomic instability, including massive aneuploidy and frequent breakpoints in the centromeric regions and in fragile sites. Loss of heterozygosity involving whole chromosomes was common, but there were no signs of previous near-haploidisation events or chromothripsis. A total of 21 fusion genes were detected, including six predicted in-frame fusions; none were recurrent. Global gene expression analysis showed 661 genes to be differentially expressed between ATC and papillary thyroid cancer cell lines, with pathway enrichment analyses showing downregulation of *TP53* signalling as well as cell adhesion molecules in ATC. Besides previously known driver events, such as mutations in *BRAF*, *NRAS*, *TP53* and the *TERT* promoter, we identified *PTPRD* and *NEGR1* as putative novel target genes in ATC, based on deletions in six and four cell lines, respectively; the latter gene also carried a somatic mutation in one cell line. Taken together, our data provide novel insights into the tumourigenesis of ATC and may be used to identify new therapeutic targets.

Key Words
- thyroid
- molecular genetics
- gene expression

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Introduction

Anaplastic thyroid cancer (ATC) is a rare and lethal disease, accounting for only 1–5% of all thyroid malignancies and yet being responsible for approximately 50% of deaths attributed to thyroid cancer (Kebebew *et al*. 2005). Patients presenting with this extremely aggressive tumour have a very poor prognosis, with a median survival of just 4–12 months from the time of diagnosis, compared with the high cure rates and long-term survival rates for patients diagnosed with a well-differentiated thyroid cancer, such as follicular thyroid cancer (FTC) or papillary thyroid

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cancer (PTC) (Are & Shaha 2006). ATC is characterised by rapid tumour growth and frequent metastasis, with half of patients presenting with distant metastases (Ain 1999).

The genetic basis of PTC and FTC has been quite well studied, showing frequent mutations in *RAS* genes, including *HRAS*, *KRAS* and *NRAS*. PTC also frequently display *BRAF* mutations and fusions involving *RET*, whereas FTC commonly harbour *PAX8–PPARG* rearrangements (Kimura *et al*. 2003, Nikiforova *et al*. 2003, Soares *et al*. 2003, The Cancer Genome Atlas Research Network 2014). However, few investigations have so far focused on the genomic landscape of ATC. Array comparative genome hybridisation studies have shown a high degree of structural and numerical chromosomal aberrations, with gains in chromosomal copy number being observed more frequently than losses (Wreesmann *et al*. 2002, Miura *et al*. 2003, Lee *et al*. 2007). Mutations are commonly seen in *TP53* (30–70% of cases), *BRAF* (30–45%), *NRAS* (15–20%), *USH2A* (18%) and *EIF1AX* (10–15%), as well as in the *TERT* promoter (50–70%) (Liu *et al*. 2013, Nikiforova *et al*. 2013, Kunstman *et al*. 2015, Jeon *et al*. 2016, Landa *et al*. 2016). To date, there have been few reports of fusion genes in ATC, with only three cases with *RET* fusions, two cases with *ALK* fusions and single cases with *NUTM1/BRD4*, *SS18/SLC5A11*, *MKRN1/BRAF*, and *FGFR2/OGDH* fusions reported in the literature (Liu *et al*. 2008, Kelly *et al*. 2014, Godbert *et al*. 2015, Kasaian *et al*. 2015, Landa *et al*. 2016), with no large-scale screening using RNA sequencing being reported.

In the present study, we investigated the genetic landscape of ATC cell lines using SNP array analysis, RNA sequencing and whole exome sequencing (WES). We found that ATC cell lines display highly complex genomes, with multiple breakpoints and large variation in copy numbers. We identified not previously implicated genes that are targeted by recurrent deletions and mutations. In addition, we found several novel in-frame gene fusions that could result in translated protein products affecting the development of ATC. Our results increase our understanding of the aetiology of this extremely aggressive disease and may be used to identify new therapeutic targets.

Materials and methods

Samples

A total of 13 newly established, low-passage thyroid cancer cell lines were included in the study (Wennerberg *et al*. 2014, Gretarsson *et al*. 2016). Ten cell lines established from patients diagnosed with ATC were ATC1 (LU-TC-1), ATC2 (LU-TC-2), ATC7 (LU-TC-7), ATC8 (LU-TC-8), ATC10 (LU-TC-10), ATC12 (LU-TC-12), ATC14 (LU-TC-14), ATC15 (LU-TC-15), ATC17 (LU-TC-17) and ATC18 (LU-TC-18), and three cell lines established from patients diagnosed with PTC were PTC4 (LU-TC-4), PTC5 (LU-TC-5) and PTC13 (LU-TC-13). For the establishment of the cell lines, patients referred to the Departments of Oncology, ENT/H&N Surgery or Endocrine Surgery at the University Hospital in Lund, Sweden, for treatment of previously untreated ATC or PTC were asked for participation in the study. Tissue sampling was performed with a conventional fine-needle aspiration technique using a 0.6–0.7 mm needle. The aspirates were directly transferred to RPMI 1640 medium with stable glutamine, supplemented with 1 mmol/L sodium pyruvate, 1× MEM non-essential amino acids, 20 µg/mL gentamicin and 10% foetal bovine serum (FBS) gold (GE Healthcare). The suspensions were immediately transferred to cell culture flasks and left to attach and grow at 37°C under a humidified atmosphere with 5% CO_2. The cells were sequentially transferred to a new flask until visibly free from fibroblasts. Cytogenetic analysis of the cell lines showed polyploid and highly complex karyotypes in all cases. RNA and DNA were extracted from the cell lines using the RNeasy Mini Kit and Gentra Puregene Cell Kit (Qiagen), respectively, according to the manufacturer's instructions. Two matched peripheral blood samples for ATC17 and ATC18 and three normal thyroid tissue samples, collected from patients undergoing a routine thyroidectomy, were also included in the study. DNA was extracted from the peripheral blood samples using the Gentra Puregene Blood Kit (Qiagen). For the tissue samples, sections of tissue measuring no more than 5 mm across one dimension were immediately transferred to a vial containing either RNAlater RNA Stabilization Solution or Allprotect Tissue Reagent (Qiagen) and stored overnight at 4°C, before removal from solution and long-term storage at –80°C. RNA was extracted from the tissue samples using the RNeasy Lipid Mini Kit (Qiagen). The study was approved by the Ethical Review Board of Lund University, reference number 522/2008, and informed consent was provided according to the Declaration of Helsinki.

SNP array analysis

SNP array analysis of all ATC cell lines except ATC17 and ATC18 and all three PTC cell lines was performed using the Illumina HumanOmni5-Quad BeadChip platform,

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containing ~5 million markers (Illumina), according to the manufacturer's instructions. SNP array analysis of ATC17 and ATC18 and their matched normal control samples was performed with the Affymetrix Genome-Wide SNP Array 6.0 (Affymetrix) according to the manufacturer's instructions. Probe positions were extracted from the GRCh37 genome build and data was analysed using the Genome studio v2011.1 (Illumina) and Nexus Copy Number 7.5 (BioDiscovery, El Segundo, CA, USA) software. For ATC17 and ATC18, constitutional copy number variants were excluded based on comparison with the matched control sample. For the remaining cases, all copy number changes <1 Mb were compared with copy number polymorphisms listed in the Database of Genomic Variants (http://dgv.tcag.ca/dgv/app/home) or Nexus and excluded from further analysis if there was substantial overlap. Regions displaying loss of heterozygosity (LOH) were included as aberrant if they comprised at least 5 Mb or were part of another rearrangement.

RNA sequencing

RNA sequencing was performed for all ATC cell lines, except ATC17 and ATC18, and all PTC cell lines. Paired-end RNA libraries were constructed using the TruSeq RNA Sample Preparation Kit v2 (Illumina) and sequenced on the Illumina HiSeq2000 platform according the manufacturer's instructions by BGI Tech Solutions (Hong Kong) (Supplementary Table 1, see section on supplementary data given at the end of this article). The data has been submitted to the Gene Expression Omnibus database (GEO; https://www.ncbi.nlm.nih.gov/geo) and is available under accession number GSE94465.

Fusion gene analysis

Potential fusion genes were identified from the RNA-sequencing data using SOAPfuse version 1.26 (http://soap.genomics.org.cn/soapfuse.html), Chimerascan version 0.4.5 (http://code.google.com/p/chimerascan) and TopHat version 2.0.7 (http://ccb.jhu.edu/software/tophat/fusion_index.html). The GRCh37 build was used as the human reference genome. Potential fusion genes for further investigation were identified using a filtering pipeline based on spanning/total read number and previous identification of SNP events. Briefly, the output list of fusion genes was first filtered to remove chimeras identified as read-through transcripts before filtering to generate two lists of potential fusion genes containing (1) chimeras with one or more spanning reads, and (2) chimeras with five or more total reads. The lists were then both filtered to remove pseudogenes, unannotated genes and fusions between gene family members. Finally, chimeras containing gene partners that were less than 100 kb apart in distance were discarded unless an imbalance was detected by SNP array analysis in one or both of the fusion partners. Also, the *C15orf57–CBX3* fusion, seen in three samples (ATC2, ATC12 and PTC4), was excluded from the results as it has been shown to result from a retrotransposition insertion in a substantial proportion of individuals (Schrider *et al.* 2013). To validate potential fusion genes, RT-PCR was performed in the corresponding cell line and three normal thyroid tissue samples. Briefly, cDNA was generated from 2.5 µg of RNA, and primers (available on request) specific to potential fusion transcripts were designed using Primer 3 (http://primer3.ut.ee/). PCR was performed according to standard methods and amplified products were sequenced using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). All cell lines were subsequently screened with RT-PCR for fusion genes that were validated in this way.

Gene expression analysis

Gene expression levels based on RNA sequencing for all ATC cell lines except ATC17 and ATC18, and all PTC cell lines, were estimated using the TCGA UNC V2 RNA-Seq Workflow (https://webshare.bioinf.unc.edu/public/mRNAseq_TCGA/UNC_mRNAseq_summary.pdf). The expected read counts in the '.genes.results' files generated by RSEM were used as the input for DESeq2 (Love *et al.* 2014), limma–voom (Law *et al.* 2014) and edgeR (Robinson *et al.* 2010), respectively. Differentially expressed genes were defined as those with a fold change >2 and FDR <0.01. Functional enrichment of differentially expressed genes in the KEGG pathways was performed by KOBAS (version 2.0) software (Xie *et al.* 2011), and KEGG pathways with *P* value <0.01 were considered significantly enriched.

Whole exome sequencing

WES was performed on all ten ATC cell lines and matched normal blood for ATC17 and ATC18 to an average read depth of ~200× (Supplementary Table 2). DNA libraries were constructed using the V5 50M Exon Kit (Agilent Technologies) and sequenced on the Complete Genomics

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sequencing platform according the manufacturer's instructions by BGI Tech Solutions (Hong Kong). Raw read data were aligned using Teramap and variants were identified using a pipeline designed and implemented by BGI Tech Solutions (Hong Kong). The eight ATC cell lines without a matched normal blood sample were filtered against genetic variations reported in the 1000 Genomes Project, 6500 Exome Project, the Single Nucleotide Polymorphism Database (dbSNP) Build 129, and RefSeq: NCBI Reference Sequence Database and then evaluated only for known gene mutations previously reported in ATC and PTC. Somatic gene mutations were identified in cell lines ATC17 and ATC18 using a pipeline designed and implemented by BGI Tech Solutions (Hong Kong). The mutational signatures in ATC17 and ATC18 were investigated using MutSigCV (Lawrence *et al*. 2013). The data has been submitted to the Sequence Read Archive (https://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?view=announcement) and is available under accession number SRP098778.

Analysis of *TERT* promoter mutations and validation of mutations

Mutations in the *BRAF* gene were validated using a standard PCR and primers designed for exon 15 as previously reported (Davidsson *et al*. 2008): BRAFEx15 For: TGCTTGCTCTGATAGGAAAATGAG and BRAFEx15 Rev: TCTCAGGGCCAAAAATTTAATCA. Mutations in the promoter region of the *TERT* gene were validated using a standard PCR as previously reported by Liu and coworkers (Liu *et al*. 2013) using the AmpliTaq Gold 360 Master Mix (Thermo Fisher Scientific). Validation of mutations detected by whole exome sequencing in the genes *TP53*, *NRAS*, *PTEN*, *PIK3CA*, *NEGR1* and *EIF1AX* was done using Sanger sequencing according to standard methods (primers available upon request).

Fluorescence in situ hybridisation (FISH) analysis

In order to investigate whether the *NEGR1* deletions were associated with chromosomal rearrangements, metaphase FISH analysis was performed according to standard methods in cell lines ATC7, ATC8, ATC10 and ATC15. Probes used were the bacterial artificial chromosomes RP11-115M14 (distal of *NEGR1*), RP11-82L20 (in 3′ part of *NEGR1*; includes exon 7), RP11-566G9 (in intron 1 of *NEGR1*) and RP11-292O17 (proximal of *NEGR1*), obtained from the BACPAC Resource Center (https://bacpacresources.org/).

RT-PCR for *NEGR1*

RT-PCR to detect *NEGR1* transcripts were done using primers in exons 1a and 7 in all ten ATC cell lines, the three PTC cell lines, three normal thyroid tissue samples, and the Human Total RNA Master Panel II (Clontech Laboratories), containing RNA from twenty normal tissues. Complementary DNA was produced from 2.5 µg RNA using M-MLV reverse transcriptase (Invitrogen) and random hexamers (Invitrogen). The primers (R_exon 1: GATGGTCAGAAAAGGGGACA, F_exon 7: TCTCCTGCTGGTACCTTGTG, Life Technologies, Invitrogen) for *NEGR1* were designed using Primer3 software (http://bioinfo.ut.ee/primer3/). PCR was done according to standard methods and amplified fragments were cut out from the gel and sequenced using the BigDye v1.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI-3130 Genetic Analyzer (Applied Biosystems). Chromas Lite 2.1.1, free online software (http://technelysium.com.au/), was used for the analysis of the *NEGR1* sequence data. In addition, RNA-Seq data was screened for junctions between exons and compared with the RT-PCR data.

Results

ATC cell lines have complex genomes and display overt signs of chromosomal instability

The SNP array analysis of ten ATC cell lines revealed highly complex genomes with multiple breakpoints and large variations in copy number within each case (Fig. 1A and B). The median number of breakpoints, defined as a change in copy number state, was 49.5 (range 9–167) (Table 1; Supplementary Table 3). There was no evidence of chromothripsis. Many of the identified breakpoints (36/601; 6.0%) were located in the centromeric regions. Breaks were most common in the centromeric regions of chromosomes 8 (5/10 cell lines; 50%), 3, 4 and 9 (4/10; 40% each), and 1, 2 and 17 (3/10; 30% each). ATC10 had the highest number of centromere breaks (n=9), while ATC 1, 2 and 7 only had one each.

Another common feature of the ATC cell lines was LOH involving whole chromosomes (wcLOH), with 5/10 cell lines having one or more wcLOH. Chromosomes 13 (4/10 cases; 40%), 18 (4/10 cases; 40%) and 17 (3/10 cases; 30%) were most commonly affected (Fig. 1C; Supplementary Table 4). However, wcLOH was only seen for a minority of chromosomes in any given case; thus, no case displayed evidence of duplication of a near-haploid stem line (Fig. 1C).

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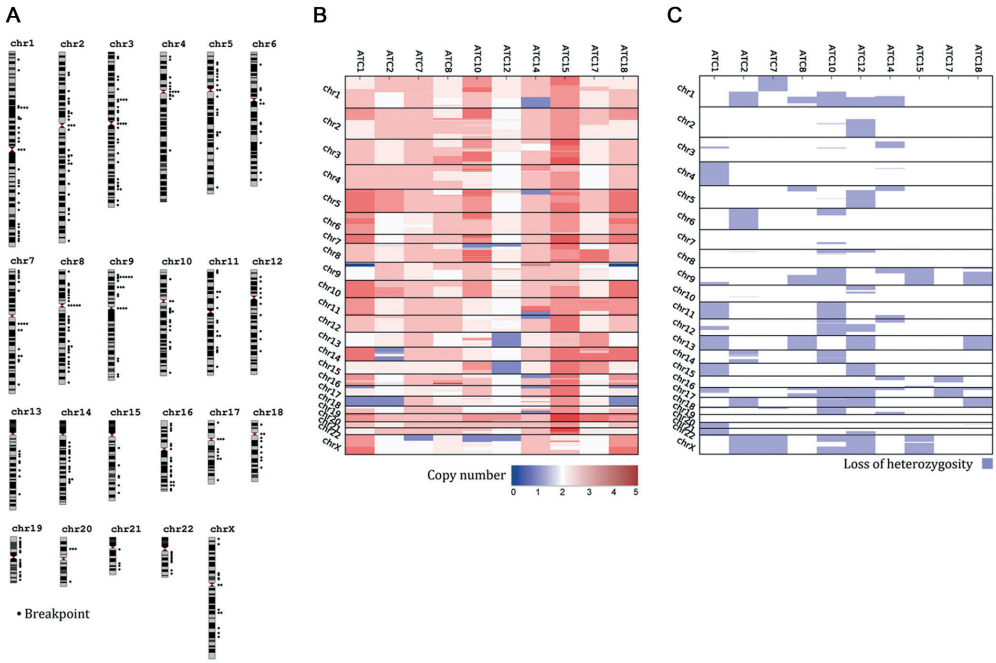

Figure 1
Many breakpoints and massive aneuploidy in ATC cell lines. (A) Breakpoint map of ATC cell lines. Breakpoints were defined as a change in copy number. A large number of breakpoints in centromeric regions were seen. (B) Heatmap of copy number changes in ATC cell lines. All cases displayed massive aneuploidy. (C) Overview of regions displaying loss of heterozygosity (LOH). Note that cell lines ATC2, ATC12 and ATC15 were established from male patients; hence the LOH.

There were a total of 157 focal (<1 Mb) regions of copy number gain and loss among the ten cell lines (median 12.5; range 2–40), with the most common type of alteration being hemizygous loss (n=123), followed by homozygous loss (n=14) (Supplementary Table 4).

Table 1 Summary of breakpoints in ten anaplastic thyroid cancer cell lines.

Cell line	Number of centromeric breakpoints* (%)	Total number of breakpoints*
ATC1	1 (1.0)	105
ATC2	1 (5.6)	18
ATC7	1 (11)	9
ATC8	6 (7.6)	79
ATC10	9 (5.4)	167
ATC12	4 (9.1)	44
ATC14	3 (8.1)	37
ATC15	7 (13)	55
ATC17	2 (6.7)	30
ATC18	2 (3.5)	57

*As determined by a change in copy number.

Regions of focal copy number gains were rare (n=20), with most of the gained regions affecting more than one gene; none of these regions were recurrent (Supplementary Table 4). There were no recurrent amplifications or regions of high copy number gain.

ATC cell lines harbour multiple out-of-frame fusion genes

A total of 21 fusion genes were found and validated among six ATC cell lines (Table 2; Supplementary Fig. 1). Six (29%) of these were expected to produce an in-frame transcript resulting in a protein product (Table 2). In addition, three fusion genes, including two in-frame chimeras, were seen in the three PTC cell lines (Table 2). Of the validated fusion genes, ten (42%) were identified by only one of the fusion transcript identifier programmes, whereas five (21%) were identified by all three programmes (Table 2; Supplementary Fig. 2).

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Table 2 Validated fusion genes identified in the ATC and PTC cell lines.

Cell line	Fusion gene	Chromosome	Inframe/ frame-shift	Partner gene previously reported in fusion gene*	Software identifying fusion
ATC1	*MYBL1/VCPIP1*	8q13.1/8q13	Frame-shift	*MYBL1* - Diffuse large B-cell lymphoma - Astrocytoma, pilocytic/juvenile (brain)	SoapFUSE, Chimerascan
ATC1	*PPP6R2/CACNA1A*	22q13.33/19p13	Frame-shift		SoapFUSE, TopHat
ATC1	*SPOP/TBX21*	17q21.33/17q21.32	Frame-shift		SoapFUSE, Chimerascan
ATC1	*AP2A2/TALDO1*	11p15.5/11p15.5-p15.4	Frame-shift	*AP2A2* - Acute myeloid leukaemia, NOS	SoapFUSE
ATC1	*DDAH1/ZNHIT6*	1p22/1p22.3	Frame-shift		SoapFUSE, Chimerascan, TopHat
ATC1	*PEX14/KIF1B*	1p36.22/1p36.2	Frame-shift		Chimerascan
ATC1	*PIAS3/XPR1*	1q21/1q25.1	Inframe		SoapFUSE, Chimerascan, TopHat
ATC1	*PLAU/SEC24C*	10q22.2/10q22.2	Frame-shift		Chimerascan
ATC2	*KIF20B/PRKG1*	10q23.31/10q11.2	Frame-shift	*PRKG1* - Adenocarcinoma (lung)	SoapFUSE, Chimerascan, TopHat
ATC2	*ATAD1/SOX5*	10q23.31/12p12.1	Frame-shift		SoapFUSE, Chimerascan
ATC7	*RGS17/HBS1L*	6q25.3/6q23.3	Frame-shift	*RGS17* - Adenocarcinoma (breast)	SoapFUSE, Chimerascan, TopHat
ATC8	*TANGO6/CDH13*	16q22.1/16q23.3	Inframe	CDH13 - Adenocarcinoma (breast) - Adenocarcinoma (lung)	Chimerascan, TopHat
ATC8	*ZNF76/PPARD*	6p21.31/6p21.2	Frame-shift		SoapFUSE, Chimerascan
ATC8	*TMCC1/PTPRG*	3q22.1/3p21-p14	Inframe	*TMCC1* - Squamous cell carcinoma (lung) - Adenocarcinoma (breast) *PTPRG* - Adenocarcinoma (breast)	Chimerascan
ATC10	*TTYH3/BRAT1*	7p22/7p22.3	Frame-shift		SoapFUSE, Chimerascan
ATC10	*MMS19/SLIT1*	10q24-q25/10q23.3-q24	Frame-shift		Chimerascan
ATC14	*KDM4B/PLIN4*	19p13.3/19p13.3	Inframe		SoapFUSE, Chimerascan
ATC14	*ZFP14/BIRC2*	19q13.12/11q22	Inframe		SoapFUSE
ATC14	*SAFB/GPI*	19p13.3-p13.2/19q13.1	Frame-shift		SoapFUSE
ATC14	*TMEM241/RPS12*	18q11.2/6q23.2	Frame-shift	*TMEM241* - Adenocarcinoma (breast)	SoapFUSE
ATC14	*DYNC2H1/CASP12*	11q21-q22.1/11q22.3	Inframe	*CASP12* - Squamous cell carcinoma (lung)	Chimerascan
PTC4	*MERTK/TG*	2q14.1/8q24	Inframe	TG - Adenocarcinoma (thyroid)	SoapFUSE, Chimerascan, TopHat
PTC4	*TOX4/TERT*	14q11.2/5p15.33	Frame-shift	*TERT* - Astrocytoma, grade III-IV (brain) - Clear cell carcinoma (kidney) - Chronic lymphocytic leukaemia - Acute lymphoblastic leukaemia/ lymphoblastic lymphoma	SoapFUSE, Chimerascan
PTC13	*STIM1/PGAP2*	11p15.5/11p15.5	Inframe	*STIM1* - Adenocarcinoma (large intestine) - Acute myeloid leukaemia, NOS	Chimerascan

*Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer (2016). Mitelman F, Johansson B and Mertens F (Eds.), http://cgap.nci.nih.gov/Chromosomes/Mitelman.
ATC, anaplastic thyroid cancer; PTC, papillary thyroid cancer.

Multiple target genes identified in ATC cell lines

The SNP array analysis revealed several target genes in ATC cell lines, based on small targeted deletions or breakpoints involving only one gene in more than one case. The deletions comprised *PTPRD* in 6/10 cell lines (60%); *NEGR1*, *AUTS2* and *FHIT* in 4/10 (40%) each; *CDKN2A* and *MACROD2* in 3/10 (30%) each; and *CNTNAP2*, *DLG2*, *IMMP2L*, *LRP1B*, *METTL15* and *NAALADL2* in 2/10 (20%) each (Fig. 2; Supplementary Table 4). Most of these deletions were hemizygous, but homozygous deletions were also observed (Supplementary Table 2). In addition,

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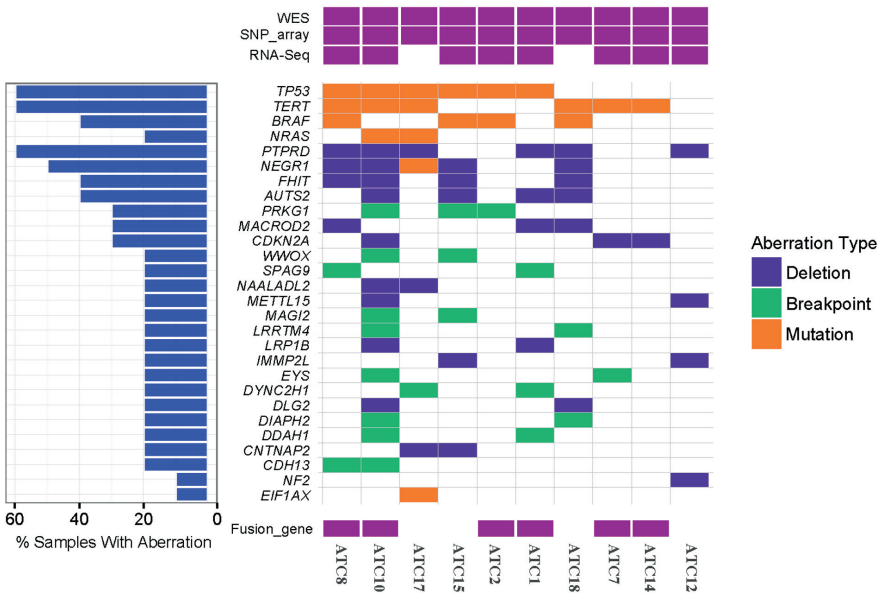

Figure 2
Genomic profile of the ten ATC cell lines. The panels display the genetic analysis performed on the ATC cell lines, the somatic gene mutations, gene deletions and breakpoints detected in the ATC cell lines, and fusion genes identified and validated by RT-PCR in the ATC cell lines.

breakpoints were recurrently seen in the genes *PRKG1* (3/10 cell lines; 30%) and *CDH13*, *DDAH1*, *DIAPH2*, *DYNC2H1*, *EYS*, *LRRTM4*, *MAGI2*, *SPAG9* and *WWOX* (2/10; 20% each) (Fig. 2; Supplementary Table 3).

Mutations

TP53 mutations were seen in six (60%) ATC cell lines, *BRAF* p.V600E mutations in four (40%), *NRAS* mutations in two (20%), and an *EIF1AX* mutation in one (10%) cell line (Fig. 2; Supplementary Tables 5 and 6). Mutations in the promoter region of the *TERT* gene were identified in 6/10 (60%) ATC cell lines; all were C228T mutations. All mutations in the above-mentioned genes were validated by Sanger sequencing (data not shown). For ATC17 and ATC18, where constitutional blood samples were available for identification of somatic mutations, the whole exome was analysed, showing a total of 71 somatic mutations in coding regions in ATC17 and 137 in ATC18 (Supplementary Table 6). ATC17 did not show a clear mutational signature, whereas ATC18 displayed predominant mutations in TpCpN trinucleotides, indicating the involvement of the APOBEC family of cytidine deaminases (Supplementary Fig. 3).

TP53 signalling and cell adhesion molecules are downregulated in ATC cell lines

Gene expression analysis of the RNA-sequencing data identified 661 genes differentially expressed between the ATC cell lines and PTC cell lines (Q value <0.01); 421 genes were downregulated in ATC and 240 genes were upregulated (Supplementary Table 7). Pathway enrichment analysis of the differentially expressed genes identified five significant pathways ($P<0.01$): the *TP53* signalling (KEGG hsa04115), the axon guidance (KEGG hsa04360), the cell adhesion molecules (CAMs) (KEGG hsa04514) and the autoimmune thyroid disease (KEGG hsa05320) pathways were downregulated, whereas the haematopoietic cell lineage pathway (KEGG hsa04640) was upregulated.

NEGR1 is a putative tumour suppressor gene involved in a high proportion of ATC cell lines

SNP array analysis showed that interstitial deletions in the *NEGR1* gene were present in 4/10 (40%) ATC cell lines. These deletions frequently occurred in a stepwise fashion (from a baseline of 3 to 5 copies of chromosome arm

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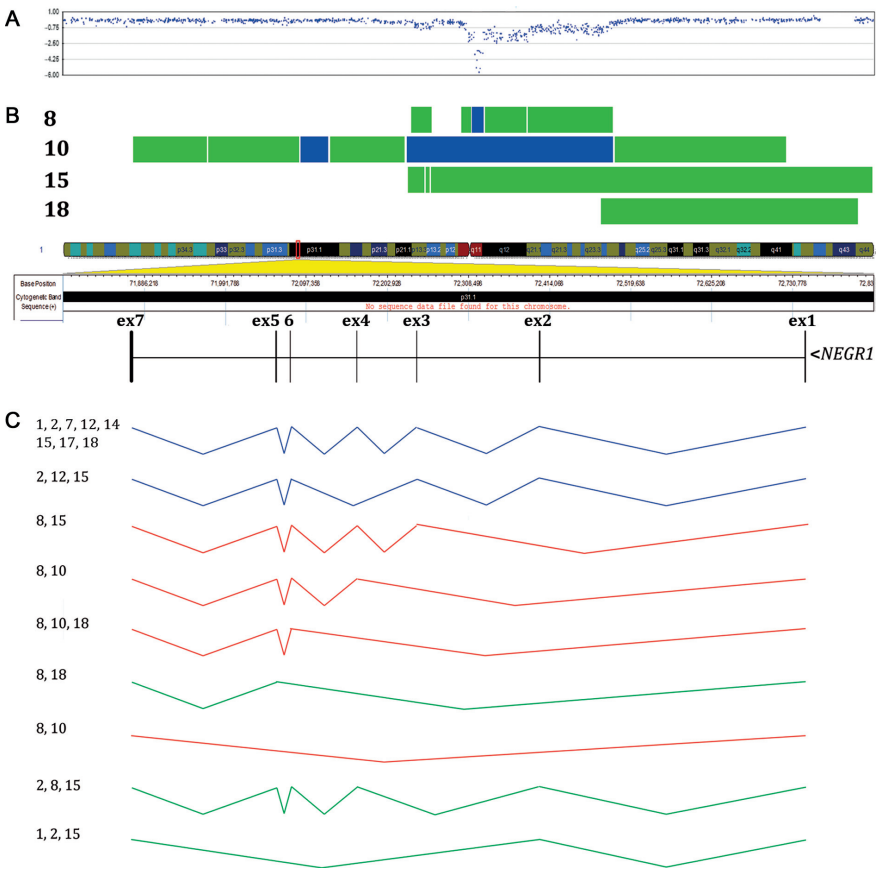

Figure 3
Deletions at the *NEGR1* locus at 1p31.1 in anaplastic thyroid cancer cell lines. (A) SNP array data from ATC8. LogR ratios are shown on the *Y* axis; each dot corresponds to one marker. Stepwise deletions are seen. (B) Deletions in the cell lines ATC8, ATC10, ATC15 and ATC18. Hemizygous deletions are shown in green; homozygous deletions in blue. (C) Splice variants of *NEGR1* detected in ATC cell lines. Two normal splice variants (NEGR1-001 and NEGR1-201 in GRCh37.p13, www.ensembl.org) are shown in blue. Of these, NEGR1-001 contains exons 1–7 and is detected in all cell lines except ATC8 and ATC10, whereas NEGR1-201 contains exons 1–3 and 5–7, and is seen in cell lines ATC2, ATC12 and ATC15. Aberrant splice variants are shown in red (out-of-frame) and green (in-frame). Cell lines harbouring the splice variants as detected by RT-PCR and RNA sequencing are shown to the left.

1p), involving different parts of the gene, but included exons 2 and 3 in 3/4 cell lines (Fig. 3). The deletions were hemizygous (i.e., at least one unaffected allele was still present) in all cell lines except ATC10, in which exons 2 and 3 were homozygously deleted. No rearrangements involving *NEGR1* were detected with metaphase FISH (Supplementary Table 8). In addition, ATC17 harboured a somatic p.V156I mutation in *NEGR1*. This mutation occurred in a position between two of the Ig-like domains, but we could not predict its effects since the structure of the NEGR1 protein has not been determined. There are three normal splice variants of *NEGR1*: NEGR1_001, including exons 1a–7; transcript NEGR1_002, including exons 1b–7; and NEGR1_201, including exons 1a–3 and 5–7. RNA-Seq did not show any overall difference in the expression level of *NEGR1* between ATC and PTC, but it showed the complete absence of expression of alternative exon 1b in all cell lines, excluding the expression of NEGR1_002. To further delineate the expression of *NEGR1*, RT-PCR was performed across the gene. Apart

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from the full-length transcript NEGR1_001, which was present in all cell lines except ATC8 and ATC10, and splice variant NEGR1_201, which was present in ATC2, ATC12 and ATC15, this analysis showed multiple extra transcripts in all cell lines with deletions (Fig. 3C and Supplementary Fig. 4A) as well as in ATC1 and ATC2 that lacked deletions detectable by SNP array analysis. Sanger sequencing showed that all these extra transcripts were the results of aberrant splicing of full exons, resulting in both in-frame and out-of-frame transcripts; at least one out-of-frame transcript predicted to lead to a truncated protein was present in all cases with deletion, as well as in ATC1 and ATC2 (Fig. 3C). To check whether these extra transcripts could be normal splice variants of *NEGR1*, we performed RT-PCR using the same primers in the three PTC cell lines, three samples from normal thyroid glands, and a tissue panel containing mRNA from 20 different tissues. The full-length NEGR1_001 transcript was seen in all investigated tissues and splice variant NEGR1_002 in prostate and foetal brain; however, no other transcripts were detected (Supplementary Fig. 4B). Taken together, our data indicate that focal deletions result in aberrant splicing of *NEGR1* in ATC, possibly leading to lower levels of normal NEGR1 protein.

Discussion

ATC has a close to 100% short-time mortality and there is no curative treatment for patients with disseminated disease (Kebebew *et al.* 2005, Are & Shaha 2006). Thus, there is an urgent need to identify possible targets for treatment. Considering that ATC is one of the most malignant diseases, surprisingly little is known about its underlying genomic landscape. Most studies have included small cohorts and have been focused on only one type of genetic aberration, such as copy number changes or sequence mutations. In the present study, we performed a full genomic characterisation, comprising parallel SNP array analyses, RNA sequencing and WES, to delineate all driver events in early-passage cells from ten new ATC cell lines. A potential problem with working with cell lines is that the cells may have acquired changes during culture that were not present in the primary tumour. Although we cannot exclude that some of the aberrations detected here were acquired during culture, we think that the majority were present in the primary tumour, based on (1) the cells analysed here were from early passages (passages 3–5), (2) the frequencies of well-known mutations, such as *NRAS*, *BRAF* and *TP53* mutations as well as *TERT* promoter mutations, in our cohort closely resemble reports of primary ATC, and (3) several of the potential new driver events identified here, in particular *PTPRD* and *NEGR1* deletions, were seen in a high proportion of the investigated cell lines. Thus, the abnormalities reported here are likely to be largely representative of primary ATC.

All ten investigated cell lines were polyploid and showed complex copy number aberration patterns, with frequent stepwise deletions and gains (Figs 1 and 3) and massive aneuploidy. Deletions were frequently seen at known fragile sites, indicating replicative stress (Bignell *et al.* 2010). There were a high number of breakpoints (median 49.5; range 9–167) in all cases as ascertained by a change in copy number; these numbers are likely an underestimate since balanced rearrangements will not be detected. Notably, the breakpoints were frequently in pericentromeric regions (Fig. 1A), similar to what has been reported previously in, e.g., colorectal cancer and head and neck squamous cell carcinomas (Stewenius *et al.* 2005). Such breaks could either result from telomere shortening leading to breakage-fusion-bridge cycles, which has been suggested to preferentially induce pericentromeric breaks, or from mitotic spindle defects leading to centromere shearing; either way, they are associated with chromosomal instability (Stewenius *et al.* 2005, Guerrero *et al.* 2010). There is no data in the literature on telomere lengths in ATC, but considering that *TERT* promoter mutations, leading to telomerase expression and presumably preventing telomere shortening, are common (found in 60% of the cell lines investigated here) it is likely that the underlying cause of the pericentromeric breaks in ATC cell lines is a malfunctioning mitotic spindle. Corver and coworkers (Corver *et al.* 2012) reported that massive loss of chromosomes, i.e., near-haploidisation, was common in FTC. Although LOH across whole chromosomes and large regions was frequently seen in the ten ATC cell lines, it always involved a minority of the chromosomes and was hence not indicative of a previous near-haploid step; we can thus exclude that this mechanism is common in ATC. Thus, on the chromosomal scale, the overall pattern of copy number changes and breakpoints detected in this study suggest that ATC exhibits replicative stress as well as genomic instability resulting from mitotic spindle defects. Considering that PTC generally appear to be relatively genomically stable (The Cancer Genome Atlas Research Network 2014), the induction of genomic instability could be one of the major factors leading to the development of ATC from a prior, more differentiated tumour.

Six of the eight investigated ATC cell lines harboured at least one fusion gene, with ATC1 displaying eight

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different chimeric genes. None of the fusion genes were recurrent and none have previously been described in ATC, although twelve of the fusion partner genes identified have previously been reported as fused to other partner genes in various solid tumours and haematological malignancies (Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer (2016). Mitelman F, Johansson B and Mertens F (Eds.), http://cgap.nci.nih.gov/Chromosomes/Mitelman). No cell line harboured any *RET* fusions, *ALK* fusions, or *NUTM1/BRD4*, *SS18/SLC5A11*, *MKRN1/BRAF* or *FGFR2/OGDH*, which has previously been reported to occur at a low frequency in ATC (Liu *et al*. 2008, Kelly *et al*. 2014, Godbert *et al*. 2015, Kasaian *et al*. 2015, Landa *et al*. 2016). Thus, our data suggest that there is no common fusion gene that drives ATC development. The high number of fusion genes detected in this study could be due to the genomic instability that generates many chromosomal breaks and some of them could thus be passenger events, in particular the out-of-frame fusions. However, considering that in-frame fusion genes are frequently potent driver events, a subset of the fusions identified here are likely to be involved in tumourigenesis.

In contrast to the fusion genes, there were several regions of recurrent focal deletions involving only one gene (Fig. 2; Supplementary Table 4), including the well-known tumour suppressor genes *PTPRD* (60%), *FHIT* (40%) and *CDKN2A* (30%). Several reports have shown previously that *FHIT* and *CDKN2A* are involved in thyroid tumourigenesis (Chang *et al*. 1998, Elisei *et al*. 1998, Zou *et al*. 1999, Lee *et al*. 2008), whereas the involvement of *PTPRD* in ATC is a novel finding. *PTPRD* encodes a receptor protein tyrosine phosphatase that is deleted in a wide range of tumours, such as neuroblastoma and glioblastoma; these deletions are believed to lead to STAT3 hyperactivation and thereby promote tumourigenesis (Molenaar *et al*. 2012, Ortiz *et al*. 2014). Another commonly targeted gene was *NEGR1* in 1p31.1, which displayed intragenic deletions in four cases and a non-synonymous mutation in an additional case; thus, in total 50% of the investigated cell lines had somatic aberrations in this gene. *NEGR1*, also known as *KILON*, encodes a glycosylphosphatidylinositol (GPI)-anchored membrane protein involved in cell adhesion (Funatsu *et al*. 1999, Kim *et al*. 2014). It functions in neuronal development (Pischedda *et al*. 2014, Sanz *et al*. 2015), and constitutional variants affecting the expression of this gene have consistently been associated with obesity in genome-wide association studies (Willer *et al*. 2009, Wheeler *et al*. 2013). However, *NEGR1* is expressed in most tissues (www.genecards.org and Supplementary Fig. 4B). *NEGR1* has previously been reported to be somatically deleted in neuroblastoma and frequently downregulated in cancer (Takita *et al*. 2011, Kim *et al*. 2014). Knockdown of this gene was shown to promote cell migration and invasion in an ovarian adenocarcinoma cell line, suggesting that it may function as a tumour suppressor gene (Kim *et al*. 2014). Although we could not detect an overall lower expression of *NEGR1* in cell lines with deletions compared with cell lines lacking such deletions, two cell lines (ATC8 and ATC10) lacked the full-length transcript (Supplementary Fig. 4A). In addition, RT-PCR across the gene showed multiple abnormal transcripts in all cell lines with deletions as well as in ATC1 and ATC2, which did not have any visible *NEGR1* deletion as ascertained by SNP array analysis (Fig. 1 and Supplementary Fig. 3). These transcripts arose from aberrant splicing of whole exons and were both in-frame and out-of-frame (Fig. 3C). Thus, although the overall expression of *NEGR1* may not be lowered by the deletions, they may lead to lesser amounts of functional protein. In contrast, the three PTC cell lines without deletions and a panel of 20 normal tissues, including thyroid gland, only showed full-length *NEGR1* transcripts (Supplementary Fig. 4). Taken together, our data suggest that deletion of *NEGR1*, leading to aberrant splicing, may be a frequent driver event in ATC. Considering that *NEGR1* is believed to function in cell adhesion, these deletions may increase invasiveness and thereby promote metastasis.

Global gene expression analyses based on the RNA-sequencing data showed similar results to a recent investigation by Kasaian and coworkers (Kasaian *et al*. 2015), with downregulation of *TP53*-regulated genes and cell adhesion molecules in ATC cell lines. The former is likely associated with the high frequency of inactivating *TP53* mutations in this cohort, whereas the latter agree well with the high metastatic potential of ATC.

Taken together, we present copy number, transcriptional and mutational data from ten newly established ATC cell lines, likely to closely resemble primary ATC in terms of the genomic landscape. We found a high degree of genomic complexity and overt signs of genomic instability that likely contribute to the malignant phenotype of these tumours. No common fusion gene was found. Besides confirming that mutations in *TP53*, *NRAS*, *BRAF* and the *TERT* promoter are common, our findings suggest that *PTPRD* and *NEGR1* deletions frequently may drive tumourigenesis in ATC.

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Supplementary data
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Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
K P, J W and L E conceived the study; E L W, A B, N R, M Y, L E and J W analysed the data; E L W, A B and K P wrote the manuscript, which was approved by all co-authors.

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Article II

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Identification of Targetable Lesions in Anaplastic Thyroid Cancer by Genome Profiling

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Abstract: Anaplastic thyroid cancer (ATC) is a rare and extremely malignant tumor with no available cure. The genetic landscape of this malignancy has not yet been fully explored. In this study, we performed whole exome sequencing and the RNA-sequencing of fourteen cases of ATC to delineate copy number changes, fusion gene events, and somatic mutations. A high frequency of genomic amplifications was seen, including 29% of cases having amplification of *CCNE1* and 9% of *CDK6*; these events may be targetable by cyclin dependent kinase (CDK) inhibition. Furthermore, 9% harbored amplification of *TWIST1*, which is also a potentially targetable lesion. A total of 21 fusion genes in five cases were seen, none of which were recurrent. Frequent mutations included *TP53* (55%), the *TERT* promoter (36%), and *ATM* (27%). Analyses of mutational signatures showed an involvement of processes that are associated with normal aging, defective DNA mismatch repair, activation induced cytidine deaminase (AID)/apolipoprotein B editing complex (APOBEC) activity, failure of DNA double-strand break repair, and tobacco exposure. Taken together, our results shed new light on the tumorigenesis of ATC and show that a relatively large proportion (36%) of ATCs harbor genetic events that make them candidates for novel therapeutic approaches. When considering that ATC today has a mortality rate of close to 100%, this is highly relevant from a clinical perspective.

Keywords: anaplastic thyroid cancer; whole exome sequencing; RNA-sequencing; formalin-fixed paraffin embedded tissues; fusion genes; somatic mutations; copy number alterations; *CCNE1*

1. Introduction

Anaplastic thyroid cancer (ATC) is an extremely aggressive tumor, with close to 100% mortality and no available cure [1–4]. The lack of curative treatments for ATC makes it important to understand the underlying tumorigenesis of this disease; however, the genomic landscape of ATC has not yet been fully delineated.

Cytogenetic analysis and array comparative genome hybridization (aCGH) of ATC has revealed high levels of aneuploidy, with chromosome numbers ranging from 65–120; however, these studies were performed on small cohorts of samples [5–11]. Recently, Pozdeyev et al. found amplifications involving *KIT* in 4q12 (4% of cases), *CCNE1* in 19q12 (4% of cases) and *CD274* (previously *PD-L1*), *PDCD1LG2* (previously *PD-L2*), and *JAK2* in 9p24 (3% of cases) using targeted sequencing. As regards

to structural rearrangements, a *STRN-ALK* translocation has been found in one case of ATC [12], but no recurrent gene fusions have been identified. Genes that are recurrently mutated in ATC include *TP53* (25–60% of cases), *BRAF* (25–90%), *USH2A* (20%), *NRAS* (15–20%), *PTEN* (15%), *NF1* (10–35%), *PIK3CA* (10–20%), *EIF1AX* (10%), *ATM* (8%), *HRAS* (7%), *KRAS* (5–10%), and *CTNNB1* (5%) [13–22]. Furthermore, *TERT* promoter mutations, leading to *TERT* expression, are seen in 15–75% of cases [13,17,18,21–23].

Most next generation sequencing studies of ATC have been done by targeted sequencing of custom gene panels [13–19]. In total, only 41 primary ATCs that were investigated with whole exome seuencing (WES) have been previously published [20,21,24], and no RNA-sequencing (RNA-seq) that is aimed at fusion gene detection is available in the literature. As the genomic landscape of ATC has thus not yet been fully explored, we applied WES and RNA-seq on primary tumor samples to identify the novel genetic events that contribute to ATC tumorigenesis and that may be used as therapeutic targets.

2. Results

2.1. Genomic Amplifications Are Common in ATC

Copy number analysis could be undertaken based on WES data in 10 cases (Table 1). Seven of the analyzed cases had large variations in chromosome copy number, as well as variant allele frequencies (VAFs) that were suggestive of polyploidy, whereas three appeared to have near-diploid genomes (Figure 1a, Table S1). A median of 16 breakpoints, defined as a change in copy number state, were detected per case (range 5–43), with a high proportion (31/187; 17%) of breakpoints occurring in centromeres; all of the cases had at least one breakpoint in a centromere (range 1–7; Figure 1b, Table S1). Chromosome 8 displayed a pattern of loss of 8p and concurrent gain of 8q, with breakpoints in the centromere in six cases (60%), possibly indicating isochromosome 8q. Two additional cases had gain of the whole chromosome 8, making gain of 8q present in 8/10 (80%) investigated cases (Table S1).

Table 1. Clinical data and genetic analyses of 14 cases of primary anaplastic thyroid cancer.

Case No.	Gender	Age	Tumor Size (cm)	T	N	M	Stage	Ki67 (%)	Copy Number Analysis	Fusion Gene Analysis	Mutation Analysis (Matched Normal)
1	F	71	5.5 × 4 × 7	T4b	N1	M1	IV C	N/A	Yes	Yes	Yes (yes)
2	M	70	6 × 8	T4a	N0	M0	IV A	35	Yes	Yes	Yes (yes)
3	F	73	8 × 6 × 5	T4b	N1b	M0	IV B	75	Yes	Yes	Yes (no)
4	M	64	4.6 × 4.3 × 7.4	T4b	N0	M0	IV B	90	Yes	No	Yes (yes)
5	M	64	8 × 7	T4b	N0	M1	IV B	60	No	Yes	No
6	F	72	5 × 5 × 7	T4b	N1b	M0	IV B	N/A	No	Yes	No
7	F	74	6 × 10 × 7	T4b	N1	M1	IV C	N/A	Yes	Yes	Yes (yes)
8	F	84	11.9 × 8.3 × 11.7	pT4b	pN1b	pM1	IV C	50	Yes	Yes	Yes (yes)
9	F	86	7 × 5.5 × 4.5	pT4b	No	M1	IV C	N/A	Yes	Yes	Yes (no)
10	F	70	5 × 3.5 × 5	T4b	N0	M0	IV B	50	No	Yes	No
11	M	84	8.5 × 6.5 × 5.5	T4b	N0	M1	IV C	N/A	Yes	Yes	Yes (yes)
12	M	49	7 × 7 × 5	T4b	N0	M0	IV B	N/A	No	Yes	Yes (yes)
13	M	76	4.8 × 3.7 × 8.3	T4b	N1b	M1	IV C	N/A	Yes	No	Yes (no)
14	F	63	8 × 5.5	T4b	N0	M0	IV B	30	Yes	Yes	Yes (yes)

N/A, data not available; T: size/extent of primary tumor; N, degree of spreading to regional lymph nodes; M, presence or absence of distant metastasis. TNM staging according to Sobin et al [25].

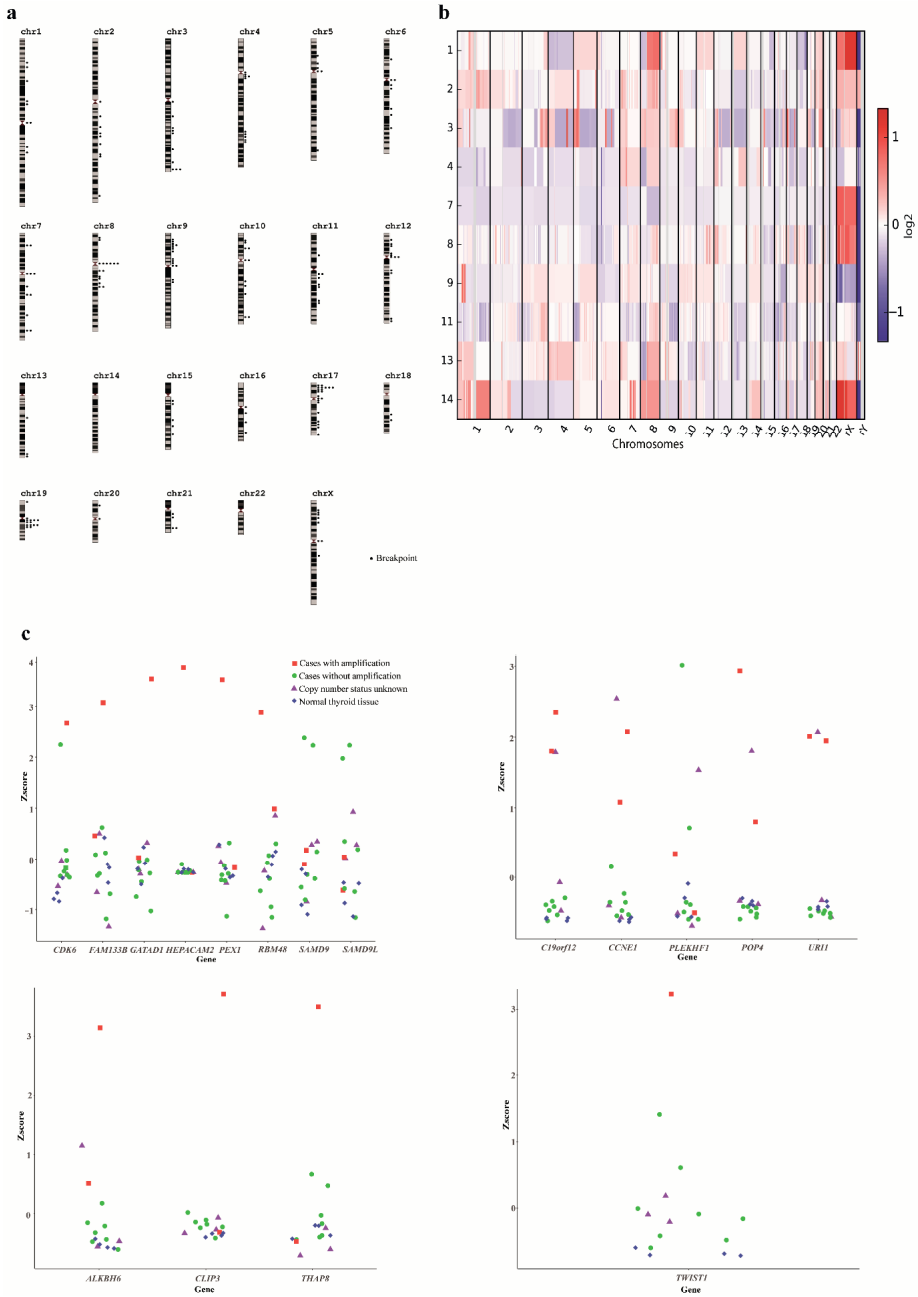

Figure 1. Detection of copy number variants in anaplastic thyroid cancer (ATC). (**a**) Breakpoint map of 10 primary ATC cases based on whole exome sequencing. Breakpoints were defined as a change in copy number state. A large number of breakpoints in centromeric regions were seen. (**b**) Heat map of copy number aberrations in 10 primary ATC cases. Polyploidy and large variations in chromosomal copy number were seen. (**c**) Expression of genes in amplified regions in all ATC cases and normal thyroid tissue.

High-level amplifications, defined as a gain of more than three extra copies over the baseline level, were seen in eight of ten (80%) cases, with recurrent amplifications in 19q12 and 19q13

(Table S1). The 19q12 amplification was seen in three cases (30%) with the minimally gained region chr19:30020714-30649335, including the *POP4*, *PLEKHF1*, *C19orf12*, *CCNE1*, and *URI1* genes. All of these genes were highly expressed in the cases with amplification as compared to cases without amplification (Figure 1c). In addition, one of the cases where no copy number analysis had been performed displayed high expression for all of these genes. Furthermore, two cases (20%) had 19q13 amplifications, with the minimally gained region chr19:36494128-36585117, including the *ALKBH6*, *CLIP3*, and *THAP8* genes. Of these, only *CLIP3* showed increased expression in cases with the amplification (Figure 1c). Among the amplifications that were only seen in single cases, case 2 had an amplification in 7q21 with the minimally gained region chr7:91974285-92987750, including the *GATAD1*, *PEX1*, *RBM48*, *FAM133B*, *CDK6*, *SAMD9*, *SAMD9L*, and *HEPACAM2* genes. All of these genes, except for *SAMD9*, *SAMD9L* and *HEPACAM2*, were highly expressed in this case (Figure 1c). Furthermore, case 2 had an amplification of chr7:18330180-19461461, including *TWIST1*, which was highly expressed in this case (Figure 1c).

2.2. Multiple Non-Recurrent Fusion Genes in ATC

A total of 21 fusion genes were identified in 5/12 cases that were investigated with RNA-seq (Table 2). Of these, 15 were seen in case 5, whereas cases 3 and 7 had two fusion genes each and cases 12 and 14 had one fusion gene each. Nine of the fusion genes were in-frame and twelve out-of-frame. None of the fusion genes were recurrent, but *FN1* was involved in two different out-of-frame fusions. For *MLXIP/PTEN* and *EP400/NCOR2* fusion genes, the reciprocal *PTEN/MLXIP* and *NCOR2/EP400* fusions, respectively, were seen; no other reciprocal fusions were detected.

Table 2. Fusion genes detected in twelve cases of primary anaplastic thyroid cancer.

Case	Fusion Gene	Chromosome	Inframe/ Frame-Shift	Software Identifying Fusion	Validated
3	*BGN/THOC7*	Xq28/3p14.1	Inframe	FusionCatcher	Not done
3	*POSTN/EIF3A*	13q13.3/10q26.11	Frame-shift	InFusion	Not done
5	*EP400/NCOR2* *NCOR2/EP400*	12q24.33/12q24.31	Inframe	FusionCatcher	Not done
5	*FN1/PABPC1*	2q35/8q22.3	Frame-shift	FusionCatcher	Not done
5	*IVNS1ABP/KYNU*	1q25.3/2q22.2	Inframe	FusionCatcher	Not done
5	*MYH9/EIF2AK3*	22q12.3/2p11.2	Frame-shift	FusionCatcher	Not done
5	*PRPF6/TENM3*	20q13.33/4q35.1	Inframe	FusionCatcher	Not done
5	*RAB23/DST*	6p11.2/6p11.2	Inframe	FusionCatcher	Not done
5	*MYH3/FZD4*	17p13.1/11q14.2	Inframe	InFusion	Not done
5	*TAOK1/NME6*	17q11.2/3p21.31	Inframe	InFusion	Not done
5	*CNTN1/CCZ1B*	12q12/7p22.1	Frame-shift	InFusion	Not done
5	*HELZ/MYH10*	17q24.2/17p13.1	Inframe	InFusion	Not done
5	*VSIG4/TRA2B*	Xq12/3q27.2	Frame-shift	InFusion	Not done
5	*OPHN1/PTRF*	Xq12/17q21.2	Frame-shift	InFusion	Not done
5	*SDC2/SRRT*	8q22.1/7q22.1	Frame-shift	InFusion	Not done
5	*HTRA1/AMZ2*	10q26.13/17q24.2	Frame-shift	InFusion	Not done
5	*GPR107/MYH10*	9q34.11/17p13.1	Inframe	InFusion	Not done
7	*MXI1/STMN1*	10q25.2/1p36.11	Frame-shift	FusionCatcher	Not done
7	*USP46/FN1*	4q12/2q35	Frame-shift	FusionCatcher	Not done
12	*ENO2/PIEZO2*	12p13.31/18p11.21	Frame-shift	InFusion	Not done
14	*MLXIP/PTEN* *PTEN/MLXIP*	12q24.31/10q23.31	Inframe	FusionCatcher	Yes

2.3. Somatic Mutations in ATC

WES detected a total of 7478 somatic coding mutations in the eight cases with matched normal samples for analysis, with a median of 60 mutations per case (range 28–6863) (Table S2). Excluding case 12, which had 6863 mutations, the remaining ten cases had a median of 52 mutations per case (range 28–247). For the three cases with no matched normal, a total of 245 mutations remained after filtering, with a median of 87 mutations per case (range 58–99) (Table S3). The most commonly mutated gene was *TP53* (6/11 cases; 55%), followed by mutations in the *TERT* promoter (four cases; 36%), *ATM* (three cases; 27%), and *ARID2*, *BRAF*, *FANCA*, *INPP4B*, *MAP3K1*, *NF2*, *PIK3CA*, *RB1*, *SMARCA4*, and

TET2 (two cases each; 18%) (Figure 2, Tables S2 and S3). Furthermore, single cases (9%) had mutations in *NRAS* and *HRAS* (Figure 2, Tables S2 and S3). In addition, case 12 displayed a mutation in *CCNE1*, which was classified as "tolerated" by SIFT and "benign" by PolyPhen2; it is thus not clear whether this had a pathogenetic effect.

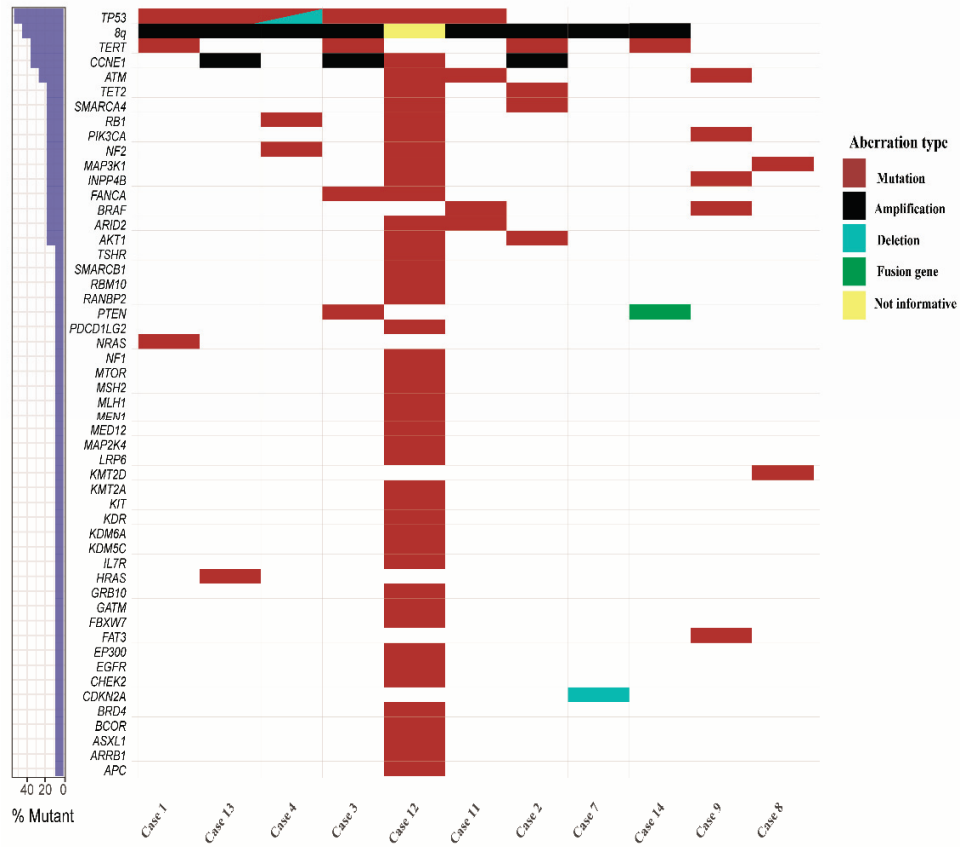

Figure 2. Genomic landscape of 11 cases of anaplastic thyroid cancer investigated by whole exome sequencing.

2.4. Associations Between Genetic Aberrations and Clinical Parameters

To see whether the genetic aberrations were associated with any clinical parameters, we performed Fisher's exact two-sided test for the recurrent abnormalites that are listed in Figure 2 vs. nodal stage and presence of distant metastases. No significant associations were detected.

2.5. Mutational Signatures in ATC

The analysis of mutational signatures could be performed in all 11 cases that were subjected to WES. The most common mutational signature was 1A/B, associated with aging, seen in six cases (55%; Figure S1). Five cases (45%) displayed signature 6, associated with defective DNA mismatch repair, four (36%) displayed signatures 2 or 13, associated with activity of the AID/APOBEC family of cytidine deaminases, four (36%) displayed signature 3, associated with failure of DNA double-strand break-repair by homologous recombination, four (36%) displayed signature 4, associated with tobacco exposure, three (27%) displayed signature 7, associated with ultraviolet light exposure, and two (18%) displayed signature 11, associated with exposure to alkylating agents (Figure S1, https://cancer.sanger.ac.uk/cosmic/signatures). Furthermore, case 12 displayed signature 14, which is associated with very

high numbers of somatic mutations. Additionally, signature 17, which was of unknown etiology, was seen in five cases (45%).

2.6. Microsatellite Instability Is Rare in ATC

WES data was analyzed to check whether ATC exhibited microsatellite instability (MSI). Only one (9%) of the eleven cases had a value that was close to the cut-off for MSI (Figure 3). Notably, this case (#12) also had mutations in *MLH1* and *MSH2*.

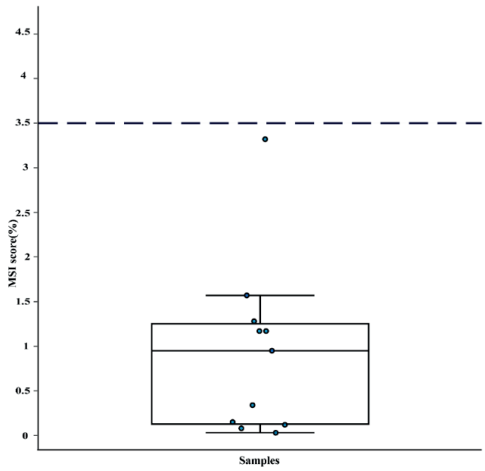

Figure 3. Microsatellite instability (MSI) scores for 11 anaplastic thyroid cancer cases. The dotted line represents the threshold to classify a case as MSI positive. Only one case was borderline MSI positive.

3. Discussion

In spite of ATC being one of the most fatal malignancies, its genetic background has not yet been fully explored. Here, we present data on copy number, fusion genes, and mutations from fourteen primary ATC samples, obtained before chemo- or radiotherapy.

Copy number analysis was done based on WES, providing a variable resolution across the genome depending on gene density. In line with previous studies using chromosome banding and aCGH [5–11], we found that at least 7/10 (70%) tumors were polyploid based on variable copy number and VAF. Furthermore, a high frequency of breakpoints in the centromeric regions were seen, similar to what we have previously reported in the ATC cell lines [26]. This has been proposed to be a sign of chromosomal instability that can arise either due to the formation of breakage-fusion-bridge cycles that are caused by telomere dysfunction or due to mitotic spindle defects [27,28]. On the chromosomal level, most of the chromosomes displayed variable copy numbers between cases. The exception was chromosome 8, which showed a loss of 8p relative to the baseline copy number in 6/10 (60%) cases and gain of 8q in 8/10 cases (80%). Thus, it is likely that a gain of 8q is a driver event in ATC; however, since this is a large region containing >1500 genes, it was not possible to identify the target gene(s).

A relatively high number of amplifications was seen. Three cases (#2, #3, and #13) harbored amplification of 19q12 with *CCNE1* as the putative target gene. CCNE1 promotes progression into the S phase of the cell cycle by interacting with cyclin dependent kinases (CDKs) [29]. *CCNE1* amplifications were recently reported in ATC [17] and they are known to occur in multiple malignancies, including ovarian, breast, and gastric cancer [30–33]. Apart from being highly expressed in all cases with the amplification, one additional case (#5) without copy number data displayed high expression of *CCNE1* and also likely had amplification. Furthermore, case 12 displayed a somatic mutation in *CCNE1*. However, this was classified as non-pathogenic and possibly a passenger event. Thus, in total, 4/14 (29%) of our cases displayed an amplification of *CCNE1*; a frequency much higher than the 4% of cases

that were reported by Pozdeyev et al. [17], although this difference could be due to the small size of our cohort. Interestingly, one of our cases (#2) also had the amplification and overexpression of *CDK6*, active in the same cellular processes as CCNE1 [34]. Although there are no drugs that are available that specifically targets CCNE1, multiple inhibitors for CDKs are under development and could be viable treatment options in *CCNE1*- and *CDK6*-amplified ATC [29,35].

Amplification of *TWIST1* was seen in case 2. TWIST1 is a transcription factor that is involved in the epithelial-to-mesenchymal transition (EMT) pathway and it is frequently overexpressed in cancer [36]. Preclinical studies in mouse models of *KRAS*-mutant lung cancer suggest that harmine, a β-carboline alkaloid, can be used to target TWIST1 with high efficacy [37].

A total of 21 fusion genes were identified, none of which were recurrent. There was a large variation in the number of fusion genes detected per case, with case 5 displaying 16 fusion genes and the remaining eleven cases investigated with RNA-seq having 0–2 fusion genes (Table 2). This is similar to what we have previously reported in ATC cell lines [26], and suggest that a large proportion of the fusion genes detected may be passenger events resulting from chromosomal breaks. Notably, *FN1* was involved in two different fusion genes—a *FN1/PABPC1* in case 5 and a *USP46/FN1* in case 7. *FN1* is a component of the extracellular matrix and it is recurrently involved in in-frame fusion genes as the 5′ partner in *FN1-ALK* and *FN1-FGFR1* in soft tissue tumors [38,39]. However, the *FN1* fusions that were detected here were both out-of-frame and therefore likely to result in a loss of the normal function of *FN1*. Furthermore, one case harbored an in-frame *PTEN/MLXIP* fusion, which has not been previously reported. *PTEN* is a well-known tumor suppressor gene that regulates the PI3K/AKT pathway [40]. Both in-frame and out-of-frame *PTEN* fusion genes have been reported to occur at a low frequency in various malignancies; the pathogenetic outcome is generally considered to be the loss of normal *PTEN* function [41–43].

The pattern of somatic mutations found in our study was similar to what has been previously reported [13–23]. An analysis of mutational signatures showed a high incidence (>30% of cases) of processes that are involved with normal aging, defective DNA mismatch repair, AID/APOBEC activity, failure of DNA double-strand break repair, and tobacco exposure. This agrees well with the results from Pozdeyev et al. [17], who reported that defective DNA mismatch repair and the activation of AID/APOBEC was common in ATC, based on targeted sequencing data from 24 cases, and also with the data from Dong et al. [24] from WES in five cases. Taken together, though, the mutational processes occurring in ATC appear to differ between cases (Figure S1), suggesting different underlying etiologies.

Case 12 was an outlier in terms of having a much higher number of somatic mutations (6863 vs. a median of 52 for the remaining cases), as well as occurring in a relatively young (49 years) patient. This case also showed a borderline value for microsatellite instability and it had mutations in both *MLH1* and *MSH2*, involved in DNA mismatch repair (Figures 2 and 3). Notably, case 12 also exhibited mutational signature 14, which was recently shown to be caused by a combination of loss of polymerase proofreading due to mutations in *POLE* or *POLD1* and defective mismatch repair [44]; in line with this, case 12 had two mutations in *POLE* (Table S2). Microsatellite instability has been proposed to be a marker for a favorable response to PD1 blockade therapy [45]; thus, this treatment could have been a viable therapeutic option for this patient.

Pozdeyev et al. [17] recently suggested that ATC may be divided into three different subtypes that are based on the mutational pattern: (1) tumors with *BRAF* V600E mutations, together with *PIK3CA*, *AKT1* or *ARID2* mutations, (2) tumors with *NRAS* mutations and *CCNE1* amplification, and (3) tumors with a high mutational burden and *MSH2/MLH1* mutations. In our cohort, cases 9 and 11 could be classified as type 1 according to this system based on having concurrent *BRAF* V600E and *ARID2/PIK3CA* mutations. Cases 2, 3, 5, and 13 had *CCNE1* amplification corresponding to type 2, none of which had *NRAS* mutation, whereas case 1 had an *NRAS* mutation; all of these could tentatively be classified as type 2. Finally, case 12 had a very high number of somatic mutations (n = 6863) and mutations in both *MSH2/MLH1,* agreeing well with type 3. The remaining cases could not be classified as either of these types based on the mutations.

Taken together, we show that a relatively large proportion (5/14 cases; 36%) of ATC harbor genetic events that make them suitable for novel therapeutic approaches, including CDK and TWIST1 inhibition, as well as PD1 blockade therapy. When considering the dismal prognosis of this disease, this should be addressed in future clinical trials.

4. Material and Methods

4.1. Patient Samples

The study initially included a total of 23 cases of ATC, which were selected on the basis of not having obtained chemo- or radiotherapy treatment prior to sampling, as well as on sample availability. Twenty-two formalin-fixed, paraffin-embedded (FFPE) samples with hematoxylin and eosin stained tumor sections were obtained from the Pathology Department, Laboratory Medicine, Skåne, Sweden. Furthermore, a fine-needle aspirate that was obtained at ATC diagnosis and a paired peripheral blood sample was included from one additional patient (case 1). A pathologist reviewed all of the FFPE blocks to confirm the presence of ATC and to determine tumor cell content. Cases with <30% tumor cells based on the pathologist's estimate ($n = 5$), and cases with no copy number aberrations or mutations that were detected by WES ($n = 4$) were excluded from further analyses, leaving 14 cases (Table 1). Three 10 µm sections were cut from each tumor and parts containing tumor and adjacent normal tissue were manually microdissected when possible. Sections were immediately put in deparaffinization solution (Qiagen, Valencia, CA, USA), followed by DNA and RNA extraction with the all prep DNA/RNA FFPE Kit (Qiagen), according to the manufacturer's recommendations. For the fine needle aspirate and the matching peripheral blood sample, DNA and RNA extractions were performed with the all prep DNA/RNA kit (Qiagen), according to the manufacturer's recommendations. The Ethical Review Board of Lund University approved the study (No. 2016/51, 1 February 2016).

4.2. Whole Exome Sequencing

WES was performed on eight matched tumor-normal samples and three tumor samples without matching normal samples (Table 1). DNA damage that was caused by formaldehyde fixation was repaired with the PreCR Repair Mix (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's recommendations. Genomic DNA was sheared via sonication while using an S220 focused-ultrasonicator (Covaris, Woburn, MA, USA) and DNA libraries were constructed using the TruSeq Exome Kit (Illumina, San Diego, CA, USA), according to the manufacturer's recommendations. The libraries were sequenced using the High Output Kit (150 cycles) on a NextSeq500 (Illumina). Raw reads were aligned to the human reference genome hg19 with the BWA-MEM algorithm [46]. Picard (http://broadinstitute.github.io/picard) was used to remove the PCR duplicates and local realignment around the indel region was performed using GATK [47]. Copy number aberrations were identified by cnvkit [48] and manually annotated; the resulting data from case 12 were too noisy for interpretation, and this case was hence excluded from copy number analysis. For cases with matched normal samples, the somatic mutations were identified using MuTect2 [49] with default settings. For tumor samples without matched normal samples, variations were identified by GATK Unified Genotyper and annotation parameters QD (variant confidence/quality by depth) < 2.0, MQ (root mean square mapping quality) < 40.0, FS (Fisher strand) 60.0, HaploTypeScore > 13.0, MQRankSum < −12.5, and ReadPosRankSum < −8.0 were used to filter the low quality variations [50]. High quality variants were further filtered by 1000 Genomes (20110521 release), ESP6500, ExAC, CG46 (popfreq_max_20150413), and 170 million variants (kaviar_20150923) provided by ANNOVAR [51] to remove the potential SNP sites. Annotation of variants were carried out with ANNOVAR [51]. The lists of somatic mutations were further filtered for a minimum coverage of 15 reads, keeping only non-synonymous mutations that were supported by ≥5 reads and a mutant allele frequency of ≥5%. Mutation signatures were identified with DeconstructSigs [52]. The genomic landscape plot was generated using GenVisR [53].

MSIsensor [54] was used for analyzing microsatellite instability. Only heteropolymer sites were included in this analysis.

4.3. RNA Sequencing

RNA-seq was performed on 12 primary ATC cases (Table 1) and normal tissue from four thyroids. For the tumor cases, the quantity and purity of RNA was measured with NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) and the quality on a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) to check for the fraction of RNA fragments that were greater than 200 nt (Dv200). mRNA libraries were constructed with an input of 20–50 ng of RNA, depending on the Dv200 value using the TruSeq RNA Access Library Prep Kit (Illumina), according to the manufacturer's recommendations. Constructed libraries were sequenced using Illumina's High Output Kit (150 cycles) on an Illumina NextSeq500. Identifications of fusion transcripts were performed using FusionCatcher [55] and InFusion [56] from raw fastq files. The list of fusion genes was filtered to remove chimeras that were identified as read-through transcripts, pseudogenes, unannotated genes, and fusions between gene family members, as well as by keeping only fusions that had unique spanning reads ≥ 3 (FusionCatcher) and ≥ 20 (InFusion). For expression analysis, RNA sequencing data were processed using the TCGA mRNA-seq pipeline (https://docs.gdc.cancer.gov/Data/Bioinformatics_Pipelines/Expression_mRNA_Pipeline/#mrna-analysis-pipeline). Briefly, the sequencing reads were aligned to the human GRCh38 genome assembly using STAR [57] and the read counts for each gene were obtained using HTSeq-count [58] and they were normalized using the fragments per kilobase of exon model per million mapped reads (FPKM) method. For fusion gene validation, RT-PCR was performed in case 14, which was the only case where cDNA could be obtained. Briefly, cDNA was synthesized while using High–Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) and SuperScript IV First-Strand Synthesis System (Thermo Fisher Scientific). Primers (available on request) were designed using Primer 3 (http://primer3.ut.ee/) specifically for the fusion transcript. PCR was performed according to standard methods and Eurofins Genomics sequenced the amplified products (Ebersberg, Germany).

4.4. Analysis of TERT Promoter Mutations

TERT promoter mutations were investigated according to Liu et al. [59] using the AmpliTaq Gold 360 Master Mix (Thermo Fisher Scientific).

4.5. Statistical Analysis

Fisher's two-sided exact test was used to investigate whether any of the detected genetic aberrations were associated with clinical parameters. Since all cases were T4, stage IV, and had high Ki67, this analysis could only be done for nodal stage and the presence of distant metastases.

5. Conclusions

In conclusion, we have performed a full-scale genomic analysis of primary ATC, showing complex copy number aberrations and polyploidy, multiple fusion genes, and a high level of mutations. A high proportion of the investigated cases were found to be candidates for novel treatments, showing that genomic analyses are highly clinically valuable in ATC.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6694/11/3/402/s1, Figure S1: Mutation signatures of 11 primary anaplastic thyroid cancer cases, showing heterogeneity in the involved mutational processes. Table S1: Copy number aberrations detected by whole exome sequencing in 10 cases of anaplastic thyroid cancer, Table S2: Somatic mutations detected in 8 paired tumor/normal samples of anaplastic thyroid cancer, Table S3: Somatic mutations detected in 3 cases of anaplastic thyroid cancer lacking a matched normal sample.

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Article IV