JOINING FORCES IN ENDOCRINE CANCER GENETICS

Molecular testing, surveillance and
treatment decision making
in clinical practice

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This introductory chapter will provide background on 1) the diagnosis and treatment options of rare endocrine tumors, and 2) the associated tumor predisposition syndromes included in this thesis: DICER1 syndrome, MEN2a syndrome, *CDC73*-related disorder and *SDHA*-associated paraganglioma

General Introduction

A few years ago I met a then 12-year-old girl, diagnosed with thyroid cancer, at the Department of Clinical Genetics of the Leiden University Medical Center. She and her parents had three important questions: "Why do I have cancer? Are other relatives at risk? And if so, can we prevent cancer?"

These questions, i.e. Why?, Who?, and How?, are the backbone of this thesis, which describes investigations of the genetic background of a wide variety of rare endocrine tumors, including those of the thyroid, parathyroid, adrenal and paraganglia. This introductory chapter will provide background on 1) the diagnosis and treatment options of rare endocrine tumors, and 2) the associated tumor predisposition syndromes included in this thesis: DICER1 syndrome, MEN2a syndrome, *CDC73*-related disorder and *SDHA*-associated paraganglioma (see Figure 1).

In order to provide answers to the questions asked by that 12-year-old girl, patient- and family-centered endocrine cancer care encourages active collaboration between the departments of endocrinology, oncology, surgery, pathology, chemistry, radiology, nuclear medicine and clinical genetics (see Figure 2).

The implementation of high-throughput DNA/RNA sequencing platforms allows novel molecular information to be used to optimize primary endocrine cancer care: firstly, via somatic

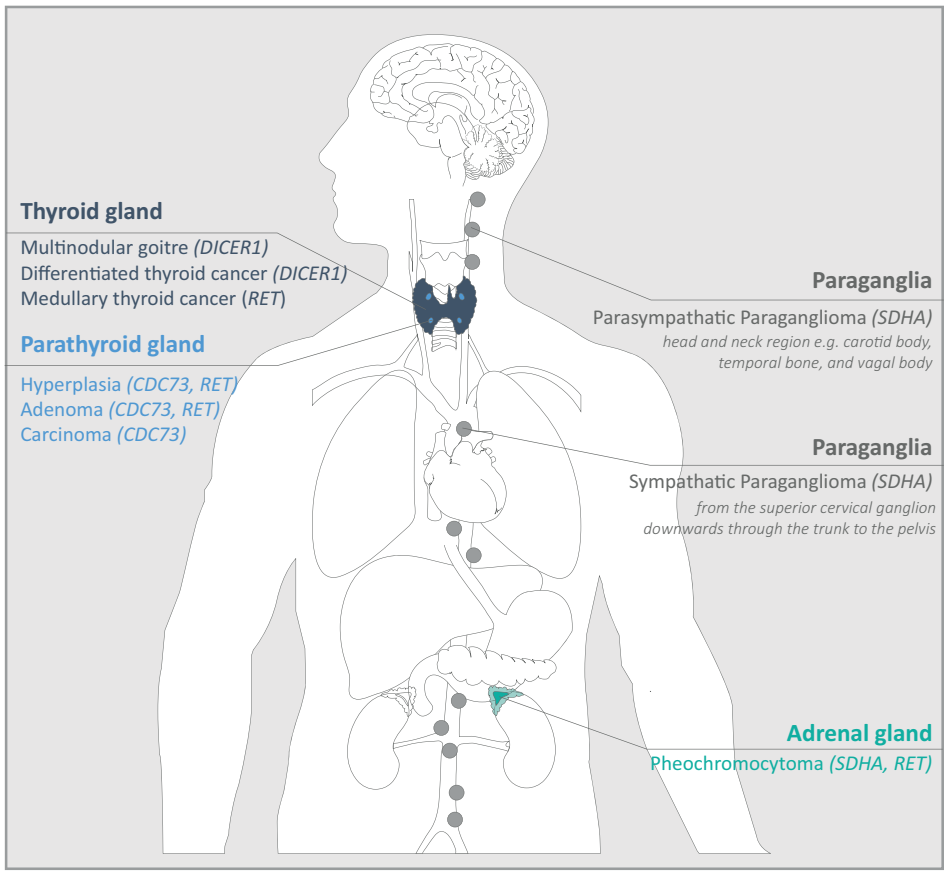


Figure 1. Endocrine tumors and related tumor predisposition syndromes investigated in this thesis.

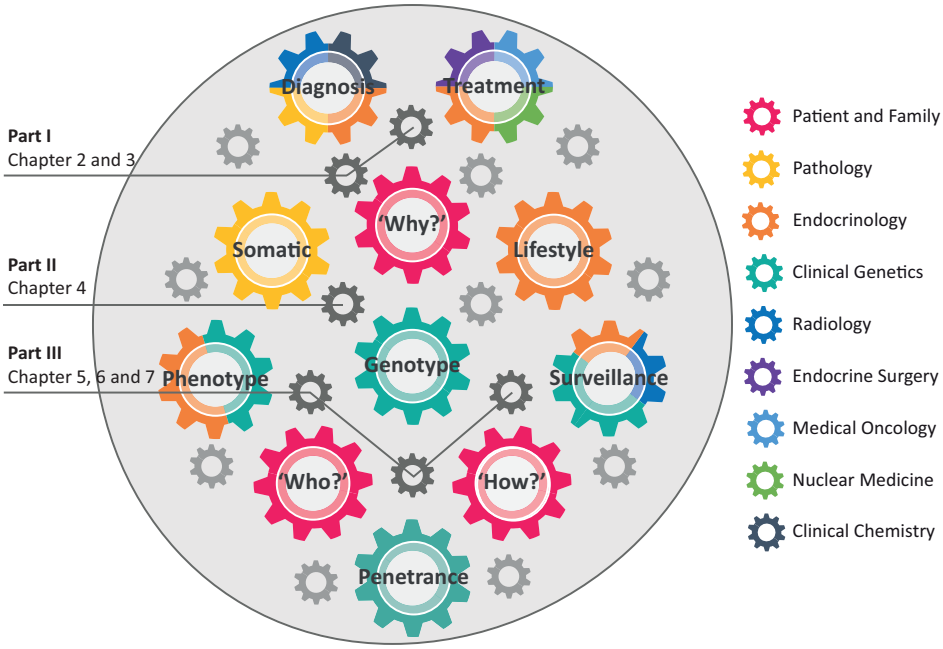


Figure 2. Joining forces in patient- and family-centered endocrine cancer care inspired by the questions: "Why do I have cancer? [Who?], Are relatives at risk? [Who?], And if so, can we prevent cancer? [How?]". Part I. The roll of molecular testing in endocrine cancer diagnostics and treatment decision making. Part II. Identification genetic predisposition in pediatric non-medullary thyroid carcinoma. Part III. Genetic counseling in endocrine tumor predisposition syndromes.

and germline molecular information that can be used in the pre- and postoperative phase for primary diagnostics and to select therapy choices. Secondly, in case of cancer recurrence molecular information increasingly stratifies for the effectiveness of targeted drugs (mainly antibody or small molecule drugs) or for the effectiveness of immunotherapeutic drugs. Finally, DNA sequencing aids in the elucidation of genetic factors underlying the increased disease susceptibility in patients and their families.

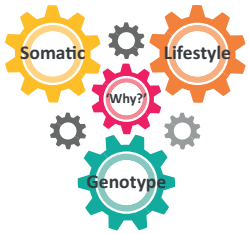
Not unlike multidisciplinary patient care, interdisciplinary efforts are increasingly important to scientific discoveries and translational research efforts. This thesis emphasizes not only local, national and international collaborations between the medical disciplines involved but also the interaction between basic and clinical research, taking research from bench to beside and back again.

Q1: WHY DO I HAVE CANCER?

Tumors evolve from benign to malignant lesions by acquiring a series of non-synonymous variants* (i.e. single nucleotide substitutions, structural variants that alter protein products).[1] This gradual accumulation of gene mutations† is attributable to hereditary, replicative and environmental factors (see textbox 1).[2]

* Genetic terminology is included in the glossary in the appendix (page - 160-164)
† In this thesis, the word *mutation* is used as a synonym for pathogenic (disease causing) variant

Textbox 1. Q1. Why do I have cancer?
A: Due to the accumulation of non-synonymous gene mutations over time, attributable to hereditary, replicative and environmental factors.
- Hereditary factors: Germline mutations that can be transmitted via eggs or sperm cell and are present in every cell of the offspring (also referred to as 'genotype' in Figure 2).
- Replicative factors: Somatic mutations that result from (unavoidable) DNA replication errors (also referred to as 'somatic' in Figure 2).
- Environmental factors: Somatic mutations that result from external mutagens such as smoking, alcohol and UV light (also referred to as 'lifestyle factors' in Figure 2).



Determining the contribution of each factor is challenging and differs among cancer types.[2] In general, the acquisition of de novo somatic variants (due to replicative and environmental factors) accounts for approximately 90% of all new cancer diagnoses. Somatic variants generally occur in cells with high proliferation rates (leading to random mistakes during normal DNA replication) and/or occur in barrier tissue through prolonged exposure to environmental carcinogens (e.g. smoking, alcohol and UV light). As a result, the number of sporadic cancers increases with age. The remaining 10% of cancer diagnosis are related to inherited mutations. In patients with a germline mutation in a tumor suppressor gene or oncogene, the first step in the development of cancer has already been taken in every cell. This differs from patients with sporadic cancers who do not harbor constitutional gene mutations and therefore must acquire multiple somatic gene mutations within a single cell before tumorigenesis can occur.[2] Unsurprisingly, one of the main indicators of a genetic predisposition to cancer is the development of one or more malignancies at an earlier than expected age. The proportion of inherited disease in the context of the total disease population might be an underestimate owing to still unidentified genetic causes or because heredity is not recognized due to an unavailable, incomplete or misdiagnosed family history and/or variable penetrance. Identification of a causative germline mutation may not only have important clinical implications for the index patient (proband), it also facilitates cascade testing and surveillance of relatives in order to prevent, or at least allow early identification of, (pre)malignant conditions.

ENDOCRINE TUMORS

All endocrine tumors are considered rare diseases. To date, a total of six to seven thousand rare diseases have been discovered and new diseases are described regularly in the medical literature. The reported number of rare diseases depends to a large extent on the degree of specificity used when classifying the different entities. Comprehensive genetic analysis, including genomics, transcriptomics and proteomics, for example by The Cancer Genome Atlas (TCGA), has recently led to a large increase in (neuroendocrine) cancer subtypes.[3,4] Interestingly, different cancer subtypes may have a partially comparable genetic background (e.g. solid tumors with *NTRK* gene fusions). This type of molecular reclassification may also extend beyond the current boundaries of organ-specific histologic tumor classification.[5] The primary reason for classification of tumors is to better assign appropriate (targeted) therapy. In 2018, the *NTRK* inhibitor, Larotrectinib, was approved by the American Food and Drug Administration (FDA) for adult and pediatric patients with advanced solid tumors harboring an *NTRK* gene fusion without a known acquired resistance mutation. In contrast to other cancer types (e.g. melanoma and ovarian cancer), molecular profiling in endocrine tumors is mainly used for primary diagnostics (i.e. subtyping and prognostic

forecasting) and has not yet been implemented for tailored treatment in clinical practice.[6] Current treatment options are limited for some endocrine cancer subtypes (e.g. advanced radioactive iodine-refractory thyroid cancer, parathyroid carcinoma and metastatic paraganglioma).

The following paragraphs will give an overview of the diagnostic procedures and treatment options for the endocrine tumors investigated in this thesis, i.e. of the thyroid, parathyroid, adrenal gland and paraganglia.

Thyroid gland

Diagnosis

Thyroid nodules are common, as around 5% of adults harbor thyroid nodules by palpation and up to 60% show nodules on ultrasound. Only a small fraction (4.0%-6.5%) of all evaluated thyroid nodules is found to be malignant.[7] Thyroid ultrasound characteristics, such as size, echogenicity, and presence of macrocalcifications and/or irregular margins, have been used to stratify the risk of malignancy in thyroid nodules and aid decision-making regarding whether further investigation is indicated.[8,9] Fine-needle aspiration is typically performed to further stratify thyroid nodules suspect for malignancy. A definitive morphological diagnosis of benign or malignant nodules can be provided by cytology examination in up to 70-75% of cases, whereas the remainder is considered undetermined (Bethesda category III and IV).[10,11] In these cases in particular the increasing use of molecular testing improves diagnosis and clinical management.[12]

Thyroid cancer (TC) is the most common endocrine malignancy and its incidence has increased appreciably over the last few decades, especially in Europe and North America. TC now accounts for 1-3% of all new malignant tumors.[13,14] Of these, the vast majority (>90%) are differentiated thyroid carcinomas (DTC) that derive from the follicular epithelial cells and have an indolent clinical course and low mortality.[15] Trends in TC incidence probably largely reflect incidental detection of asymptomatic disease through the increasing use of medical imaging modalities.[16] The incidence of DTC is about three to four times higher among females than males and shows distinct age-related patterns regarding gender and different histological subtypes.[16]

Histological subtypes can be distinguished based on morphological features and molecular background.[17] Papillary thyroid carcinoma (PTC, 85-90%) is the most common subtype and specific nuclear features are important diagnostic hallmarks. Many morphological variants of PTC have been described.[18] Classic PTCs are associated with somatic *BRAF*^{V600E} variants or gene rearrangements (*RET-PTC*, *NTRK*- and *ALK)*.[19] The follicular variant of PTC (FVPTC) more commonly harbors *RAS* or *BRAF* non-V600E variants.[20] These mutations, leading to activation of the mitogen-activated protein kinase (MAPK) signaling pathway, are almost always mutually exclusive.[21] Follicular thyroid carcinoma (FTC, 5-10%) is diagnosed by minimal or wide follicular cell invasion of the tumor capsule and/or blood vessels and has frequently been linked to somatic mutations (*RAS* or *PTEN)* or *PAX8-PPARγ* gene rearrangements.[19] Hürthle cell carcinomas are characterized by oncocytic cells as a result of mitochondrial abundance and are associated with whole chromosome loss accompanied by endoreduplication or genomic doubling, in the absence of alterations in the abovementioned genes.[22,23] In contrast to PTC, poorly differentiated (PDTC, 2%) and anaplastic thyroid carcinomas (ATC, 2%) are aggressive tumors that have undergone dedifferentiation due to additional or relatively frequent somatic *TERT*, *TP53, CTNNB1* and/or *PIK3CA* mutations.[24] Medullary thyroid cancer (MTC, 2%) originates from the calcitonin-producing parafollicular cells and is associated with (germline and somatic) activating *RET* proto-*oncogene* mutations or somatic *RAS* mutations. [25,26]

Treatment

Treatment decisions are guided by the extent of disease and include lobectomy or total thyroidectomy with or without radioactive iodine (RAI) therapy to treat persistent loco-regional, nodal disease or distant metastases not amenable to surgery.[27] These treatments are highly effective in the majority of DTC patients and the 10-year survival rate ranges between 80 and 95%.[13,14] However, up to 5% of DTC patients become refractory to RAI (RAI-R). The 10-year survival rate in these patients is about 20-40%, due to frequently unresectable metastatic lesions.[15,28] The survival rates for less common TC histological subtypes range from 65% for MTC after 10 years, less than 20% for PDTC at 5 years, and less than 10% for ATC at 6 months after the initial diagnosis.[26,29] A range of targeted treatments have been approved by the FDA for the treatment of advanced RAI-R DTC, ATC and MTC. Several clinical trials are currently investigating the potential of (primarily) alternative kinase inhibitors (e.g. NTRK-, ALK-, BRAF- and RET- inhibitors).[30]

Parathyroid gland

Diagnosis

Hyperparathyroidism (i.e. increased parathyroid hormone levels in blood; HPT) results either from autonomous hyperfunction of the parathyroid glands themselves (primary hyperparathyroidism; pHPT) or secondary/tertiary to an underlying condition (e.g. vitamin D deficiency or kidney failure). HPT is typically characterized by the quartet *stones, bones, groans, and psychiatric overtones* referring to the presence of renal stones, osteoporosis, gastrointestinal symptoms and depression, respectively. Nevertheless, most patients are asymptomatic. pHPT is a relative common endocrine disease, with a prevalence of 1-4 per 1000, a female predominance (3:1) and a peak incidence in the sixth decade of life.[31] Benign, sporadic parathyroid adenomas (PA) are the most common cause of pHPT (~85%). A further 15% of pHPT is attributable to multi-gland disease (including hyperplasia and double adenomas) and less than 1% is due to a parathyroid carcinoma (PC).[32,33] The lack of specific discriminating clinical, biochemical and radiological features makes distinguishing between PA and PC challenging. However, discriminating the two conditions is of the utmost importance as it determines the extent and radical nature of initial surgery, which is in turn the major determinant of prognosis.[34] Pre-operative features that should raise suspicion of PC are: calcium >3mmol/L, PTH >3 times upper limit, parathyroid lesion >3cm and a family history of PC.[35] Intraoperative findings that suggest carcinoma are firm, large grayish to white irregularly-shaped tumors that can be adherent or invade surrounding structures. Even the histological diagnosis remains in some cases difficult and the diagnosis of PC is often made retrospectively, after tumor recurrence or metastasis.[36,37] The criteria to unequivocally diagnose PC include: capsular invasion, vascular invasion, invasion in surrounding tissue and/or distant metastasis.[38] Parathyroid lesions without unequivocal histological signs of PC but with some features of malignancy (e.g. fibrotic bands, questionable capsular invasion, increased mitotic figures) are defined as atypical adenoma and might require closer follow-up. Inactivating *CDC73* mutations are a major driver of PC (~70%) and in one-third of cases the mutations are found in the germline.[39,40] In contrast, these mutations are extremely rare in sporadic PAs.[41] When found, they were typically associated with unusual histologic features, such as cystic appearance.[42] Immunohistochemical staining of the protein product of *CDC73*, parafibromin, and somatic *CDC73* mutation analysis can be useful in the differential diagnosis of PC and may serve as a prognostic factor.[43,44] *MEN1*, *CCND1/cyclin D1* and the *CDKI* genes have been established as primary tumorigenic drivers in PAs.[40]

Treatment

Surgery is the most common treatment for pHPT and provides a cure in about 95% of all cases. The extent of surgery (focused vs. bilateral exploration, selective vs. extensive parathyroidectomy) depends on the differential diagnosis and possible underlying hereditary setting. Most patients with PC achieve long-term survival (5-year mortality ~10%) after surgical resection.[33,35] However, following multiple operations, systemic therapy may be required for recurrent or metastatic disease. Radiotherapy and cytotoxic regimes have not been proven to be effective and current treatment focuses on controlling hypercalcemia. Chapter 8 will discuss the future perspectives for metastatic PC treatment, based on recent comprehensive genetic profiling studies.[45-48]

Paraganglia and adrenal medulla

Diagnosis

Paragangliomas (PGLs) are rare neuroendocrine tumors (*i.e.,* 2-5/1.000.000/year) and carry the highest degree of heritability among human neoplasms.[49,50] PGLs are classified according to their anatomical location (intra or extra-adrenal PGL) and whether they are of sympathetic or parasympathetic origin. Head and neck paragangliomas (HNPGL) emerge from the parasympathetic nervous system and are usually benign, slow-growing non-secreting tumors.[51,52] Common sites include the carotid body, the temporal bone, and the vagal body. Pheochromocytoma (PHEO) and sympathetic paraganglioma (SPGL) are catecholamine-secreting tumors, with associated clinical features such as high blood pressure, a rapid heartbeat, flushed skin, sweating, headache and tremors.[53] PHEOs are derived from the chromaffin cells of the adrenal medulla and SPGLs are found in close relationship to the peripheral sympathetic nervous system, from the level of the superior cervical ganglion downwards through the trunk to the pelvis.[54] Diagnostic workup generally includes measurement of metanephrines (i.e. the O-methylated metabolites of catecholamines) levels in blood and/or urine, one or more anatomic or nuclear imaging tests (i.e. CT, MRI, MIBG, and/or PET) for differential diagnosis and to accurately define the location of the lesion, and might also include germline genetic testing.[49,54] Immunohistochemistry for SDHB and SDHA has been shown to be a valuable additional tool in the histopathological analysis of these tumors, and can be considered a surrogate marker for molecular analysis.[55]

Treatment

Treatment of PGL depends on the location and origin of the tumor. For PHEO and SPGL surgical resection is generally the treatment of first choice due to excess production of hormones. For non-producing, slow-growing HNPGL watchful waiting might be more appropriate. Metastases are more often present in SPGL compared to PHEO or HNPGL.[51] Patients with metastatic disease have limited treatment options[56] and a markedly variable prognosis (reported 5-year survival rates range between 24% and 85%).[38] Recently, an integrated analysis identified several molecular markers that were associated with an increased risk of metastatic disease and which may serve as potential drug targets.[4] Chapter 8 will discuss the future perspectives for metastatic PGL treatment.

In summary, (somatic) genetic information has the potential to improve endocrine tumor classification, prognostic forecasting, and the development of personalized treatment. Furthermore, molecular analysis of tumor tissue can be used as a pre-test tool for the identification of patients at high risk for a genetic predisposition syndrome.

Q2: ARE RELATIVES AT RISK?

Identification of the causative gene variant in a cancer patient offers his/her relatives the possibility of pre-symptomatic genetic testing, i.e. at-risk family members can be screened for the presence of the mutation to establish 'who' has inherited an increased cancer risk (mutation carrier vs. non–mutation carrier). Most cancer predisposition syndromes follow an autosomal dominant inheritance pattern in which the patient's first-degree relatives (i.e. parents, children, and siblings) have a 50% risk of carrying the causative mutation. Successful implementation of genetic testing in diagnostics requires accurate estimates of variant pathogenicity classification, phenotype and disease penetrance.

Although an increasing proportion of cases can now be attributed to inherited gene mutations, a substantial fraction of suggestive hereditary cases (i.e. young onset, multiple tumors and/or strong family history) are still genetically unaccounted for. For individuals with clinical features suggestive of a hereditary cancer syndrome, but without a mutation in the known predisposition genes, predictive testing of family members, genetic counseling and preventive medical management are hampered.

ENDOCRINE TUMOR PREDISPOSITION SYNDROMES

Among the first hereditary tumor predisposition syndromes to be recognized were Multiple Endocrine Neoplasia (MEN) type 1-2 and von Hippel-Lindau syndrome.[57,58] Depending on the specific endocrine tumor type, 10-30% of cases are associated with genetic factors, in which up to 15 different genes per tumor type may be implicated.[34,50] The relatively large role of inherited DNA variants in endocrine tumors compared to other cancer types (e.g. 5%-10% in breast cancer) has been suggested to be a counterpart of the relatively low contribution of somatic mutations. The latter is the result of both fewer replicative alterations (due to relatively low proliferation rates[59]) and the limited influence of environmental factors.[2]

While endocrine neoplasia syndromes show many features commonly seen in familial disease (early onset, family history, multifocal neoplasia, multiorgan involvement), some of these syndromes are considered to be phenotypically complex and heterogeneous. Moreover, endocrine predisposition syndromes commonly present with *de novo* mutations. The latter presentation can make them difficult to recognize and classify on purely clinical grounds.

Due to an active international research community, over time the number of endocrine tumor syndromes and associated genes has expanded significantly.[60] Furthermore, new disease patterns have emerged following the identification of non-endocrine tumors and other clinical features as part of hereditary endocrine tumor syndromes, and with the occurrence of endocrine tumors in non-classical endocrine tumor syndromes.[60]

The following paragraphs provide an overview of genetic predisposition for the endocrine tumors and syndromes discussed in this thesis:

- Thyroid cancer, focusing on DICER1 syndrome and MEN2a syndrome (*Figure 3*)
- Parathyroid tumors, focusing on *CDC73*-related disorder (*Figure 4*)
- Paraganglioma, focusing on *SDHA*-associated paraganglioma (*Figure 4*)

Genetic predisposition of thyroid cancer

DTC can manifest as part of a tumor predisposition syndrome, including PTEN hamartoma tumor syndrome, DICER1 syndrome (see below), Carney complex, familiar adenomatous

Table 1. Hereditary syndromes associated with non-medullary thyroid cancer

Syndrome	Gene (locus)	Inheritance	Thyroid phenotype*	Penetrance thyroid phenotype	Syndromic features
PTHS / Cowden	*PTEN* (10q23.31)	AD	FTC > PTC MNG	~10%	e.g. breast- uterine-, colon cancer, hamartomas, macrocephaly
Carney complex	*PRKAR1A* (17q24.2)	AD	MNG PTC, FTC	~60% ~5%	e.g. myxoma, lentigines, endocrine overactivity
DICER1 (Figure 3)	*DICER1* (14q32.13)	AD	MNG PTC, FTC	~35% ~5%	e.g. pleuropulmonary blastoma, cystic nephroma, Sertoli–Leydig cell tumor
FAP	*APC* (5q22.2)	AD	CMV-PTC	~2-10%	e.g. polyposis, colon cancer
Werner	*WRN* (8p12)	AR	PTC, FTC, ATC	~18%	Adult progeria
Pendred	*SLC26A4* (7q21–34)	AR	MNG FTC	~80% ~1%	Congenital deafness

PTHS; *PTEN* hamartoma tumor syndrome, FAP; familiar adenomatous polyposis; AD; autosomal dominant, AR; autosomal recessive, PTC; papillary thyroid carcinoma, FTC; follicular thyroid carcinoma, CMV-; cribriform-morular variant, ATC; anaplastic thyroid carcinoma, MNG; multi nodular goiter, *predominant phenotype.

polyposis, Werner syndrome, and Pendred syndrome (see Table 1).[61,62] However, DTC occurs as a minor component in these syndromes and the majority of apparently hereditary DTC is still genetically unaccounted for. While genome-wide association studies (GWAS) have identified associations with polymorphisms at various loci, additional studies are needed to determine their role in DTC tumorigenesis.[63-65] While the majority of patients with MTC have sporadic disease, 25-30% of cases are diagnosed with MEN2 syndrome (see below) resulting from germline *RET* mutations.[26]

DICER1 syndrome

First reported in 2009, DICER1 syndrome is a rare autosomal dominant inherited disorder that predisposes to a variety of cancerous and noncancerous tumors of mostly pediatric and adolescent onset (see Figure 3).[66] The *DICER1* gene encodes a ribonuclease III enzyme that is crucial for the cleavage of noncoding small RNA precursors to generate mature micro-RNAs (miRNAs), which in turn post-transcriptionally regulate expression of many genes.[67] *DICER1* genetics is consistent with a tumor suppressor two-hit model, whereby a germline inactivating mutation is coupled to a missense "hotspot" mutation within the functional ribonuclease (RNase) IIIb domain in tumor DNA.

Pleuropulmonary blastoma (a rare pediatric lung tumor; PPB), cystic nephroma (CN), ovarian Sertoli-Leydig cell tumor (SLCT) and thyroid neoplasia are the hallmark tumors of DICER1 syndrome.[68] Due to the phenotypic rarity of associated tumors (e.g. PPB, CN and SLCT), the prevalence of DICER1 syndrome was assumed to be low. However, it has recently been estimated that the population incidence of germline *DICER1* mutations could be as high as ~1:2,529 to 1:10,600, based on publicly-available germline whole-exome sequence datasets.[69] The TCGA

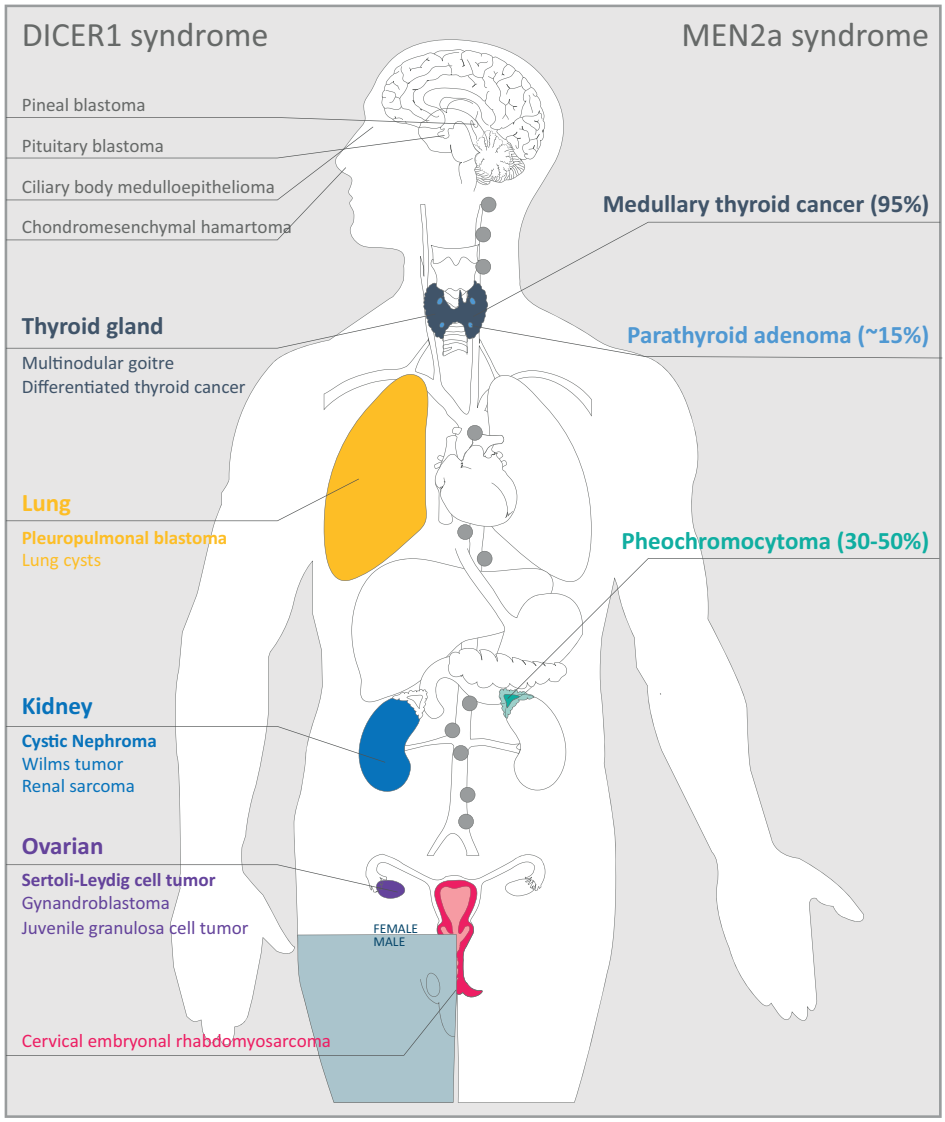


Figure 3. DICER1 syndrome (left) and MEN2a syndrome (right) associated tumors. Clinical hallmarks in bold. Between brackets; estimated MEN2a disease penetrance, DICER1 syndrome disease penetrance is unknown.

database showed germline *DICER1* mutations in ~1:4600 adult cancer cases.[70] The penetrance of each of the *DICER1*-related conditions is not fully understood, but is suggested to be low-to-moderate.[71] Despite reduced disease penetrance, identification of *DICER1* mutation carriers is important, since clinical surveillance is focused on early detection of PPB, and early tumor stages are associated with lower mortality.[72] Large international prospective studies are needed to evaluate and optimize current screening guidelines.[73,74]

Multiple endocrine neoplasia type 2a

MEN2a syndrome is caused by heterozygous germline *RET* mutations, and is characterized by the presence of MTC (>95%), PHEO (40-50%) and/or pHPT (10-20%), see Figure 3.[75] Furthermore, a small number of patients may present with cutaneous lichen amyloidosis or Hirschsprung's disease. Approximately 10% of all cases are caused by *de novo* mutations.[76] Current treatment and surveillance recommendations, from the American Thyroid Association (ATA), are based on the classification of specific *RET* mutations into risk levels according to genotype-phenotype correlations.[77]

Genetic predisposition for parathyroid tumors

A genetic predisposition for pHPT can be found in approximately 10% of pHPT cases and to date, pathogenic variants in at least 11 genes have been associated with hereditary pHPT.[78] The most commonly identified hereditary syndromes associated with pHPT are listed in Table 2, and include MEN type 1, 2a, or 4, *CaSR*-, and *CDC73*-related disorders (see below).[79-81] Disease penetrance and phenotype (predominantly parathyroid hyperplasia, PA or PC) varies among the different syndromes. Therefore, early identification of hereditary pHPT is crucial for optimal clinical and surgical management, e.g. minimal invasive procedure or bilateral neck exploration with (sub) total parathyroidectomy.[34]

CDC73-related syndrome

Inactivation of the *CDC73* tumor suppressor gene (formerly known as *HRPT2* and encoding parafibromin) predisposes heterozygous mutation carriers to pHPT and less frequently, ossifying fibromas of the jaw and/or a variety of benign and malignant renal/uterine lesions (see

Table 2. Hereditary syndromes associated with primary hyperparathyroidism

Syndrome	Gene (locus)	Inheritance	Parathyroid phenotype*	pHPT penetrance	Mean age pHPT	Syndromic features
MEN1	*MEN1* (11q13)	AD	Hyperplasia	95%	20-25y	e.g. pituitary adenoma, pNET, carcinoid
MEN2	*RET* (10q11.21)	AD	Adenoma	20-40%	35–41y	MTC, PHEO
MEN4	*CDKN1B* (12p13.1)	AD	Hyperplasia	High?	36–79y	*Similar to MEN1*
HPT-JT (Figure 4)	*CDC73* (1q31.2)	AD	Adenoma (e.g. cystic, atypical), carcinoma	80-95%	early adulthood	Ossifying fibroma jaw, renal- and uterine lesions
FIHP	*CASR*^ (3q21.1)	AD	Adenoma	High?		None

FIHP; familiar isolation hyperparathyroidism, AD; autosomal dominant, pHPT; primary hyperparathyroidism, y; years; pNET; pancreatic neuro-endocrine tumor (secreting or non-secreting), MTC; medullary thyroid carcinoma; PHEO; pheochromocytoma,^ *CASR* mutations are also associated with other health conditions, *predominant phenotype.

Figure 4).[34,82] pHPT onset is typically in late adolescence or early adulthood and penetrance has been reported to be as high as 80-95%.[34] In contrast to sporadic cases and other hereditary pHPT syndromes, PCs may be found in up to 15-20% of patients with germline *CDC73* mutations.[34] The majority of germline (and somatic) *CDC73* mutations are frameshift and nonsense variants found in exons 1, 2 and 7, although missense variants as well as (small) deletions and insertions

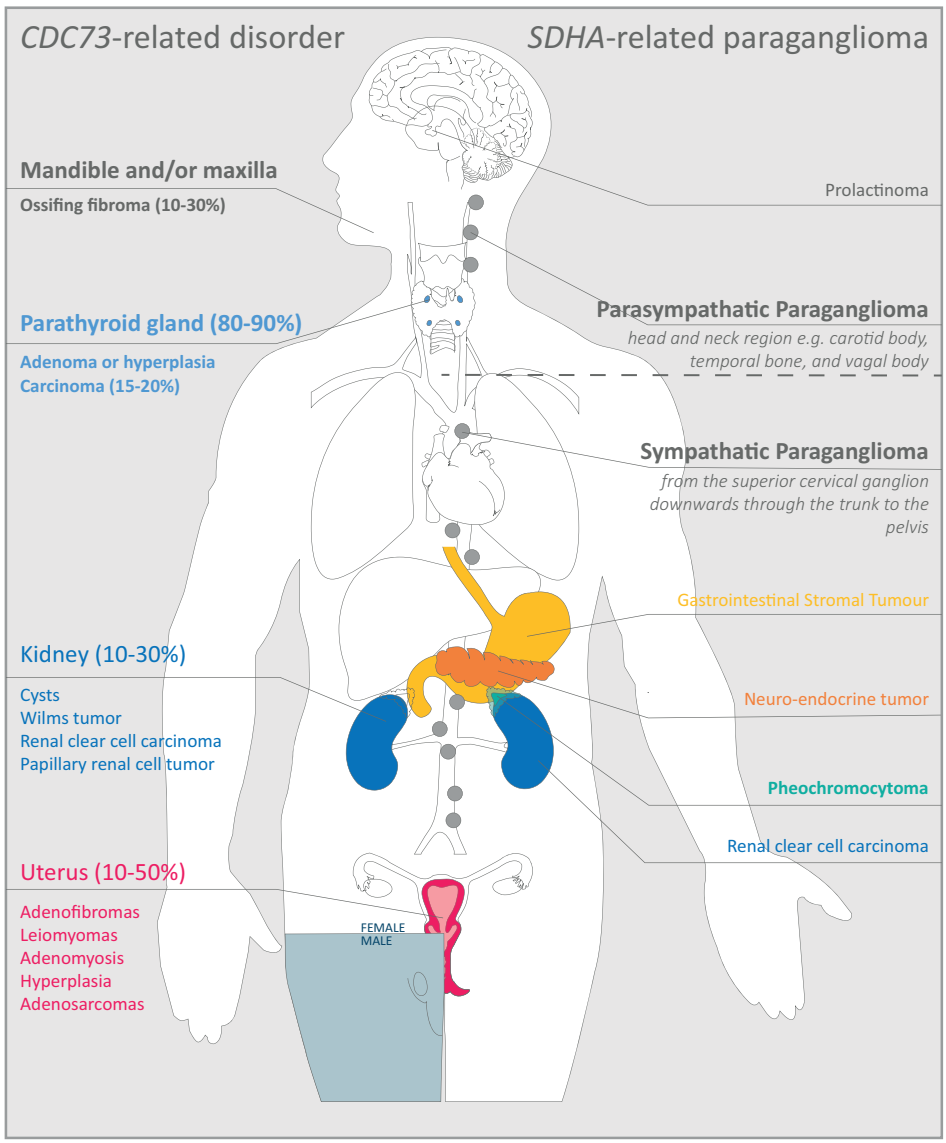


Figure 4. *CDC73*-related disorder (left) and *SDHA*-related paraganglioma (right) associated tumors. Clinical hallmarks in bold. Between brackets; estimated *CDC73*-related disorder disease penetrance. *SDHA*-related disease penetrance unknown.

have been reported.[83-85] No clear phenotype-genotype relationship has been identified in the approximately 120 index *CDC73* mutation carriers described to date.[34]

Genetic predisposition to paraganglioma

About one third of the PGL patients reportedly carry germline mutations in a growing list of susceptibility genes.[86] The best described genes, summarized in Table 3, are: *NF1, RET, VHL, SDHD, SDHC, SDHB, SDHAF2, SDHA (see below), TMEM127* and *MAX*. Germline mutations in the succinate dehydrogenase (*SDH*) genes are the most common genetic cause of PGLs, occurring in up to 15% of all PGL patients and half of all familiar cases.[50,87] In the last decade at least 12 additional genes have been associated with PGL, mostly in case reports (*BAP1, DNMT3A, EGLN1, KIF1Bβ, IHD, FH, MITF, MEN1, MDH2, PHD1, PHD2/EPAS1, and SLC25A11*) and it is likely that further rare and/or low-penetrant genes will be identified.

Table 3. Hereditary syndromes associated with paraganglioma and pheochromocytoma

Syndrome	Gene (locus)	Year report^	Inheritance	Mutation yield	PGL vs PHEO*	Multiple	Metastatic risk	Syndromic features
PGL1	*SDHD* (11q23)	2000	Paternal	8-9%	HNPGL	~50%	Low	Gastro intestinal stromal tumor, prolactinoma, RCC, pNET
PGL2	*SDHAF2* (11q13)	2009	Paternal	<0.1%	HNPGL	~90%	Low	
PGL3	*SDHB* (1q21)	2001	AD	10-25%	PGL	~20%	~50%	
PGL4	*SDHC* (1p35-36)	2000	AD	2-8%	PGL	~20%	Low	
PGL5 (Figure 4)	*SDHA* (5p15)	2010	AD	0.6-3%	HNPGL	Rare	Low	
	MAX (14q23)	2011	Paternal	~1%	PHEO	~60%	~25%	None
	TMEM127 (2q11)	2010	AD	~2%	PHEO	~25%	Low	None
NF1	*NF1* (17q11.2)	1990	AD *(cave de novo)*	<5%	PHEO	~15%	Low	Neurofibromas, café au lait macules, freckling
VHL	*VHL* (3p25-26)	1993	AD *(cave de novo)*	2-11%	PHEO	~40%	<5%	Hemangioblastomas, RCC, pNET
MEN2 (Figure 3)	*RET* (10q133.1)	1994	AD *(cave de novo)*	<5%	PHEO	~60%	<5%	MEN2a: MTC, pHPT MEN2b: neuromas, marfanoid habitus

AD; autosomal dominant, PGL; paraganglioma, HNPGL; head and neck paraganglioma; PHEO; pheochromocytoma, pNET; pancreatic neuro-endocrine tumor, RCC; renal cell carcinoma, MTC; medullary thyroid carcinoma; pHPT; primary hyperparathyroidism, ^direct association between gene and disease, *predominant phenotype.

SDHA-associated paraganglioma

In 2010, a direct association between germline *SDHA* mutations and PGL was reported.[88] The clinical phenotype seems to be comparable with the other SDH genes; e.g. predominately characterized by PGLs, with an additional risk of developing other tumor types such as clear cell renal cancer (RCC), gastrointestinal stromal tumors (GIST) and more rarely, neuroendocrine tumors (NET) and pituitary adenomas (see Figure 4)[89-91] Moreover, germline *SDHA* variants were recently identified in children and adults with various cancers, although a direct association has not been proven.[92] *SDHA* variants are also observed at an unexpectedly high frequency in the general population (Genome Aggregation Database cohort, public available genomic database), with ~1% and ~0.1% harboring a rare missense or loss of function variant, respectively.[93] To date, 39 unique (likely) pathogenic *SDHA* variants have been reported in about 100 index PGL patients, most of which were nonsense or frameshift variants, with the remainder made up of splice site and missense variants.[94-96] Of the index cases, half presented with HNPGL, whereas the remainder manifested either with PHEO or SPGL. The mean age at diagnosis was 40 years (range 15-81), with an equal gender distribution. Germline *SDHA* mutations have been associated with an increased risk of metastatic disease.[95] Notably, few patients reported a positive family history for (possibly) *SDHA*-associated disease, suggesting that the overall penetrance is substantially lower compared to the other SDH genes. The latter conclusion is supported by the high *SDHA* variant frequency in the general population.[93]

Q3: HOW CAN WE PREVENT CANCER?

Ideally, mutation carriers should be enrolled in specific surveillance programs that have been designed to improve their prognosis. In addition, genetic risk factors can be addressed in clinical practice by educating families and their treating physicians about early signs of disease. Collaboration between among others the departments of endocrinology, oncology, surgery, pathology, chemistry, radiology, nuclear medicine and clinical genetics is of the utmost importance. However, the advantages of early tumor detection should be weighed against the disadvantages of tumor screening, e.g. false positive and negative results, potential risk due to the screening modality itself (e.g. radiation), anxiety, negative emotional impact and healthcare costs.

In summary, with the implementation of high-throughput DNA/RNA sequencing platforms, somatic and germline genetic information may provide answers to the question *"Why do I have cancer?"*. Furthermore this molecular information has the potential to improve endocrine tumor classification, prognostic forecasting, the development of personalized treatment and the identification of patients at high risk for tumor development. Identification of endocrine predisposition syndromes, i.e. *Are other relatives at risk?,* cannot be seen separately from the question *"Do these relatives need to undergo surveillance?"*. Current challenges in known tumor predisposition syndromes include accurate estimates of variant pathogenicity, disease penetrance, genotype-phenotype relationships and the variable phenotypes within families, and from there to tailored treatment and surveillance guidelines. Clinical information on the rare endocrine tumor syndromes studied in this thesis, e.g. *DICER1*-related TC, *CDC73*-related disorder, and *SDHA*-associated PGL, has so far been limited to small case series.

OBJECTIVES AND OUTLINE OF THIS THESIS

The main objectives of this thesis were:

1. To investigate the role of molecular testing in TC diagnostics and treatment decision making.
2. To improve knowledge of the genetic background of pediatric non-medullary TC by:
   - determining the contribution of mutations in known cancer predisposition genes, and
   - identifying novel TC susceptibility genes.
3. To further delineate the genotype and phenotype of known endocrine tumor predisposition syndromes, i.e**.** DICER1 syndrome, MEN2a syndrome, *CDC73*-related disorder and *SDHA*-associated PGL.

Thesis outline:

Part I. The role of molecular testing in endocrine cancer diagnostics and treatment decision making

In **Chapter 2** we perform genetic characterization of 10 *DICER1*-related TC and report on follow-up of affected individuals. In **Chapter 3** we determine the contribution of somatic gene fusions in RAI-R TC, with the intention to stratify for targeted therapy.

Part II. Identification of genetic predisposition in pediatric non-medullary thyroid carcinoma

Chapter 4 describes the first results of a whole genome study investigating the contribution of mutations in known cancer predisposition genes and novel TC susceptibility genes in pediatric patients with non-medullary TC.

Part III. Genetic counseling in endocrine tumor predisposition syndromes

In **Chapter 5** we describe the clinical manifestations and penetrance in *CDC73*-related disorders and formulate recommendations to improve case detection in pHPT. In **Chapter 6** we estimate the contribution of germline *SDHA* mutation in PGL patients, assess the clinical manifestations and determine the age-related penetrance. **Chapter 7** describes an unusual case of apparent non-penetrance in a family with MEN2a.

Part IV: General discussion

Chapter 8 summarizes the main findings this thesis in the context of the current literature. Moreover, future perspectives for genetic testing will be discussed in a broader context.

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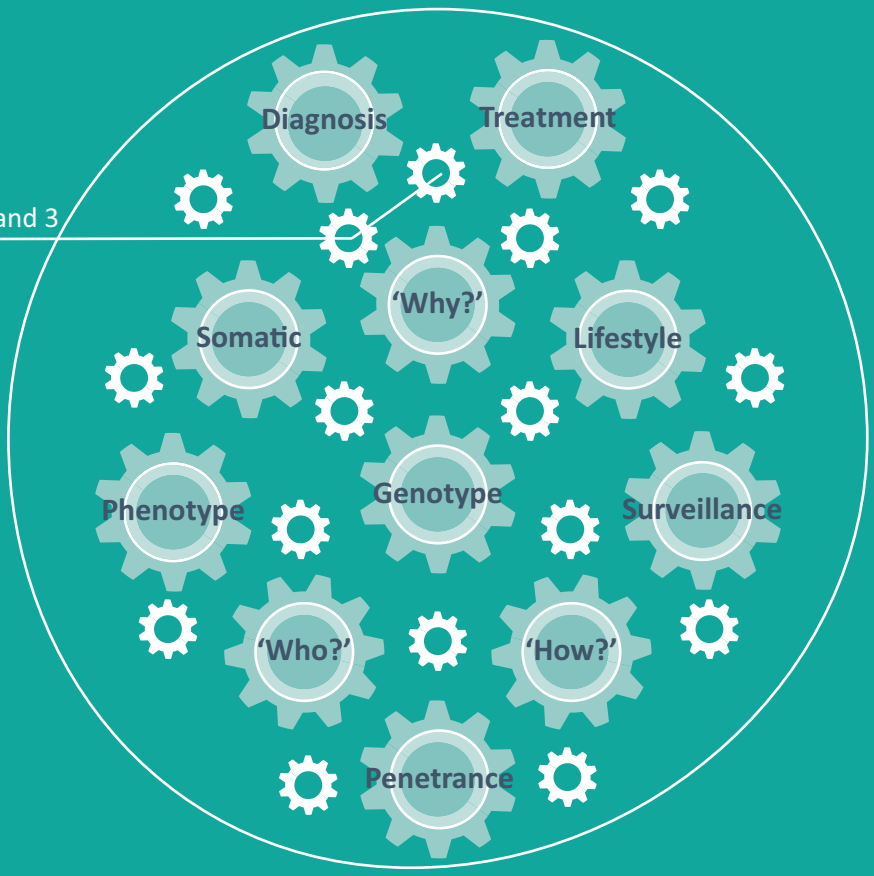
Part I
Chapter 2 and 3



PART I

THE ROLE OF MOLECULAR TESTING IN ENDOCRINE CANCER DIAGNOSTICS AND TREATMENT DECISION MAKING

This chapter describes the genetic characterization of 10 DICER1-related thyroid carcinomas and report on follow-up of affected individuals.

Clinical and Molecular Characteristics May Alter Treatment Strategies of Thyroid Malignancies in DICER1-syndrome

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J Clin Endocrinol Metab. 2019 Feb 1;104(2):277-284

ABSTRACT

Context

DICER1 syndrome is a rare autosomal-dominantly inherited disorder that predisposes to a variety of cancerous and noncancerous tumors of mostly pediatric and adolescent onset, including differentiated thyroid carcinoma (DTC). DTC has been hypothesized to arise secondarily to the increased prevalence of thyroid hyperplastic nodules in syndromic patients.

Objective

To determine somatic alterations in DICER1-associated DTC and to study patient outcomes.

Design

Retrospective series.

Setting

Tertiary referral centers.

Patients

Ten patients with germline pathogenic *DICER1* variants and early-onset DTC.

Methods

Somatic *DICER1* mutation analysis, extensive somatic DNA variant and gene fusion analyses were performed on all tumors.

Results

Median age at DTC diagnosis was 13.5 years and there was no recurrent or metastatic disease (median follow-up, 8 years). All thyroid specimens showed diffuse nodular hyperplasia with at least one focus suspicious of DTC but without infiltrative growth, extrathyroidal extension, vascular invasion, or lymph node metastasis. Most of the individual nodules (benign and malignant) sampled from the 10 tumors harbored distinct *DICER1* RNase IIIb hotspot mutations, indicating a polyclonal composition of each tumor. Furthermore, nine of 10 DICER1-related DTCs lacked wellknown oncogenic driver DNA variants and gene rearrangements.

Conclusion

On the basis of our clinical, histological, and molecular data, we consider that most DICER1-related DTCs form a low-risk subgroup. These tumors may arise within one of multiple benign monoclonal nodules; thus, hemi-thyroidectomy or, more likely, total thyroidectomy may often be required. However, radioiodine treatment may be unnecessary given the patients' ages and the tumors' low propensity for metastases. (*J Clin Endocrinol Metab 104: 277–284, 2019*)

INTRODUCTION

DICER1 syndrome is a rare autosomal-dominantly inherited disorder that predisposes to a variety of cancerous and noncancerous tumors of mostly pediatric and adolescent onset.[1] The *DICER1* gene encodes a ribonuclease III enzyme involved in cleaving noncoding small RNA precursors to generate mature miRNAs, which in turn, posttranscriptionally regulate expression of many genes.[2]

Pleuropulmonary blastoma (PPB; a rare pediatric lung tumor), cystic nephroma, and ovarian Sertoli-Leydig cell tumor are the hallmark tumors of DICER1 syndrome. The broad tumor spectrum includes rare entities such as botryoid embryonal rhabdomyosarcoma of the uterine cervix, ciliary body medulloepithelioma, pineoblastoma, pituitary blastoma, and nasal chondromesenchymal hamartoma.[3] Furthermore, patients with DICER1 syndrome are at increased risk of developing multinodular goiter (MNG) compared with family controls and differentiated thyroid cancer (DTC) compared with population data from the National Cancer Institute SEER program.[4] It is possible that the increased risk of thyroid malignancy in *DICER1* heterozygotes is secondary to the greatly increased prevalence of benign hyperplastic thyroid nodules (i.e., MNG) in this syndrome. Alterations in *DICER1* are consistent with a two-hit tumor suppressor model, whereby a germline loss-of-function variant is followed by a second somatic mutation. However, in contrast to the typical two-hit model, in the case of *DICER1*, the second hit is most often a missense "hotspot" variant within the sequence encoding the RNase IIIb domain.[5] Studies have shown that somatic *DICER1* hotspot variants are present in benign and malignant thyroid nodules from patients with germline pathogenic *DICER1* variants[4,6,7], as well as those with sporadic adolescent-onset DTC.[8] Furthermore, different somatic *DICER1* variants may be present in distinct thyroid nodules resected from the same individual.[6]

In contrast to sporadic thyroid carcinomas in which point mutations (e.g., of *BRAF* and *RAS* genes), as well as gene fusions (e.g., *RET-PTC 1-12*, *PPARg-PAX8*, *ALK*, and *NTRK*), lead to tumorigenesis and progression through activation of the mitogen-activated protein kinase pathway [9-12], limited data are available on the acquired genetic alterations that induce malignant transformation of DICER1-associated MNG.[13] In this study, we performed genetic characterization of 10 DICER1-related thyroid carcinomas and report on follow-up of the affected persons.

PATIENTS AND METHODS

Study population and design

We studied 10 patients from eight families with germline pathogenic *DICER1* variants who had young-onset nodular thyroid hyperplasia containing at least one reported focus of DTC, diagnosed between 2004 and 2017. Clinical information, pathology reports, and details of medical history were collected from the treating physicians with full patient and/or parental consent. The study was approved by the local ethical committee of the Leiden University Medical Centre (approval no. P14.312).

Histological analysis

The tumors were reviewed by pathologists at the referring institutions and by our central reference pathologist (H.M.).

Molecular analysis

Total nucleic acid (i.e., undivided DNA and RNA) was isolated from formalin-fixed paraffin-embedded tissue cores (0.6-mm diameter and variable length) or microdissected tissue regions

using a fully automated extraction procedure.[14] Broad DNA variant and gene fusion analyses were performed using the following methods. Somatic *DICER1* variant analysis of the RNase IIIa and RNase IIIb domains was performed by conventional Sanger sequencing at either Radboud University Medical Centre or McGill University and Genome Quebec Innovation Centre (primers available on request). Somatic DNA variant analysis was performed using a customized next-generation sequencing AmpliSeq Cancer Hotspot Panel (Thermo Fisher Scientific, Waltham, MA) targeting 50 genes (including *BRAF*, *NRAS*, *HRAS*, *KRAS*, *TP53*, *PTEN*, and *PIK3CA*), as previously described.[15] *TERT* promotor variant (NM_ 198253.2; c.-57A.C, c.-124C.T and c.-146 C.T) analysis was performed by Sanger sequencing.

Gene fusion analysis was performed using the FusionPlex comprehensive thyroid and lung kit, version 2, for Ion Torrent (ArcherDX, Boulder, CO), which captures relevant exons from 34 genes (including *RET*, *NTRK1-3*, and *ALK*) according to the manufacturer's protocol. Data analysis was performed using the online Archer Analysis software, version 5.0 (analysis. archerdx.com). Only "strong-evidence" fusions called by the software were reported. This relatively new method was first validated on 56 formalin-fixed paraffin-embedded DTC samples (data not shown).

RESULTS

Clinical characteristics

In total, 10 patients (from eight different families) with DICER1-related thyroid carcinomas were included in this study. Details on six of these cases have been previously published (Table 1). [6,16-19] The mean age (±SD) at DTC diagnosis was 14.7± 6.2 years (range, 7 to 28 years), with a female predominance (70%). Median follow-up after thyroid cancer diagnosis was 8 years (range, 1 to 13 years). All patients in our series underwent total thyroidectomy and eight were treated with adjuvant radioactive iodine according to guidelines or expert opinion at the time. Six patients were diagnosed with at least one other DICER1-related tumor before the DTC diagnosis (Table 1).

Histological characteristics

Each of the 10 thyroid specimens showed diffuse nodular hyperplasia with multiple, discrete, well-circumscribed, and occasionally encapsulated nodules. In seven cases, at least one focus of follicular variant of papillary thyroid carcinoma (FVPTC) was considered during re-evaluation. The diagnosis of thyroid cancer was based primarily on nuclear features such as nuclear enlargement and overlap, irregularly shaped follicles, presence of nuclear clearance, and few mitotic figures. In three of these cases, the lesion was encapsulated or well demarcated without solid features. As such, the diagnosis of noninvasive follicular thyroid neoplasm with papillary-like nuclear features [20,21] was also considered. In the remaining four FVPTC samples (with no clear capsule or demarcation), dominant lesions in the context of hyperplasia was considered given the subtle nuclear characteristics. Poorly differentiated thyroid carcinoma was diagnosed in two patients (patients 4 and 6). No infiltrative growth, vascular invasion, extrathyroidal extension, or lymph node or distant metastasis were identified in any of the cases. On retrospective analyses, the histology of patient 8's right thyroid lobe lesion, which was resected 8 years earlier and which was classified and treated as benign (dominant lesions in the context of hyperplasia), had similar features to the presumed-malignant lesion from the left lobe (Fig. 1). An overview and detailed histology of all tumors is available in Supplemental Fig. 1 and full histological images are available at www.hereditarypathology.com upon request.

Molecular characteristics

We sampled between one and 11 regions from each of 10 thyroid specimens, totaling 35 regions (18 samples were classified as DTC and 17 were classified as hyperplastic nodules). Somatic *DICER1* variants were identified in 15 of 18 previously classified carcinoma samples and in 16 of 17 investigated benign nodules. We found a total of 11 distinct *DICER1* variants affecting five different residues within the RNase IIIb domain (namely, p.Glu1705, p.Asp1709, p. Glu1809, p.Glu1810, and p.Glu1813). Furthermore, loss of heterozygosity of the wild-type allele was present in both lesions from patient 4 who has a predisposing mosaic RNase IIIb hotspot mutation. In patient 8's tumor, we identified the same c.5438A.T somatic *DICER1* variant in the dominant lesion [classified as FVPTC (T1)] and in the surrounding hyperplasic lesion (L10). No additional known thyroid carcinoma diver DNA variants were found in the FVPTC (Fig. 1, II; Table 1).

Remarkably, in 14 of the 15 investigated carcinoma samples, neither common thyroid carcinoma driver DNA variants, nor gene rearrangements were identified. One pathogenic *TP53* variant was identified in a poorly DTC (patient 6). TERT promotor variants, associated with more aggressive carcinoma, were not present in the seven investigated tumors, including both poorly differentiated tumors.

DISCUSSION

In this study, we investigated the clinical, histological, and molecular characteristics of 10 thyroid tumors from young patients with germline/mosaic pathogenic *DICER1* variants. Somatic *DICER1* RNase IIIb hotspot variants were identified in most reported carcinomas and adjacent benign nodules. Secondary somatic *DICER1* variants were therefore not discriminative between benign and malignant disease. However, the identification of these distinct somatic variants in separate presumed-malignant nodules sampled from individual patients' lesions indicates that the tumors are polyclonal lesions, as has been seen in hyperplastic nodules. [4,6] Furthermore, nine of the 10 DICER1-related thyroid carcinomas lacked well-known oncogenic driver DNA variants (e.g,. *BRAF*, *RAS*) and gene rearrangements (e.g., *RET/PTC1-12*, *PPARg-PAX8*, *ALK*, and *NTRK*) that are frequently observed in sporadic thyroid carcinomas. Consistent with our findings, TERT promotor variants have been found to be rare in sporadic pediatric DTC (absent in all 77 tested cases). [22,23] In addition to these molecular findings, occasional ambiguous histological features and lack of extrathyroidal extension, infiltrative growth, vascular invasion, or lymph node or distant metastasis (at a mean follow-up of 8 years), may prompt reconsideration of the diagnosis of carcinoma in a subset of these DICER1-related tumors. Even if these tumors are classified as carcinomas, it appears their malignant potential is limited, and these data lead us to conclude that most DICER1-related DTCs form a low-risk subgroup. Whether this is also the case for DICER1-related poorly differentiated DTC should be determined.

Twelve independent studies (including the current study) have reported thyroid cancer in a total of 31 patients with germline pathogenic *DICER1* variants and/or DICER1 syndrome–related features (Supplemental Table 1). [1,4,7,16-18,24-28] As in previous studies, a subset of our patients (n = 3) had a history of extensive radiation as part of standard PPB diagnosis and treatment. We did not identify gene rearrangements in lesions from these patients despite such alterations being common in thyroid neoplasia from patients with a history of exposure to ionizing radiation through treatment or nuclear power plant accidents. [29,30] Furthermore, research has not suggested that DICER1-associated thyroid cancer is more invasive or less responsive to therapy. [4] On the contrary, recurrent or persistent disease has not been described in any patients reported to date, with a median follow-up of >5 years.

Table 1. The clinical, histological and molecular characteristics of ten *DICER1* mutation carriers with reported thyroid carcinoma

		Histology (macroscopic/microscopic)			Somatic molecular analysis		
ID	Sex / age at Dx DTC	Thyroid histology# (see suppl. Figure 1)	Multi-focal	Lesion (size, mm)	*DICER1*	Other DNA variant	Gene fusion
3	M/11	PTC	Y	T1	c.5113G>A, p.Glu1705Lys	ND* (no *BRAF/ RAS* variants in FusionPlex)	None identified
4	F/10	PDTC	Y	T1 (4mm)	LOH	None identified	None identified
				T2 (2mm)	LOH	None identified	None identified
5	F/15	FVPTC (or NIFTP)	N	T1 (17mm)	c.5437G>A, p.Glu1813Lys	None identified	None identified
6	F/14	PDTC	Y	T1 (5mm)	c.5437G>C, p.Glu1813Gln	*TP53:* c.1027_1033del 7bp, p.Glu343_ Asn345del fs	None identified
				L1 (12mm)	c.5437G>C, p.Glu1813Gln	ND	ND
7¥	F/23	FVPTC (or DHL)	Y	T1a (3mm)	c.5125G>A, p.Asp1709Asn	ND	ND
				T1b (18mm)	c.5125G>A, p.Asp1709Asn	None identified	ND
				T2 (20mm)	c.5126A>G, p.Asp1709Gly	ND	ND
				T3 (15mm)	c.5437G>A, p.Glu1813Lys	None identified	None identified
		DHL		L1 (20mm)	c.5438A>T, p.Glu1813Val	ND	ND
				L2 (1mm)	c.5126A>G, p.Asp1709Gly	ND	ND
				L3 (2mm)	c.5428G>T, p.Asp1810Tyr	ND	ND
				L4 (4mm)	c.5429A>T, p.Asp1810Val	ND	ND

	Clinical Information					
	hTERT	Personal history (age at Dx)	Follow up DTC	Family history	Germline *DICER1* variant	Reference
	ND	PPB type II (2y), CN (2y), Askin tumour (13y)	5y	PPB, CN, MNG, PitB	c.2379T>G, p.Tyr793*	de Kock *et al*. JCEM, 2014a (case 3) and ANP, 2014b (individual V-1)
	None identified ND	Bilateral renal and lung cysts (2y), Pineoblastoma (7y), bilateral SLCT (13y, 15y), CBME (17y)	12y	None	c.5437G>C, p.Glu1813Gln (mosaic)	de Kock *et al*, JMG 2016 (case 2)
	None identified	Lung cysts	2.5y	MNG	c.3999C>A, p.Cys1333*	Not previously published
	None identified ND	None	12y	MNG, SLCT	c.2256+1G>C, Splice variant	Not previously published
	ND None identified ND None identified ND ND ND ND	None	13y	MNG, PPB and ID 8	c.988G>A, p.Gln330*	Not previously published

Table 1. (continued)

		Histology (macroscopic/microscopic)			Somatic molecular analysis		
ID	Sex / age at Dx DTC	Thyroid histology# (see suppl. Figure 1)	Multi-focal	Lesion (size, mm)	*DICER1*	Other DNA variant	Gene fusion
8¥	F/28	FVPTC (or DHL)	N	T1 (3mm)	c.5438A>T, p.Glu1813Val	None identified	None identified
		DHL (R)*		L1-2 (5-15mm)	c.5113G>A, p.Glu1705Lys	ND	ND
				L3-4 (5mm)	c.5126A>G, p.Asp1709Gly	ND	ND
		DHL (L)		L5 (2mm)	c.5429A>T, p.Asp1810Val	ND	ND
				L6-10 (2-15mm)	c.5438A>T, p.Glu1813Val	ND	ND
9^	F/13	FVPTC (or DHL)	Y	T1 (12mm)	None identified	None identified	None identified
				T2 (5mm)	c.5126A>G, p.Asp1709Gly	ND	ND
				T3 (5mm)	c.5127T>G, p.Asp1709Glu	None identified	None identified
				T4 (6mm)	None identified	None identified	None identified
		DHL		L1 (2mm)	c.5113G>A, p.Glu1705Lys	ND	ND
10^	M/17	FVPTC (or NIFTP)	Y	T1 (2mm)	None identified	None identified	ND
				T2 (4mm)	c.5427_5428delinsTT, p.Asp1810Tyr	None identified	None identified
		DHL		L1 (7mm)	None identified	None identified	ND

Abbreviations: Dx, diagnosis; M, male; F, female; y, year; PPB; pleuropulmonary blastoma; MNG, multinodular goitre; CBME, ciliary body medulloepithelioma; CN, cystic nephroma; SLCT, Sertoli-Leydig cell tumour; PitB, pituitary blastoma; FVPTC, follicular variant papillary thyroid carcinoma; DHL, dominant hyperplastic lesion; NIFTP, non-invasive follicularthyroid

	Clinical Information				
hTERT	Personal history (age at Dx)	Follow up DTC	Family history	Germline *DICER1* variant	Reference
ND	None	1y	See ID 7	c.988G>A, p.Gln330*	Not previously published
ND					
ND					
ND					
ND					
None identified	None	8y	ID 10	c.1363del, p.Val455fs	Diets *et al*. Clin Cancer Res. 2018 (sister ID21)
ND					
None identified					
ND					
ND					
ND	MNG (13)	7y	See ID 9	c.1363del, p.Val455fs	Diets *et al*. Clin Cancer Res. 2018 (ID21)
None identified					
None identified					

neoplasm with papillary-like nuclear features; PTC, papillary thyroid carcinoma; PDTC, poorly differentiated thyroid carcinoma; L, left side; R, right side; LOH, loss of heterozygosity; ND, not done. # All in the context of diffuse nodular hyperplasia with multiple, discrete, well-circumscribed and occasionally encapsulated nodules; ¥Cousins; ^Siblings

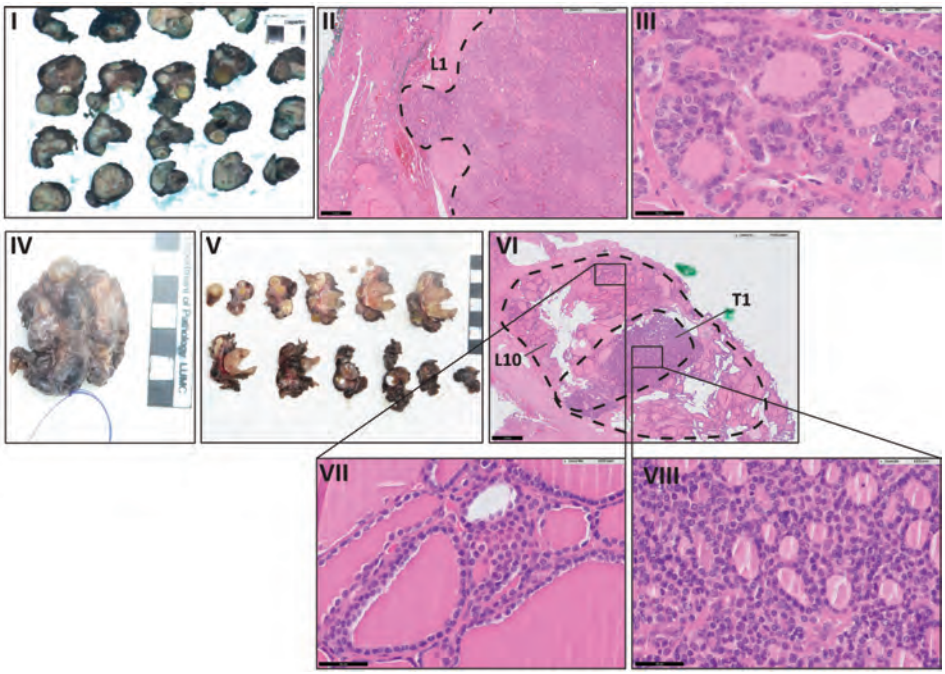

Figure 1. Histology patient ID#8. Panel 1, macroscopy hemi thyroidectomy right; Panel II and III, hematoxylin and eosin stain (HE) (×25 /×200) showing hyperplastic thyroid nodule with a somatic *DICER1* RNase IIIb variant (p.Glu1705Lys), no further DNA variant or gene fusion analysis was performed; Panel IV-V hemi thyroidectomy left, Panel VI, HE (×25) showing 2mm follicular variant papillary thyroid carcinoma (T1) in the context of a non-encapsulated hyperplastic nodule (L10) both with the same somatic *DICER1* RNase IIIb variant (p.Glu1813Val); Panel VII, HE L10 (×200) showing irregularly enlarged colloid-filled follicles, bordered by a flattened epithelium; Panel VIII, HE T1 (×200) showing nuclear features such as nuclear enlargement, indentations and presence of nuclear clearance.

DICER1 is involved in the production of miRNAs, which, in turn, posttranscriptionally regulate gene expression; therefore, we cannot rule out that malignant transformation in DICER1-related thyroid neoplasms may be driven by variations in gene expression without alterations of DNA sequence. Dysregulated miRNAs are associated with cancer initiation and progression in several tumor types .[31] miRNAs can act as both tumor suppressors and oncogenes; each miRNA has multiple mRNA targets, and each mRNA can be the target of multiple miRNAs. More than 100 miRNAs, both upregulated and downregulated, are reported in DTC, but only a few are described in the majority of the studies. Furthermore, some differentially expressed miRNAs have been described as being both upregulated and downregulated in different studies.[32] Recent, small RNA-sequencing studies reported 13 common upregulations (including miR-146b-5p, miR-221-3p, and miR222-3p) and 17 downregulations (including miR-7-3p, miR-204-5p, and miR-1179) in sporadic papillary thyroid carcinoma. The DICER1 RNase IIIb domain is responsible for cleaving the hairpin loop structure from precursor miRNAs to generate mature 5p miRNAs. In DICER1-related PPB and ovarian Sertoli-Leydig cell tumors, the presence of somatic RNase IIIb domain variants, in combination with germline loss of-function *DICER1* mutations, results in a substantial reduction in expression of 5p-derived miRNAs.[33,34] Two *DICER1*-mutated DTCs

from The Cancer Genome Atlas database showed similar overall skewed expression patterns (lower 5p and higher 3p miRNA levels) and no upregulation of commonly upregulated miRNAs in DTC (Supplemental Fig. 2).

Previous observations illustrate the complex role of miRNAs in thyroid tumorigenesis; for example, DICER1 protein levels seem to be higher in sporadic DTC, whereas *DICER1* mRNA expression is lower when compared to matched normal thyroid tissue.[35] Multiple redundant pathways and feedback loops complicate the analysis, as shown by the co-occurrence of decreased expression of *DICER1* and the let-7 miRNA family in one study[35]—*DICER1* mRNA expression is typically inversely related to let-7 levels. Moreover, let-7 was found to reduce RAS levels [36], thus interacting with the mitogen-activated protein kinase pathway, a pathway commonly altered in DTC.

In a mouse model, the arrest of mature miRNA generation in the thyroid induced progressive loss of function and cell dedifferentiation, but the mice did not have increased thyroid size or presence of nodules.[37] Despite the differences observed between human and mouse models, these studies show that *Dicer1* is required for the long-term maintenance of thyroid follicular organization and thyrocyte differentiation.

Childhood DTC is a rare disease, but is the most common endocrine malignancy in children and is the third most common solid tumor, accounting for 0.5% to 3% of all pediatric malignancies. Data from the SEER registry have shown an increased incidence of pediatric DTC, as is the case in adults. Children frequently present with more advanced disease (e.g., lymph node involvement at diagnosis, distant metastases, and multifocal disease) compared with thyroid cancer in adults.[38] Despite the excellent prognosis for pediatric patients with DTC (30-year mortality rate, <5%), morbidity caused by the treatment remains considerable.

Overdiagnosis and thus overtreatment of indolent thyroid tumors is a concern.[39] Diagnostic classification and treatment guidelines are being adapted to address this issue. The term "noninvasive follicular thyroid neoplasm with papillary-like nuclear features" was recently introduced to accommodate certain encapsulated or sharply demarcated lesions with nuclei reminiscent of papillary thyroid carcinoma that were previously classified as noninvasive encapsulated FVPTC.[21]

Until recently, the treatment of pediatric thyroid cancer was predominately based on guidelines for adult patients. In 2015, the American Thyroid Association published the first management guidelines for children with thyroid nodules and DTC.[40] Treatment decisions are guided by the extent of disease and include lobectomy or total thyroidectomy with or without radioactive iodine (RAI) therapy to treat persistent locoregional, nodal disease or distant metastases not amenable to surgery.

All patients in our series underwent total thyroidectomy and eight were treated with adjuvant RAI despite the absence of invasive growth, nodal or distant metastases. This raises concern of unnecessary exposure of a number of these young patients to adverse effects of radiation, which may include the development of second primary cancers (e.g., chronic myeloid neoplasms).[41] Other commonly reported complications of RAI treatment are salivary and lacrimal gland dysfunction, transient gonadal dysfunction, and diastolic dysfunction.[42] Even if the diagnosis of malignancy in patients with DICER1 syndrome is maintained, the behavior of the different, relatively small, distinct lesions (as indicated by the different somatic *DICER1* variants) may be indolent and the risk of recurrent disease and/or metastasis per locus seems low, based on reports published thus far. The American Thyroid Association guidelines do not recommend RAI therapy for pediatric patients with small tumors who do not have persistent locoregional disease, nodal disease, or distant metastases.[40] Furthermore, it is not known whether ionizing radiation may be more harmful in patients with DICER1 syndrome compared with sporadic cases.

In conclusion, on the basis of our clinical, histological, and molecular data, we consider that most DICER1-related DTCs form a low-risk subgroup. Because these tumors may arise from within one of multiple benign monoclonal nodules that constitute a lesion, hemithyroidectomy or total thyroidectomy could often be required, but radioiodine treatment may be unnecessary given the patients ages and the tumors' low propensity for metastases.

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40. Francis GL, Waguespack SG, Bauer AJ, et al. Management Guidelines for Children with Thyroid Nodules and Differentiated Thyroid Cancer. Thyroid 2015;25:716-59.
41. Clement SC, Peeters RP, Ronckers CM, et al. Intermediate and long-term adverse effects of radioiodine therapy for differentiated thyroid carcinoma--a systematic review. Cancer treatment reviews 2015;41:925-34.
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SUPPLEMENTAL DATA

Supplemental Table 1. Overview published patients with (suspect) DICER1-related thyroid carcinoma

Reference	ID	Gender/age (at Dx DTC, years)	Reported thyroid histology	Treatment thyroid tumor
de Kock *et al.* JCEM 2014	**Case 1***	**F/9**	**FTC**	**TT+RAI**
	Case 2	**F/7**	**FVPTC**	**TT+RAI**
	Case 3	**M/11**	**Bilateral papillary carcinoma within follicular adenoma**	**TT+RAI**
	Case 4**	F/6	FTC	TT+LND+RAI
	Case 5***	F/16	miFTC associated to a vesicular adenoma	TT
Schultz *et al.* PatholCaseRev 2014		F/8	FVPTC	TT
Puckett *et al.* Journal of Pediatric Sx Case Reports 2015	Mother	F/?	PTC	TT
Rutter *et al.* JCEM 2016	Mother	F/18	DTC	TT
	Patient A	F/12	DTC	TT
	Patient B	F/14	DTC	TT
	Brother	M/?	Multifocal PTC	TT
Durieux *et al.* Virchows Arch 2016	Case 1	F/18	(E)FVPTC	TT+LND
	Case 2	F/12	FTC	TT+LND+RAI
de Kock *et al.* J Med Genet. 2016	**Case 2**	**F/10**	**PTC**	**TT + RAI**
Yoshida *et al.* Hum Pathol. 2017		F/15	FTC	Hemithyroidectomy
Khan *et al.* JCEM 2017	NCI-77-02-004	F/41	PTC	Not specified
	NCI-63-01-001	M/18	FVPTC	Not specified
	NCI-63-02-002	F/43	miFTC	Not specified
	NCI-64-02-00	F/30	Thyroid carcinoma, papillary, macro follicular type	Not specified

Germline *DICER1* variant	Somatic *DICER1* variant thyroid tumor	Other DICER1-related conditions (age at Dx, years)	Treatment history
c.3505dupT	**p.Glu1813Asp**	**Type II PPB(2); relapsed PPB(4)**	**Sx, CTx and PBSCT**
c.3579_3580delCA	**p.Glu1813Gly**	**Type I PPB(1); CBME(6)**	**Sx and CTx**
c.2379T>G	**p.Glu1705Lys**	**Type II PPB and CN(2.7)**	**Sx and CTx**
Not tested	Not tested	PPB(3)	Sx, CTx and BMT
Not tested	Not tested	PPB(3y); cERMS(7); Bladder undifferentiated RMS(12); MNG(16)	Sx and CTx
Yes (not specified)	p.Glu1813Val	Type II PPB(5); NCMH(13.5); SLCT	Sx and CTx
Yes (not specified)	Not tested	SLCT(unknown age)	Sx and CTx
c.5441C>T	Not tested	SLCT(7 and 18)	Unknown
c.5441C>T	p.Asp1709Gly	SLCT(12), CN	None (SLCT after DTC diagnosis)
c.5441C>T	p.Gly1809Arg	None	None
c.5441C>T	p.Asp1709Gly and p.Asp1810His	None	None
Not tested	p.Glu1813Gln	SLCT(17)	Not specified
Not tested	p.Glu1813Gln	SLCT(15)	None (SLCT after DTC diagnosis)
c.5437G>C (mosaic)	**Loss of heterozygosity**	**Bilateral renal and lung csts (2), Pineoblastoma (7), bilateral SLCT (13, 15), CBME (17)**	**Not specified**
c.5426_5442del GGGATATTTTTGAGTCGinsCA	ASK: p.Glu1705Lys; FTC: p.Glu1813Asp	Anaplastic sarcoma of the kidney (ASK)	Sx, CTx, RTx and PBSCT
c.3515_3525del11insA	Not tested	Thyroid nodules(22.6); MNG(26.7)	None
c.3726C>A (p.Tyr1242*)	p.Gly1809Glu	Type II PPB (4)	Sx and CTx
c.3726C>A (p.Tyr1242*)	Not tested	MNG(22); PPB type Ir(39)	None
c.3675C>G (p.Y1225*)	p.Glu1705Lys and p.Asp1709Gly	MNG(16)	None

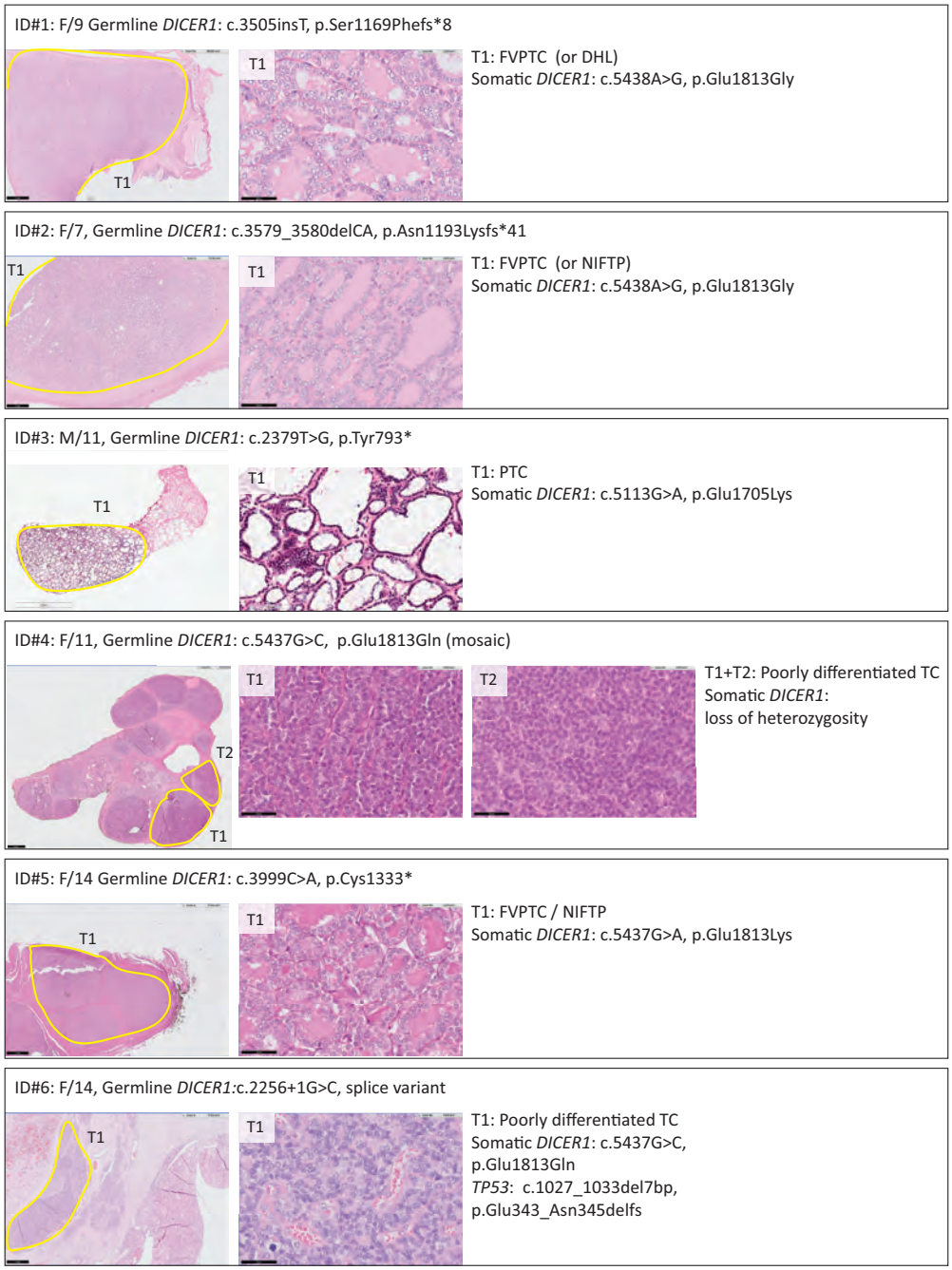
Supplemental Table 1. (continued)

Reference	ID	Gender/age (at Dx DTC, years)	Reported thyroid histology	Treatment thyroid tumor
	IPPBR 5501	F/17	FTC	Not specified
	IPPBR 5502	F/15	FVPTC	Not specified
	IPPBR 5503	F/10	Follicular thyroid carcinoma, follicular with areas of papillary	Not specified
	IPPBR 5504	F/8	PTC	Not specified
	IPPBR 5505	F/9	FVPTC	Not specified
	IPPBR 5507	F/10	FVPTC	Not specified
Gullo *et al.* Am J Clin Pathol 2018		F/12	DTC	TT
Diets *et al.* Clin Cancer Res. 2018	**ID 21**	**M/17**	**PTC**	**TT + RAI**
	Sister ID 21	**F/13**	**FTC**	**TT + RAI**

Abbreviations: Dx, diagnosis; M, male; F, female; FVPTC, follicular variant papillary thyroid carcinoma; PTC, papillary thyroid carcinoma; DTC, differentiated thyroid carcinoma; FTC, follicular thyroid carcinoma; miFTC, minimal invasive FTC, TT, total thyroidectomy; RAI, radioactive iodine treatment; PPB, pleuropulmonary blastoma; MNG, multinodular goitre; CBME, ciliary body medulloepithelioma; CN, cystic nephroma; SLCT, Sertoli-Leydig cell tumour;

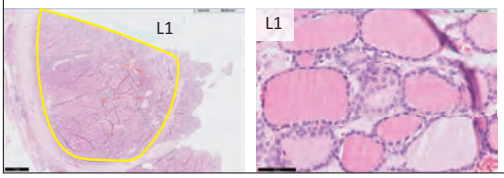
Germline *DICER1* variant	Somatic *DICER1* variant thyroid tumor	Other DICER1-related conditions (age at Dx, years)	Treatment history
Yes (not specified)	Not tested	Type II PPB (2)	Sx and CTx
not tested	Not tested	Type II PPB (3)	Sx, CTx and RTx
not tested	Not tested	Type II PPB (1)	Sx and CTx
Yes (not specified)	Not tested	Type I PPB (1)	Sx and CTx
Yes (not specified)	Not tested	Type II PPB (5)	Sx and CTx
not tested	Not tested	Pineoblastoma(?); PPB type Ir(17)	CTx and RTx
(p.Arg1060Ilefs*7)	p.Glu1813Gly and p.Asp1810Asn	Cervix ERMS(7)	Sx and CTx
c.1363del, (p.Val455fs)	**p.Asp1810Tyr**	**MNG(13)**	**None**
c.1363del (p.Val455fs)	**p.Asp1709Gly and p.Asp1709Glu**	**None**	**None**

Sx, surgery; CTx; chemotherapy, RTx; radiotherapy; BMT, bone marrow transplantation; PBSCT, peripheral blood stem cell transplantation
In bold, patients reported in this manuscript. * also Shin *et al*. Thyroid 2012; ** also Oue *et al*. PediatrBlood Cancer 2007; *** also Rome *et al*. PediatrBlood Cancer 2008



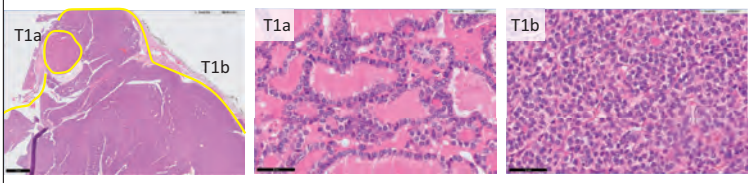
Supplemental Figure 1. Histology DICER1-associated thyroid carcinoma. Left panels overview tumor / lesion in 5 or 10x magnification, right panel(s) details 200x magnification. M; male, F; female, FVPTC; follicular variant papillary thyroid carcinoma, PTC; papillary thyroid carcinoma, TC; thyroid carcinoma; NIFTP; noninvasive follicular thyroid neoplasm with papillary-like nuclear features, T; tumor, L; lesion

ID#6: F/14, Germline *DICER1*:c.2256+1G>C, splic variant

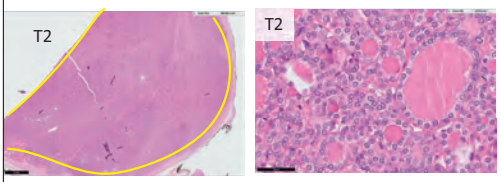


L1: Hyperplastic nodule:
Somatic *DICER1*: c.5437 G>C, p.Glu1813Gln

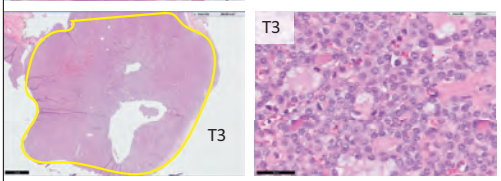
ID#7: F/23 Germline *DICER1*: c.988G>A, p.Gln330*



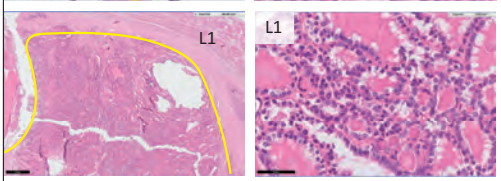
T2a+b: FVPTC (or DHL)
Somatic *DICER1*:
c.5125G>A, p.Asp1709Asn



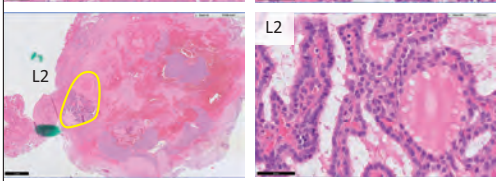
T2: FVPTC (or DHL)
Somatic *DICER1*: c.5126A>G, p.Asp1709Gly



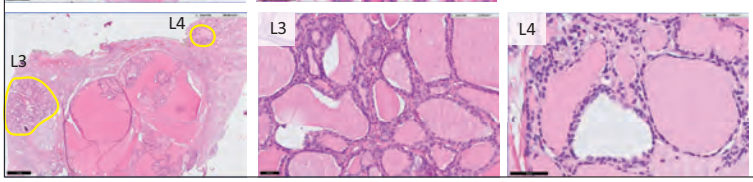
T3: FVPTC (or DHL)
Somatic *DICER1*: c.5437G>A, p.Glu1813Lys



L1: Hyperplastic nodule:
Somatic *DICER1*: c.5438A>T, p.Glu1813Val



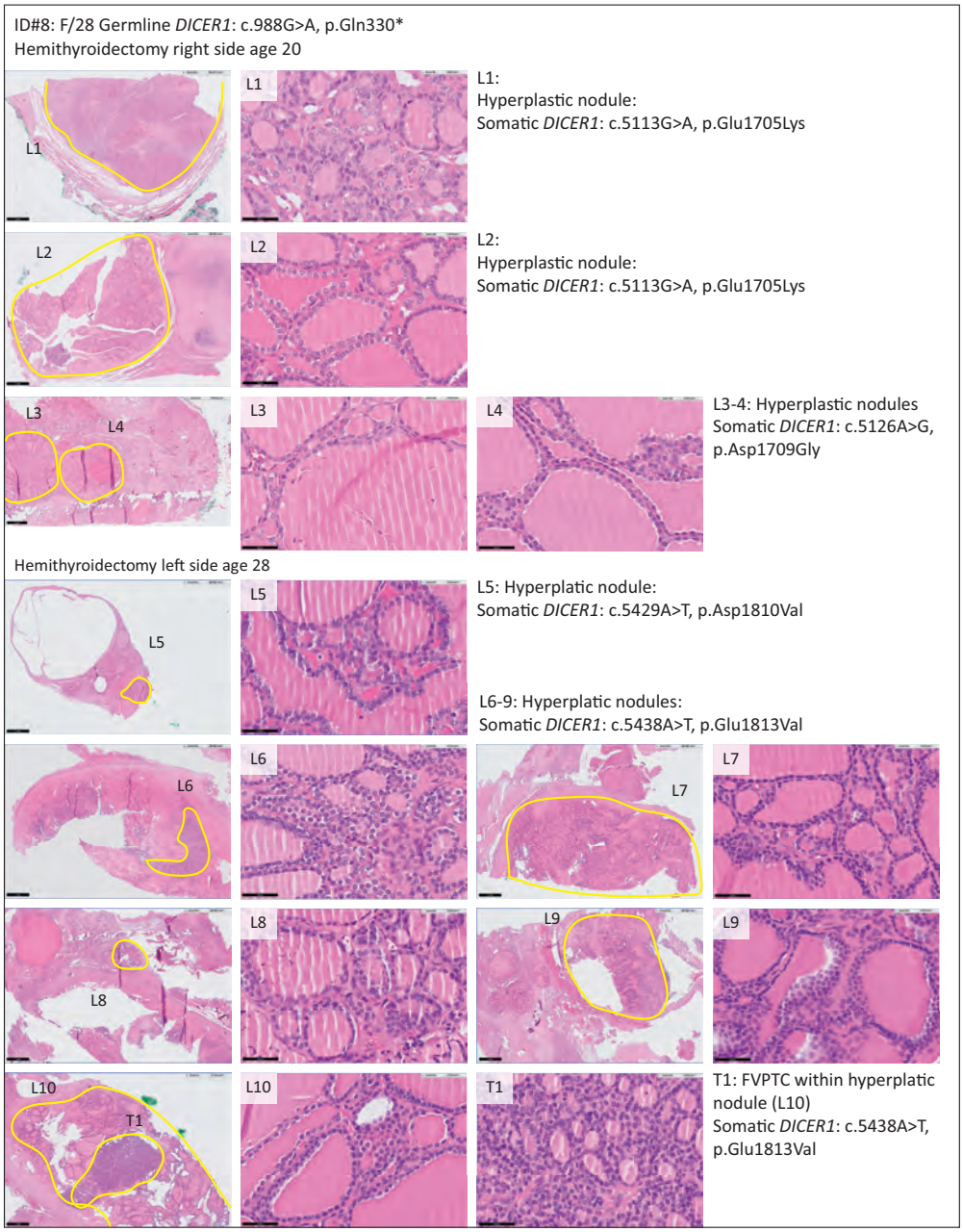
L2: Hyperplastic nodule:
Somatic *DICER1*: c.5426A>G, p.Asp1709Gly



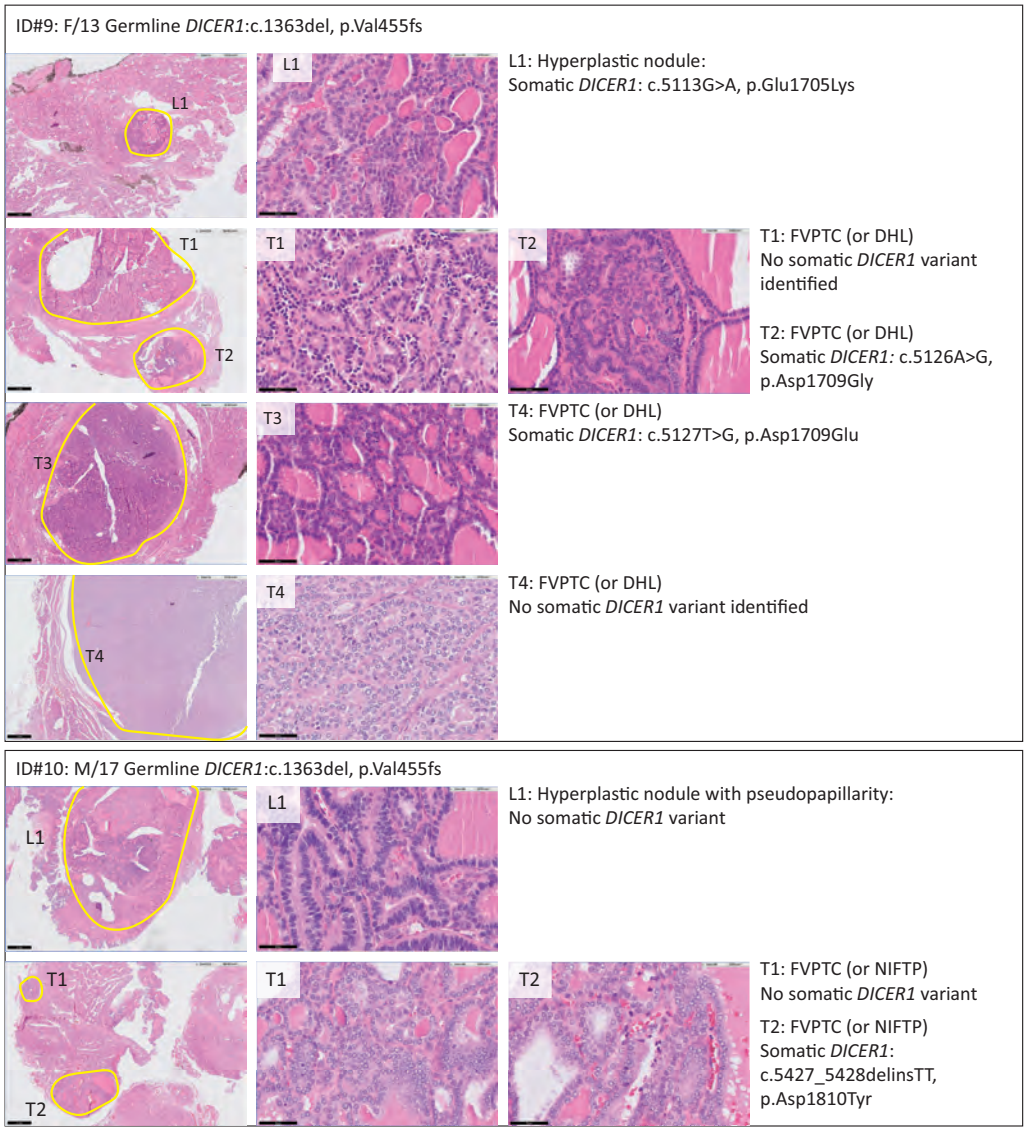
L3: Hyperplastic nodule:
Somatic *DICER1*:
c.5428G>T, p.Asp1810Tyr

L4: Hyperplastic nodule:
Somatic *DICER1*:
c.5429A>T, p.Asp1810Val

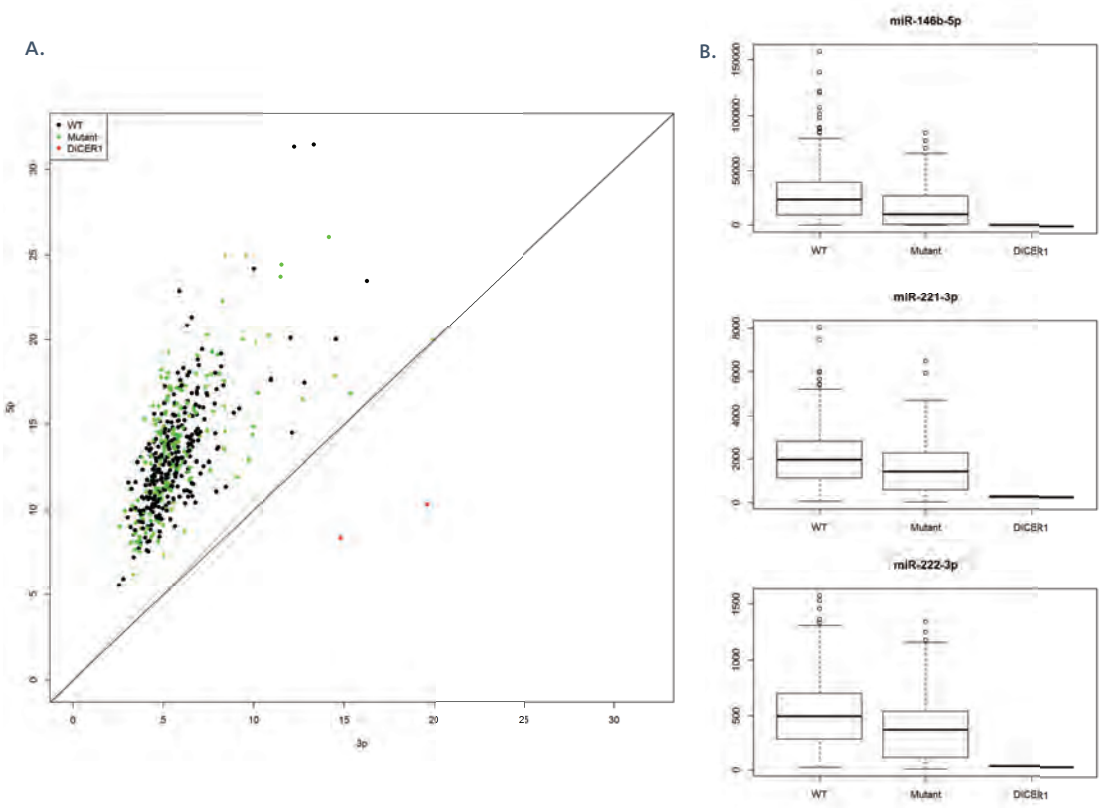
Supplemental Figure 1. (continued)



Supplemental Figure 1. (continued)



Supplemental Figure 1. (continued)



Supplemental Figure 2. miRNA expression DTC TCGA database. **A.** Lower overall normalized median expression of 5p miRNAs compared to non-*DICER1* mutated differentiated thyroid carcinoma (reads per million). **B.** miRNA that are commonly upregulated in differentiated thyroid carcinoma (miR-146b-5p, miR-221-3p and miR-222-3p) seems to be lower in DICER1-related thyroid carcinoma (reads per million). miRNA data obtained from http://firebrowse.org, analysis with R version 3.4.3. WT= no *BRAF*, *RAS*, *EIF1AX*, or *DICER1* mutation; Mutant = *BRAF*, *HRAS*, *NRAS*, *KRAS* or *EIF1AX*; *DICER1* = one loss of function DICER1 mutations and one *DICER1* hotspot RNaseIIIb domain mutation.

This chapter describes the contribution of somatic gene fusions in radioactive iodine-refractory thyroid cancer, with the intention to stratify for targeted therapy.

Targetable Gene Fusions Identified in Radioactive Iodine-Refractory Thyroid Carcinoma

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Eur J Endocrinol. 2019 Apr 1;180(4):235-241

ABSTRACT

Objective

Gene alterations leading to activation of the MAPK pathway are of interest for targeted therapy in patients with advanced radioactive iodine-refractory (RAI-R) thyroid carcinoma. Due to technical reasons gene fusion analysis in RNA isolated from formalin-fixed tumor tissues has till now been limited. The objective of the present study was to identify targetable gene rearrangements in RNA isolated from formalin-fixed RAI-R thyroid carcinomas.

Design

Retrospective study in 132 patients with RAI-R thyroid carcinoma (59 papillary-, 24 follicular-, 35 Hürthle cell-, and 14 anaplastic thyroid carcinoma).

Methods

Total nucleic acid (undivided DNA and RNA) was isolated from formalin-fixed tissue. Extensive gene fusion analysis was performed in all samples that tested negative for pathogenic *BRAF*, *NRAS*, *HRAS* and *KRAS* variants.

Results

Seven targetable gene fusions were identified in the remaining 60 samples without known DNA variants. This includes frequently reported gene fusions such as *CCDC6/RET* (PTC1), *PRKAR1A/RET* (PTC2) and *ETV6/NTRK3*, and gene fusions that are less common in thyroid cancer (*TPM3/NTRK1*, *EML4/ALK* and *EML4/NTRK3)*. Of note, most gene fusions were detected in papillary thyroid carcinoma and MAPK-associated alterations in Hürthle cell carcinomas are rare (2/35).

Conclusion

Targetable gene fusions were found in 12% of RAI-R thyroid carcinoma without DNA variants, and can be effectively identified in formalin-fixed tissue. These gene fusions might provide a preclinical rationale to include specific kinase inhibitors in the treatment regimen for these patients. The latter intends to restore iodine transport and/or take advantage of the direct effect on tumor cell vitality once progressive disease is seen. *(Eur J Endocrinol.180: 235-241, 2019)*

INTRODUCTION

Thyroid cancer (TC) is the most common endocrine malignancy with an increasing incidence over the past decades, accounting for 3.4% of all new malignant tumors.[1] Differentiated thyroid cancer (DTC) is the most common subtype and includes papillary thyroid carcinoma (PTC, 80%), follicular thyroid carcinoma (FTC, 10-15%) and more rare subtypes like Hürthle cell carcinoma (HCC, <5%).[2] Pathological subtypes of PTC include classical or conventional PTC (cPTC), follicular variant of PTC (FVPTC) and many rare subtypes. Anaplastic thyroid carcinoma (ATC) derives from follicular cells that have undergone dedifferentiation and represents less than 2% of all TCs. The current treatment for DTC includes total thyroidectomy and postoperative radioactive iodine (RAI) to ablate the remaining thyroid tissue and eliminate possible (micro) metastases.[3] These treatments are highly effective in the majority of DTC patients and therefore the 10-year survival rate ranges between 80 and 95%. However, nearly 5% of DTC patients become refractory to RAI (RAI-R) through a dedifferentiation process. The 10-year survival rate in these patients is less good (20-40%) due to usually aggressive unresectable metastatic lesions.[4,5]

Point mutations (e.g. *BRAF* and *RAS* genes) as well as gene fusions (e.g. *RET-PTC* 1-12 and *NTRK*) leading to activation of the mitogen-activated protein kinase (MAPK) pathway are crucial for tumorigenesis and progression in thyroid tumors.[6-10] These mutations are almost always mutually exclusive.[6] The fraction of protein kinase gene fusions is higher in thyroid carcinoma (8.5%) compared to other tumor types (1-4.5%).[11] Over the last decade, improved understanding of genetic pathways involved in thyroid tumorigenesis enabled the development of promising targeted therapies. [12,13]

The ability to detect gene fusions in RNA isolated from formalin-fixed, paraffin-embedded (FFPE) tumor tissues has been limited till now due to technical reasons. In this study, we succeeded in extensive gene fusion analysis on FFPE material intending to stratify RAI-R cases for targeted therapy.

SUBJECTS AND METHODS

Sample selection, DNA/RNA extraction and mutation analysis

FFPE tissue blocks were collected from 132 patients in the Netherlands with recurrent RAI-R thyroid carcinoma (primary tumor or lymph node metastasis). For the current study RAI-R was defined as either persistent or progressive disease on radiological images despite extensive RAI treatment or one or more measurable lesions that did not demonstrate RAI uptake on any RAI scan. All patient samples were handled in accordance with the Dutch medical ethical guidelines described in the Code for Proper Secondary Use of Human Tissue established by the Dutch Federation of Medical Sciences. That Code agrees with an augmented system of 'opt-out' for further use in scientific research of coded human tissue, unless there are special circumstances. The current study, including the used 'opt-out' policy, was approved by the Medical Ethical Committee of the Leiden University Medical Centre, protocol no. B16.012. All patients were informed about the secondary use of tissue for research and none of the patients included in this study signed an objection form. In total 34 patients were previously included in phase II trials with Sorafenib[14] and/or Everolimus[15,16] (Clinical-Trials.gov #NCT00887107 and #NCT01118065 respectively). For this manuscript, patient data were anonymized.

Total nucleic acid (undivided DNA and RNA) was isolated from FFPE tissue cores (0.6 mm diameter and variable length) using a fully automated extraction procedure.[17] DNA variant analysis (e.g. *BRAF, NRAS, HRAS* and *KRAS)* has been performed with either a customized AmpliSeq

Cancer Hotspot Panel or with Sanger sequencing, depending on the time period as previously described.[18,19] Samples without DNA variants tested with Sanger sequencing were re-analyzed using the gene fusion data (see 'data analysis' section below). Additional *TERT* promoter variant (NM_198253.2; c.-57A>C, c.-124C>T and c.-146 C>T) analysis was performed in 85 samples by Sanger sequencing. Tested cases did not significantly differ based on age of onset, gender, histological subtype and genetic alterations distribution from non-tested cases.

Gene fusion analysis

Gene fusion analysis was performed in all samples that tested negative for pathogenic *BRAF, NRAS, HRAS* and *KRAS* variants. The whole procedure was executed using the FusionPlex® comprehensive thyroid and lung kit v2 for Ion Torrent (ArcherDX Boulder, Colorado), according to the manufactures' protocol. If available, up to 200ng total nucleic acid were used for cDNA synthesis. The PreSeq RNA Quality Control (QC) assay was performed on 1µL cDNA using the VCP primer mix (ArcherDX) and iTaq Universal SYBR green supermix (Bio-Rad Laboratories). In this study, we proceeded with all samples irrespectively of the QC value. The cDNA fragments were prepared for the adaptor ligation with an end repair/dA-Tailing reaction. Molecule-level barcoding (or unique molecule identifier tagging) and sample-level barcoding (also known as index tagging) are both incorporated during Archer MBC ligation. In the first and second PCR a specific primer set was used to cover relevant exons in 34 genes including: *ALK* (exon 5'; 2,4,6,10,16-23,(intron19)), *AXL* (exon 3';18-20), *BRAF* (exon 5'; 7-11, exon 3'; 7,8,10), *CCND1* (exon 5'; 1-4, exon 3'; 1,2,4), *FGFR1* (exon 5';2, 8-10,17,exon 3' ; 17), *FGFR2* (exon 5';2,5,7-10 , exon 3'; 17), *FGFR3* (exon 5'; 3,5,8-10, exon 3'; 17, (intron17)), *MET* (exon 5'; 2,4-6, 13,14,16,17,21, exon 3'; 2), *NRG1* (exon 5'; 1,2,3,6), *NTRK1* (exon 5'; 2,4,6,8,10-13), *NTRK2* (exon 5'; 5,7,9,11-17), *NTRK3* (exon 5'; 4,7,10,13-16), *PPARG* (exon 5'; 1,2,3,5), *RAF1* (exon 5'; 4-7, 9-12), *RET* (exon 5'; 2,4,6,8,9-14), *ROS1* (exon 5'; 2,4,7,31-37) and *THADA* (exon 3'; 24-30, 36,37). This method enables to detect known gene fusions as well as novel gene fusion partners. Final libraries were diluted 1:100 and quantified using Ion Library TaqMan® Quantitation Kit (Thermo Fisher Scientific). The libraries were pooled (concentration 60 pM, loaded on a chip (Ion Chef™ System) and sequenced on an Ion Proton sequencer (Thermo Fisher Scientific).

Data analysis

Data analysis was performed using the online Archer Analysis software v5.0 (http://analysis.archerdx.com). Only 'strong-evidence' fusions within the software annotation were reported. Furthermore, *BRAF/RAS* point mutations were reported based on DNA/RNA reads. The total number of reads and the fractions of unique reads / RNA reads were documented for all samples as possible quality indicators.

Confirmation of fusion transcripts

Identified gene fusions were validated using different methods. In the majority of cases the presence of the fusion was confirmed with the FusionPlex on a second sample from the same patient (in most cases a lymph node metastasis). In one sample the presence of the fusion was confirmed with Sanger sequencing, using the following primers: 5'-CATTCTTCCACCCTGGAAAC-3' (forward *ETV6* exon 4), and 5'- GCTGAGTCCTCCTCACCACT-3' (reverse *NTRK3* exon 13). Paraffin sections of the sample with *EML4-ALK*-fusions were immunostained for ALK fusion protein using standard procedures (Clone D5F3; 1:250 dilution, Cell Signaling Technology).

Statistical analysis

To describe the characteristics of the study population, the mean age at diagnosis with range was calculated. The median was estimated for the gene fusion test characteristics with a skewed distribution. Continuous variables were analyzed using an independent sample t-test or one-way ANOVA. Dichotomous variables were compared using the chi-squared test. The Kaplan–Meier method was used to estimate the survival function from lifetime data. Statistical significance was set at $P < 0.05$ and the analyses were conducted using SPSS 23.0 (SPSS).

RESULTS

We analyzed in total 132 RAI-R thyroid tumors including 52 PTC, 7 FVPTC, 24 FTC, 35 HCC and 14 ATC as illustrated in Fig. 1. The mean age (± SD) at diagnosis of TC was 60 ± 12 years (range 16-84 years). In this study population, gender was evenly distributed, while it has previously been reported that the incidence of DTC is significantly higher in women compared to men. Age of diagnosis and gender did not significantly differ between the histological subtypes or genetic alteration (Supplementary Table 1, see section on supplementary data given at the end of this article). The 5-year overall survival rates were 55% in PTC, 43% in FVPTC, 56% in FTC, 31% in HCC and 7% ATC (Supplementary Fig. 1a).

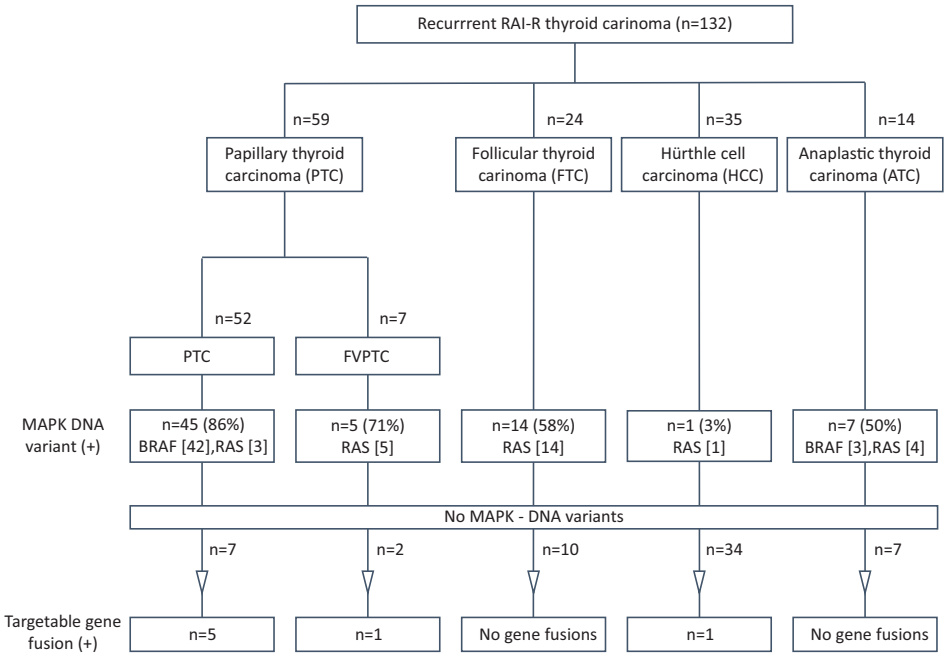

Figure 1. Flow chart of study population. In total, 132 RAI-R thyroid carcinomas with valid results for gene fusion and/or DNA variant analysis were included. We identified 45 BRAF, 27 RAS pathogenic variant(s) and 7 targetable gene fusions.

Abbreviations: PTC; papillary thyroid carcinoma, FVPTC; follicular variant PTC, FTC; follicular thyroid carcinoma, HCC; Hürthle cell carcinoma, MAPK; mitogen-activated protein kinase pathway, RAI-R;radioactive iodine refractory

The well-known driver variant *BRAF*^{V600E} was identified in 42 out of 52 PTCs (81%) and in three ATCs. Pathogenic *N-/H-/KRAS* variants were identified in PTC, FVPTC, FTC, HCC and ATC (6, 71, 58, 3 and 29%, respectively). Pathogenic *TERT* promoter variants were found in 43 out of in total 87 tested samples, predominantly c.-124 C>T (n = 40) and less frequent c.-146 C>T (n = 3). Furthermore, one variant of uncertain significance was identified (c.-160 C>T). TERT variants were identified in all histological subtypes (that is in PTC, FVPTC, FTC, HCC and AT; 20/33 = 61%, 2/4 = 50%, 9/17 = 53%, 5/21 = 24%, and 7/12 = 58%, respectively). TERT variants were present in samples with or without other genetic alterations that is in combinations with *BRAF* or *RAS* variants, gene fusions and in cases with undetected genetic drivers; 18/28 = 64%, 8/16 = 50%, 3/5 = 60% and 14/38 = 37% respectively.

Targetable gene fusions were identified in 7 out of 60 samples (12%) without pathogenic *BRAF/RAS* variants. Clinicopathological and molecular characteristics of patients with targetable gene fusions are described in Table 1. The following gene fusions were identified in classical PTC: *CCDC6–RET* (RET/PTC1), *PRKAR1A-RET* (RET/PTC2), *ETV6-NTRK3* and *EML4-ALK*. Furthermore, a *TPM3-NTRK1* gene fusion was identified in a variant PTC that we described as a 'sclerotic cribriform PTC without morulae'. One FVPTC harbored an *EML4-NTRK1* gene fusion and another *ETV6–NTRK3* gene fusion was identified in an HCC. Gene fusions were identified more frequently in PTC compared to FTC, HCC and ATC. All identified *NTRK* and *ALK* rearrangements maintained the entire kinase domain and lacked the transmembrane localization domain. Immunohistochemical staining showed ALK overexpression in the *EML4-ALK* sample (Supplementary Fig. 2).

The median number of total reads in all samples was 2.123.361 (range 8.435 - 5.648.828) and the median % of unique reads and RNA reads was 11.1% (range 5.9 - 69.5%) and 33.7% (range 1.8 - 72.4%), respectively. These parameters did not significantly differ among gene fusion positive and fusion negative cases.

Genetic alteration leading to activation of the MAPK pathway were eventually identified in 96% of PTC, 86% of FVPTC, 58% of FTC, 6% of HCC and 50% of ATC. In total 53 tumors lacked gene alterations, including 10 FTC and 33 HCC. There was no statistical difference in overall survival between the different molecular backgrounds that is *BRAF* mutant, *RAS* mutant, gene fusion positive and DNA variant / gene fusion negative cases (Supplementary Fig. 1b).

Table 1. Clinicopathological and molecular characteristics of patients with targetable gene fusions

ID	Sex	Age at Dx	Histology	Gene fusion (exon no.)	*TERT* variant	Status	Follow-up
1	F	56y	Classical PTC	*CCDC6* (e1) - *RET* (e12)	c.-124 C>T	DOD	4y
2	F	16y	Classical PTC	*PRKAR1A* (e8) - *RET* (e12)	Not tested	AWD	6y
3	M	73y	Classical PTC	*ETV6* (e4) - *NTRK3* (e13)	Not tested	DOD	2y
4	M	60y	Classical PTC	*EML4* (e13) - *ALK* (e21)	c.-124 C>T	AWD	5y
5	M	65y	FVPTC	*EML4*(e2) - *NTRK3* (e14)	Wild type	AWD	8y
6	F	60y	Sclerotic cribriform PTC without morulae	*TPM3* (e6) - *NTRK1* (e11)	c.-124 C>T	DOD	2y
7	M	75y	Hürthle cell carcinoma	*ETV6* (e3) - *NTRK3* (e13)	Wild type	DOD	0y

Dx; diagnosis thyroid carcinoma, M; male, F; female, y; years, PTC; papillary thyroid carcinoma, FVPTC; follicular variant PTC, DOD; death of disease, AWD; alive with disease.

Four patients now identified with a targetable gene fusion were already deceased, due to tumor progression despite Sorafenib, Everolimus or other treatments (age of death 62-75 years). The other three, recently diagnosed patients with recurrent disease did not report extensive disease related complaints and were therefore not yet further treated with kinase inhibitors, according to the standard procedures in our center. However, these patients might benefit from therapeutic approaches with targeted inhibitors in case of tumor progression.

DISCUSSION

We identified targetable *RET, NTRK, BRAF* or *ALK* gene fusions in 7 out of 60 (12%) formalin-fixed thyroid carcinomas from patients with recurrent RAI-R disease without pathogenic *BRAF/RAS* variants. Remarkably, gene fusions were more common in PTC compared to other histological subtypes. The advent of extensive gene fusion analysis on routinely processed FFPE tissues allowed stratification for targeted therapies for advanced thyroid cancer. This could be beneficial for patients whose tumors are either resistant to RAI immediately after surgery or show recurrent disease during follow up. Our study showed that extensive gene fusion analysis on FFPE thyroid carcinoma samples is effective and feasible.

Although the genetic landscape of differentiated- and less differentiated thyroid carcinoma has been extensively studied [6,9,10], this is one of the largest series with recurrent RAI-R DTC in which molecular analysis has been performed. However, the number within different histological subtypes is still limited. For that reason, comparing the outcome of the different molecular backgrounds stratified by the histological subtype was not possible.

In samples with a low number of (unique) reads and/or low fraction of RNA, the present gene fusions cannot be excluded with certainty. On the other hand, in one sample considered to be of low quality (only 5% of RNA reads) a validated *TPM3-NTRK3* fusion has been found. Further evaluation of quality parameters in molecular diagnostics should lead to consensus criteria to prevent that low-quality samples are incorrectly reported to be negative for gene fusions.

Fifty-tree tumors lacked apparent driver mutations. Of note, as shown previously, HCCs were clearly overrepresented in this group (n = 35) and activating variants in MAPK genes are known to be rare in this subtype.[20,21] Recent studies showed that sequential loss of whole chromosomes is a dominant driver of the oncogenesis of HCC.[22-24] Furthermore, previous studies have shown that dysregulated miRNAs are related to cancer initiation and progression in several tumor types.[25]

Understanding how genetic alterations contribute to the disease process is essential for the development of novel prognostic and therapeutic strategies. Identification of gene fusion transcripts leading to the activation of the transduction signaling pathways are of interest for targeted therapy, intending to restore iodine transport and/or take advantage of the direct effect on tumor cell vitality once progressive disease is seen. While progress has been made with the discovery of kinase inhibitors, the efficacy may be limited because of the development of resistance to treatment and severe side effects.[26] Lenvatinib and Sorafenib are small molecule multi-targeted tyrosine kinase inhibitors (TKIs) and so far the only registered agents for the treatment of advanced DTC.[27,28] A number of selective inhibitors have been developed and characterized in preclinical and clinical studies in other tumor types. In November 2018, the American Food and Drug Administration approved Larotrectinib for patients with solid tumors that have a neurotrophic receptor tyrosine kinase (NTRK) gene fusion. *NTRK* gene fusions are present in 1-2% of all PTCs,[12] while our study showed even a larger contribution of these fusions in RAI-R PTC (3/60 = 5%). Similar approaches could be feasible in DTCs with rearrangements involving *ALK,* 0.6-2.2%

of all PTC and 1% in our series.[29,30] Furthermore, LOXO-292 and BLU-667, selective and potent RET inhibitors,[31,32] are currently being studied in Phase1/2 trials. LOXO-292 demonstrates robust anti-tumor activity in *RET* fusion positive thyroid cancer, according to interim clinical data reported at the 2018 American Thyroid Association annual meeting. Recent preclinical and clinical studies with selumetinib, vemurafenib and dabrafenib, showed re-differentiation, increased iodine uptake and retention in *BRAF*-mutated tumors.[33-35] Further studies are needed to investigate the most effective strategy; however, combination therapy appears to be a reasonable strategy to avoid resistance. Additional (targetable) alterations include variants in the phosphoinositide 3-kinase (PI3K) pathway (e.g. *PIK3CA, PTEN, MTOR, TSC1* and *TSC2*). Everolimus, an inhibitor of the downstream mammalian target of rapamycin (mTOR) serine/ threonine protein kinase, has shown to be a promising agent in recurrent RAI refractory (RAI-R) disease.[15] Other PI3K/AKT/mTOR pathway inhibitors for the treatment of advanced solid cancers are currently tested in clinical trials.[36] For current trails, see http://www.cancer.gov/about-cancer/treatment/ clinical-trials/search.

It has been suggested that *ETV6-NTRK3* rearrangements are caused by radiation exposure, based on *in vitro* studies and case series of patients who suffered from the Chernobyl accident.[37] *NTRK3* fusions were also more frequently found in pediatric PTC, associated with more extensive disease and aggressive pathology.[38] We identified three *NTRK3* fusions in patients between 65 and 76 years old. To our knowledge, none of them had a history of extensive radiation exposure.

In conclusion, targetable gene fusions were found in 12% of recurrent RAI-R thyroid carcinoma without MAPK-related DNA variants and can be effectively identified in routinely processed FFPE tissue. These gene fusions might provide a rationale to treat these patients with specific kinase inhibitors, intending to restore iodine transport and/or take advantage of the direct effect on tumor cell vitality once progressive disease is seen.

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SUPPLEMENTAL DATA

Supplemental Table 1. Study population characteristics

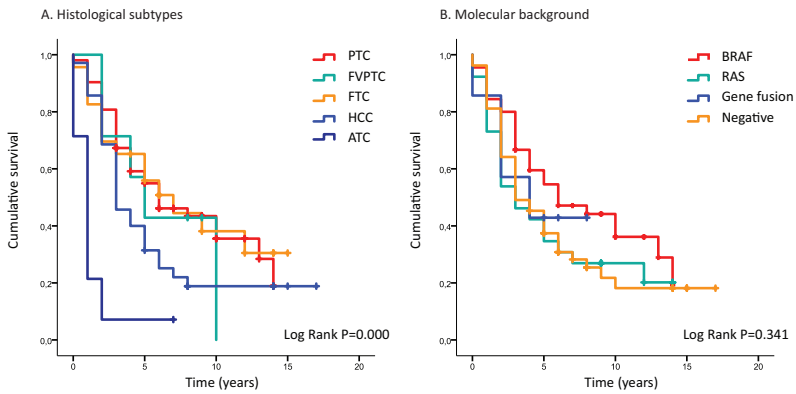
	Total	PTC	FVPTC	FTC	HCC	ATC	P-value*
No.	132	52	7	24	35	14	
Mean age Dx (range, y)	62 (16-84)	63 (16-84)	64 (47-76)	62 (36-82)	62 (40-78)	62 (36-79)	0.99
Male gender, no. (%)	67 (51)	23 (44)	4 (57)	11 (49)	19 (54)	10 (71)	0.43
MAPK-related DNA variant							
No. (% total)	72 (55)	45 (87)	5 (71)	14 (58)	1 (3)	7 (50)	
Mean age Dx (range, y)	63 (31-84)	64 (31-84)	63 (47-76)	61 (36-82)	66	82 (43-78)	0.96
Male gender, no. (%)	34 (47)	21 (47)	2 (40)	7 (50)	0 (0)	4 (57)	0.86
BRAF p.V600E no. (% total)	45 (34)	42 (81)	0	0	0	3 (21)	
Mean age Dx (range, y)	64 (31-84)	64 (31-84)	NA	NA	NA	61 (45-76)	0.70
Male Gender (%)	22 (49)	20 (48)	NA	NA	NA	2 (67)	0.52
RAS no. (% total)	27 (20)	3 (6)	5 (71)	14 (58)	1 (3)	4 (29)	
Mean age Dx (range, y)	62 (36-82)	59 (40-77)	62 (47-76)	61 (36-82)	66	62 (43-78)	0.99
Male Gender, no. (%)	12 (44)	1 (33)	2 (40)	7 (50)	0 (0)	2 (50)	0.88
Gene fusion							
No. (% mut. negative)	7 (12)	5 (71)	1 (50)	0	1 (3)	0	
Mean age Dx (range, y)	58 (16-75)	53 (16-73)	65	NA	75	NA	0.64
Gender	4 (57)	2 (40)	1 (100)	NA	1 (100)	NA	0.35
Apparently no MAPK-related DNA variant of gene fusion							
No. (% total)	53	2	1	10	33	7	
Mean age Dx (range, y)	62 (36-79)	54 (50-58)	68	62 (46-76)	62 (40-78)	61 (36-79)	0.81
Gender	29 (55)	0 (0)	1 (100)	4 (40)	18 (54)	6 *86)	0.15

Abbreviations: No; number patients, Dx; diagnosis, PTC; papillary thyroid carcinoma, FVPTC; follicular variant papillary thyroid carcinoma, FTC; follicular thyroid carcinoma, HCC; Hürthle cell carcinoma, ATC; anaplastic thyroid carcinoma, y; years, MAPK; mitogen-activated protein kinase pathway
* Chi-square test for categorical values (gender), independent T-test of One-Way ANOVA for continues values (age of diagnosis), SPSS version 23

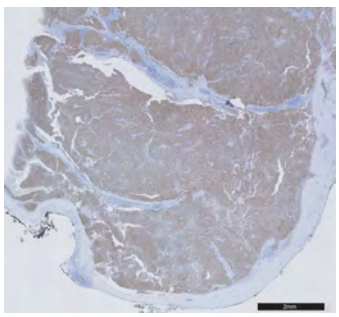
Supplemental Table 2. Identified *RAS* variants in RAI-R DTC

	Total	PTC	FVPTC	FTC	HCC	ATC
NRAS p.G13R	1			1		
NRAS p.Q61R	14	1	4	7		2
NRAS p.Q61K + p.Q61L	3	1		1		1
NRAS p.Q61K	1			1		
NRAS p.Q61H	1			1		
NRAS p.Q61?	1			1		
HRAS p.Q61R	4			3		1
HRAS p.A59T	1				1	
KRAS p.G12D	1		1			

Abbreviations: PTC; papillary thyroid carcinoma, FVPTC; follicular variant papillary thyroid carcinoma, FTC; follicular thyroid carcinoma, FTC-OV; follicular thyroid carcinoma oncocytic variant, ATC; anaplastic thyroid carcinoma; NRAS (exon 3) NM_002524.4; HRAS (exon 3) NM_001130442.1; KRAS (exon 2) NM_033360.2

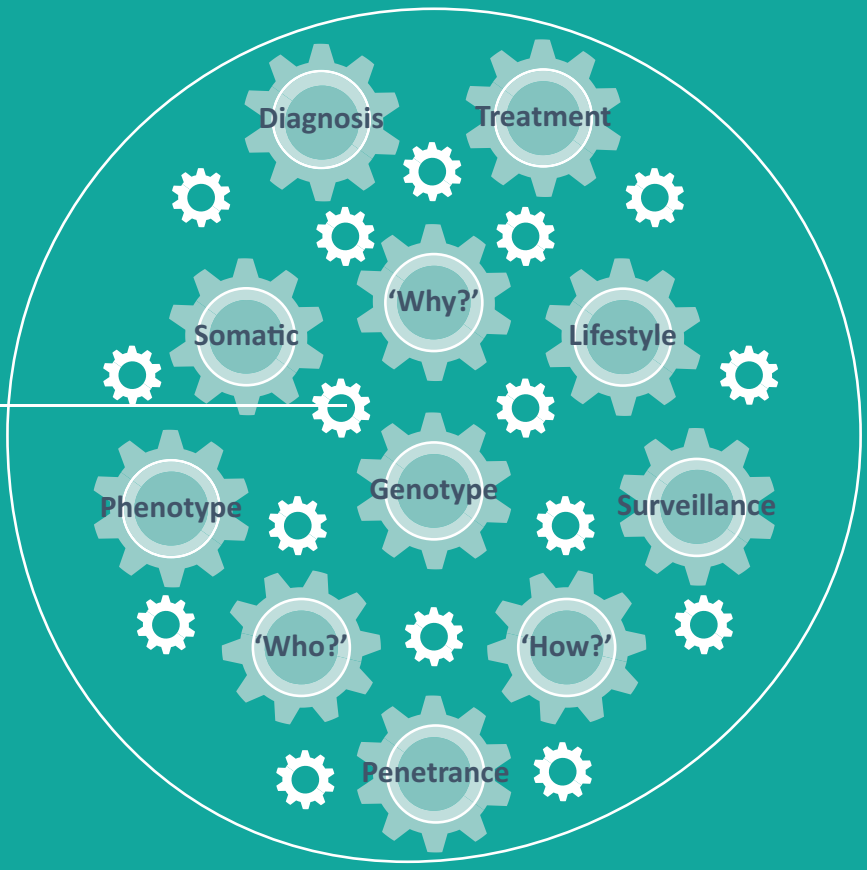


Supplemental Figure 1. Kaplan-Meier curves overall survival. **A.** Histological subtypes: Papillary thyroid carcinoma (PTC); follicular variant papillary thyroid carcinoma (FVPTC), follicular thyroid carcinoma (FTC); Hürthle cell carcinoma (HCC), anaplastic thyroid carcinoma (ATC) **B.** The molecular background subtypes: *BRAF* mutant, *RAS* variant, gene fusion, negative *Patients were not treated based on mutation status, in total 34 patients were previously included in phase II trials with Sorafenib [15] and/or Everolimus [16, 17] (Clinical-Trials.gov #NCT00887107 and #NCT01118065 respectively).



Supplemental Figure 2. ALK immunohistochemical staining. ALK overexpression in tumor ID4 with *EML4-ALK* gene fusion (ALK clone D5F3)

Part II
Chapter 4



PART II

IDENTIFICATION OF GENETIC PREDISPOSITION IN PEDIATRIC NON-MEDULLARY THYROID CARCINOMA

This chapter describes the first results of our study investigating the Genetic background of Non-medullary Thyroid cancer in Pediatrics (GeNoThyPe) using whole genome sequencing. So far 33 genes are analyzed in 64 out of 100 pediatric thyroid cancer patients. The plans for further genetic analyses are described at the end of this chapter.

Germline Mutations in Predisposition Genes in Pediatric Non-Medullary Thyroid Cancer

K. van der Tuin, T.P. Links, H. Morreau, F.J. Hes

Manuscript in preparation

ABSTRACT

Background

Most children who develop non-medullary thyroid cancer (NMTC) are so far genetically unaccounted for. Identification of NMTC predisposition genes may improve the understanding of tumorigenesis, give direction for patient care, and enable genetic counselling of patients and families. The main objective of this study was to 1) determine the contribution of germline mutations in known cancer predisposition genes, and 2) identify novel thyroid cancer susceptibility genes.

Method

Whole genome sequencing (WGS) has so far been performed in 64 out of 100 patients with pediatric NMTC. The first analysis included a subset of 32 tumor predisposing genes.

Results

We identified pathogenic germline variants in *DICER1* and *APC* in five of the 64 patients (8%). DICER1- and APC-related thyroid neoplasia appeared to differ morphologically from sporadic disease.

Discussion

Our first analysis showed relatively frequent (8%) causative germline pathogenic variants in a subset of known cancer predisposition genes, including *DICER1* and *APC*. Based on distinct thyroid histology, pathologists may play a crucial role in recognizing features for selecting patients for genetic testing. Further and in depth WGS data analysis is needed to determine the contribution of other (novel) thyroid cancer susceptibility genes.

INTRODUCTION

Childhood thyroid carcinoma (TC) is a relatively rare disease, responsible for 0.5-3% of all pediatric malignancies.[1] Moreover, data from the SEER registry have shown an increasing incidence of pediatric, adolescent and young adult TC.[2] Among non-medullary thyroid cancer (NMTC) in children, classic papillary thyroid cancer (PTC) is the commonest (63%), followed by follicular variant of papillary (23%) and follicular thyroid cancer (FTC, 10%).[1] Poorly differentiated thyroid cancer (PDTC) is rare, while anaplastic thyroid cancer or Hürthle cell cancer are practically nonexistent in children.[1]

NMTC presents in children at more advanced stages of disease (extra thyroidal extension, lymph node and distant metastases) as compared to adults.[3] Furthermore, pediatric NMTC is associated with high rates of recurrence (7%), persistent disease (8%) and postoperative complications (>30%).[3] On the other hand, there is a good general prognosis, with a disease-specific mortality <2%.[1,3] Nevertheless, pediatric and young adult patients treated for NMTC have an increased risk of certain second primary malignancies.[4] It is supposed that these second primary malignancies are induced by the effect of radioactive iodine treatments.[5,6] However, we cannot eliminate the role of genetic background in the development of both malignancies.

NMTC can manifest as part of a tumor predisposition syndrome (TPS) in rare cases, including *PTEN* hamartoma tumor syndrome (PTHS), DICER1-syndrome, familiar adenomatous polyposis (FAP), Werner syndrome, Carney complex and Pendred syndrome. However, in all of these syndromes NMTC occurs as a minor component.[7] The distinct thyroid pathology in some of these syndromes should alert the pathologist to a possible predisposition syndrome.[8] An estimated 5% of patients with NMTC have a family history of non-syndromic NMTC.[9] Several large case-control studies have reported the heritability of familiar NMTC (FNMTC) to be one of the highest of all cancers (3-10 fold increased risk).[10-12] The genetic inheritance of non-syndromic FNMTC remains largely unknown, but it is believed to be autosomal dominant with incomplete penetrance and variable expression. With the introduction of new techniques in molecular genetics, several potential loci for FNMTC gene have been identified.[13] However, the causative genes predisposing to FNMTC have not been yet identified. Therefore, currently, most children who develop NMTC are genetically unaccounted for. The frequency of different germline mutations in tumor predisposition genes in unselected children with NMTC has, to the best of our knowledge, not been systematically studied in a large cohort. Previous studies have relied mainly on candidate-gene approaches in selected patients, approaches which are, by design, limited. With the introduction of next-generation sequencing (NGS), the last decades have seen remarkable advances in our understanding of the genetic contribution to disease. Identification of 'novel' NMTC predisposition genes may improve the understanding of tumorigenesis, give direction for patient care, and enable genetic counselling of patients and families.

The main objective of this study was to improve knowledge of the genetic background of pediatric NMTC by 1) determining the contribution of mutations in known cancer predisposition genes, and 2) identifying novel thyroid cancer susceptibility genes using whole genome sequencing by further and in depth WGS data analysis. The methods and results of the first part are discussed in the next paragraphs.

PATIENT AND METHOD

Study population and design

All Dutch patients with an established diagnosis of NMTC during childhood (<18 years old) between January 1970 and December 2013 and treated in The Netherlands were eligible for

inclusion in the study entitled "Late effects of treatment and pathophysiological background in the Netherlands". The results of this nationwide follow-up study have recently been published.[3] Written informed consent for collection of molecular data next to clinical and pathological report was obtained from a subset of patients at age 18 years or older. The medical ethical committees of the primary investigator and collaborating hospitals approved the clinical research proposal (UMCG 2012/183). The current genetic study was approved by the local medical ethical (LUMC B17.042). Patients are informed by the attending physician of any pathogenic mutations in TPS genes if surveillance is recommended, as indicated in the informed consent forms. Secondary findings are discussed in an expert team and in rare cases with the medical ethical committee. Reference to the latter is standard in our center when dealing with diagnostic whole-exome sequencing.

Genetic analysis – whole genome sequencing

The method and workflow is summarized in Figure 1 (part 1). Genomic DNA was extracted from peripheral blood leukocytes according to standard procedures. Whole genome sequencing was performed by Macrogen (Seoul, Republic of Korea) on the Illumina HighSeq X Ten (2x 150bp) after quality control (QC) and library preparation (TruSeq PCR-Free library). DNA fragments were mapped to hg19 by Isaac aligner. Variant calling included SNP/InDel calling by Isaac and CNV/SV analysis by Control-EREEG/Manta, annotated to hg19 coordinates, dbSNP138, dbSNP142, 1000G, ESP6500 by SnpEff.

Cancer predisposition genes selected for analysis

To determine the contribution of mutations in known cancer predisposition genes, we divided these genes in three subsets of whom the first two groups are analyzed for this report (see Table 1). The first group included 15 genes, of which germline variants are (possibly) associated with NMTC

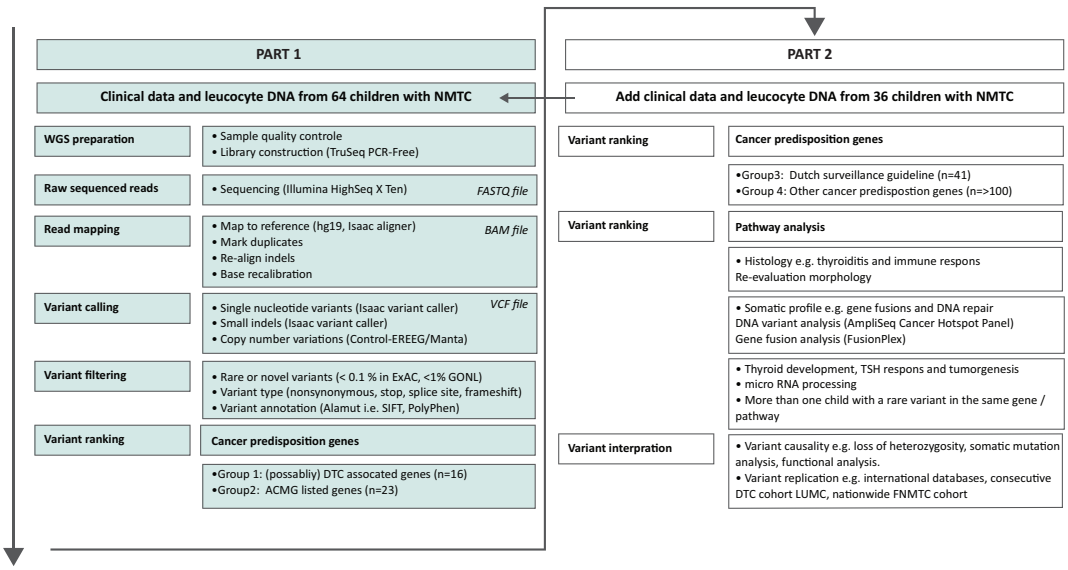


Figure 1. Full project work flow. The method and results of part 1 is described in this chapter, furthermore the plan of investigation of part 2 is described on the end of this chapter.

Table 1. Cancer predisposition genes selected for analysis

Group 1	Group 2	Group 3							
Thyroid	*ACMG*	*All reported cancer predisposing genes*							
APC	*APC*[*]	*A2ML1*	*CDKN2A*[§]	*EXT2*	*HOXB13*	*NF2*[*§]	*PTPN11*	*RTEL1*	*SRY*
DICER1	*BRCA1*	*ACD*	*CDKN2B*	*FAN1*	*HRAS*	*NHP2*	*PTPRJ*	*RUNX1*	*STAT3*
FOXE1	*BRCA2*	*AIP*	*CDKN2C*	*FANCA*	*ITK*	*NKX2-1*[*]	*RAD50*	*SBDS*	*STK11*[*§]
MEN1	*MEN1*[*]	*AKT1*	*CEBPA*	*FANCB*	*KIF1B*	*NOP10*	*RAD51B*	*SDHA*[§]	*SUFU*
NKX2-1	*MLH1*	*ALK*	*CENPJ*	*FANCC*	*KIT*	*NOTCH2*	*RAD51C*	*SDHAF2*[*§]	*TERC*
PRKAR1A	*MSH2*	*APC*[*§]	*CFTR*	*FANCD2*	*KLLN*	*NRAS*	*RAD51D*	*SDHB*[*§]	*TERF1*
PTEN	*MSH6*	*ARMC5*	*CHEK2*[§]	*FANCE*	*KRAS*	*NSD1*	*RAD54L*	*SDHC*[*§]	*TERF2IP*
SDHB	*MUTYH*	*ATM*[§]	*COL17A1*	*FANCF*	*LZTR1*	*NTHL1*	*RAF1*	*SDHD*[*§]	*TERT*
SDHC	*NF2*	*ATR*	*CREBBP*	*FANCG*	*MAP2K1*	*NTRK1*	*RASAL1*	*SEC23B*[*]	*TGFBR1*
SDHD	*PMS2*	*AXIN2*	*CTC1*	*FANCI*	*MAP2K2*	*OGG1*	*RB1*[*§]	*SEMA4A*	*TGFBR2*
SEC23B	*PTEN*[*]	*BAP1*[§]	*CTNNA1*	*FANCL*	*MAX*[§]	*PALB2*[§]	*RECQL*	*SERPINA1*	*TINF2*
SRGAP1	*RB1*	*BARD1*	*CYLD*	*FANCM*	*MC1R*	*PALLD*	*RECQL4*	*SFTPA1*	*TMEM127*[§]
SRRM2	*RET*	*BLM*	*DDB2*	*FAS*	*MDH2*	*PARK2*	*REST*	*SFTPA2*	*TNFRSF11A*
TSHR	*SDHAF2*	*BMPR1A*	*DDX11*	*FH*[§]	*MEK1*	*PAX5*	*RET*[*§]	*SH2B3*	*TP53*[*§]
WRN	*SDHB*[*]	*BRAF*	*DICER1*[*§]	*FLCN*[§]	*MEK2*	*PCNA*	*RHBDF2*	*SH2D1A*	*TRIM37*
	SDHC[*]	*BRCA1*[*§]	*DIS3L2*	*FOCAD*	*MEN1*[*§]	*PDGFRA*	*RINT1*	*SHOC2*	*TSC1*[*§]
	SDHD[*]	*BRCA2*[*§]	*DKC1*	*FOXE1*[*]	*MET*	*PHOX2B*	*RIT1*	*SLX4*	*TSC2*[*§]
	STK11	*BRIP1*	*EGFR*	*G6PC3*	*MITF*	*PIK3CA*	*RMRP*	*SMAD4*	*TSHR*[*]
	TP53	*BUB1*	*EGLN1*	*GATA1*	*MLH1*[*§]	*PMS2*[*§]	*RPL11*	*SMAD9*	*USB1*
	TSC1	*BUB1B*	*ELANE*	*GATA2*	*MPL*	*POLD1*[*§]	*RPL15*	*SMARCA4*	*VHL*[*§]
	TSC2	*BUB3*	*EPCAM*[§]	*GDNF*	*MRE11A*	*POLE*[*§]	*RPL35A*	*SMARCB1*	*WAS*
	VHL	*CASR*	*ERCC1*	*GFI1*	*MSH2*[*§]	*POLH*	*RPL5*	*SMARCE1*	*WRAP53*
	WT1	*CBL*	*ERCC2*	*GPC3*	*MSH3*	*POT1*	*RPS10*	*SOS1*	*WRN*[*]
		CDC73[§]	*ERCC3*	*GPC4*	*MSH6*[*§]	*PRF1*	*RPS17*	*SOS2*	*WT1*[*§]
		CDH1[§]	*ERCC4*	*GREM1*	*MTAP*	*PRKAR1A*[*]	*RPS19*	*SPINK1*	*XPA*
		CDK4[§]	*ERCC5*	*HABP2*	*MUC5B*	*PRSS1*	*RPS24*	*SPRED1*	*XPC*
		CDKN1A	*ERCC6*	*HAX1*	*MUTYH*[*§]	*PTCH1*[§]	*RPS26*	*SQSTM1*	*XRCC2*
		CDKN1B	*EXO1*	*HNF1A*	*NBN*	*PTCH2*	*RPS29*	*SRGAP1*[*]	*XRCC3*
		CDKN1C	*EXT1*	*HNF1B*	*NF1*[§]	*PTEN*[*§]	*RPS7*	*SRRM2*[*]	

ACMG; American College of Medical Genetics and Genomics (includes cancer predisposition genes that may require medical intervention aimed at preventing or significantly reducing morbidity and mortality) [*]genes already analyzed in the former step. [§]Dutch clinical surveillance (concept) guidelines available

according to literature. The second group included 23 (partly overlapping) cancer predisposition genes, listed by the American College of Medical Genetics and Genomics (ACMG) that may require medical intervention aimed at preventing or significantly reducing morbidity and mortality.[14] The third group includes 199 additional genes possibly associated with cancer predisposition, however so far without clear clinical implications.

Variant filtering

Variants were classified within five tiers: class 5, pathogenic; class 4, probably pathogenic; class 3, of uncertain significance; class 2, probably benign and class 1, benign according to the ACMG guidelines for interpretation.[15] Filtering of predicted pathogenicity of gene variants was mandatory, using bioinformatics prediction pipelines as well as data base analyses. Using variant databases (ExAC and the Genome of the Netherlands project (GONL)) frequent variants (MAF >0.1-1%) have been excluded. Next, (probably) benign variants based on evolutionary non-conservation (Phylop>2) and protein prediction tools (i.e. SIFT, PolyPhen-2, Mutationtaster) were excluded.

RESULTS

Clinical characteristics

The clinical characteristics of the so far 64 investigated pediatric NMTC patients are summarized in Table 2. Mean age at diagnosis was 15.6 years (range 7-18) with large female predominance (9:1). At diagnosis, lymph node metastases were present in 30 patients (47%) and distant metastases in 5 patients (8%). Total thyroidectomy was performed in all patients and in 61 patients followed by radioactive iodine treatment. According to the pathology reports, PTC accounts for 75%, FTC for 20% and poorly differentiated thyroid carcinoma (PDTC) for 5% in our cohort. At last known follow-up, 4 patients had persistent disease (6%) and 7 patients recurrent disease (11%). Overall survival was 100% after a median follow-up of 15 years (range 5-44 years).

Table 2. Clinical and histological characteristics study population

	All patients (n=64)	0-10 year (n=5)	11-14 year (n=21)	15-18 year (n=38)
Gender, n (%)				
Male	9 (14)	3 (60)	3 (14)	3 (8)
Female	55 (86)	2 (40)	18 (86)	35 (92)
Age at diagnosis, year				
Median (range)	15 (7-18)	10 (7-10)	12.6 (11-14)	17.2 (15-18)
Primary tumor size, cm				
Median (range)	2.5 (0.3-6.0)	3.75 (2.5-5.0)	2.75 (1.0-5.5)	2.5 (0.3-6.0)
Localization, n (%)				
Unilateral	39 (61)	1 (20)	13 (62)	25 (66)
Bilateral	16 (25)	2 (40)	5 (24)	9 (24)
Other ^	5 (8)	1 (20)	1 (5)	3 (8)
Unknown	4 (6)	1 (20)	2 (10)	2 (5)
Multifocality, n (%)				
Yes	17 (27)	2 (40)	4 (19)	11 (29)
No	31 (48)	2 (40)	12 (57)	17 (45)
Unknown	16 (25)	1 (20)	5 (24)	10 (26)

Table 2. (continued)

	All patients (n=64)	0-10 year (n=5)	11-14 year (n=21)	15-18 year (n=38)
TNM classification, version 7, n (%)				
T				
T1-2	40 (63)	2 (40)	13 (62)	25 (66)
T3-4	13 (20)	1 (20)	6 (29)	6 (16)
Tx	11 (17)	2 (40)	2 (10)	7 (18)
N				
N0	30 (47)	1 (20)	9 (43)	20 (53)
N1	30 (47)	4 (80)	11 (52)	15 (40)
Nx	4 (6)	0 (0)	1 (5)	3 (8)
M				
M0	54 (84)	3 (60)	17 (81)	34 (90)
M1	5 (8)	1 (20)	3 (14)	1 (3)
Mx	5 (8)	1 (20)	1 (5)	3 (8)
Primary surgery, n (%)				
Total thyroidectomy	39 (61)	3 (60)	15 (71.4)	21 (55)
Hemi-thyroidectomy[*]	25 (39)	2 (40)	6 (28.6)	17 (45)
Lymph node dissection, n (%)				
None	30 (47)	1 (20)	10 (48)	19 (50)
Central LND	4 (6)	2 (40)	0	2 (5)
LND incl. lateral levels	23 (36)	2 (40)	9 (43)	12 (32)
Unknown	7 (11)	0 (0)	2 (10)	5 (13)
Histology[¥], n (%)				
Papillary	48 (75)	4 (80)	14 (67)	30 (79)
Classic	24	3	7	14
Follicular	14	1	3	10
Other / mixed variant	10	0	4	6
Follicular	13 (20)	1 (20)	5 (23)	7 (18)
Poorly differentiated	3 (5)	0	2 (10)	1 (3)
Outcome, n (%)				
Remission	53 (83)	4 (80)	17 (81)	32 (84)
Persistent	4 (6)	1 (20)	2 (10)	1 (3)
Recurrence	7 (11)	0 (0)	2 (10)	5 (13)

n; number of cases, TNM; tumor, node, metastasis, 'x' indicates that information about that characteristic was not available, LND; lymph node dissection; [^]e.g. isthmus, thyroglossal duct, [*]in all cases a complementary contralateral hemithyroidectomy was performed, [¥]according to pathology report.

Genetic analysis

So far we completed the analysis of the first two selected gene groups (see Table 1). We identified causative germline pathogenic variants in five of the 64 patients (8%), including four *DICER1* and one *APC* variant. Furthermore one germline *PTEN* variant of uncertain significance was identified. The clinical, histological and molecular data of these six patients are summarized in Table 3 and described below.

DICER-related NMTC

In total three different pathogenic germline *DICER1* variants were identified in four index cases. None of them had a personal history of any DICER1-related tumor (see phenotype description in Figure 2). **Case 1** (*DICER1*, c.2270T>A): a 14-year-old female diagnosed with a PDTC. Tumor tissue was not available for re-evaluation and additional somatic mutation analysis. One first degree relative was operated for a lung lesion but histology is unknown. **Case 2** (*DICER1*, c.2256+1G>C): a 14-year-old female diagnosed with a PDTC published previously (case 6).[16] Her family history was suggestive for DICER1 syndrome including autosomal dominant inherited MNG and a cousin with a SLCT. Somatic mutation analysis revealed a somatic *DICER1* variant affecting the RNase IIIb domain consistent with a two-hit tumor suppressor model, whereby in the case of DICER1-related disease, a germline loss-of-function variant is followed by a somatic missense variant.[16-18] Furthermore a somatic pathogenic *TP53* variant was identified, consistent with P53 immunohistochemical overexpression. **Case 3** (*DICER1*, c.3301_3302insA): a 15-year-old female diagnosed with a difficult to classify thyroid neoplasm, initially classified as PTC. Re-evaluation showed diffuse nodular hyperplasia with multiple, discrete, well-circumscribed, and occasionally encapsulated nodules, consistent with the diagnosis DICER1-related thyroid neoplasm. Few dominant lesions showed

Table 3. Patients with a tumor predisposition syndrome

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
Sex	F	F	F	F	F	F
Age (y) at TC Dx	14	14	15	14	16	15
Gene	*DICER1*	*DICER1*	*DICER1*	*DICER1*	*APC*	*PTEN*
Germline variant	c.2270T>A, p.L757*	c.2256+1G>C, splice variant	c.3301_3302insA, p.(Ser1101Tyrfs*3)	c.3301_3302insA, p.(Ser1101Tyrfs*3)	c.2434_2437del, p.(Asp812Ilefs*7)	c.421C>T, p.His141Tyr
Variant classification	Pathogenic	Pathogenic	Pathogenic	Pathogenic	Pathogenic	Uncertain significant
Personal history	None	None	None	None	None	None
Family history	Lung lesion	MNG, SLCT	TC	None	None	None
Thyroid histology	PDTC	PDTC	MNG	FTC	CMV-PTC	PTC
Thyroiditis	NA	No	No	NA	No	Yes
Immunohistochemistry	NA	TP53 positive	NA	NA	Beta-catenin positive	PTEN weak positive
Somatic variant	NA	*DICER1*, *TP53*	NA	NA	NA	NA

Dx; diagnosis, y; years, TC; thyroid cancer, MNG; multi nodular goiter, SLCT; Sertoli-Leydig cell tumor ovarian, PDTC; poorly differentiated thyroid carcinoma, FTC; follicular thyroid carcinoma, PTC; papillary thyroid carcinoma CMV-; cribriform morular variant, NA; not applicable

	DICER1 syndrome	Familiar adenomatous polyposis (FAP)
Gene (locus)	*DICER1* (14q32.13)	*APC* (5q22.2)
Inheritance	Autosomal dominant	Autosomal dominant
Syndromic features	e.g. pleuropulmonary blastoma, cystic nephroma, ovarian Sertoli–Leydig cell tumor	e.g. polyposis, colon cancer
Thyroid phenotype (penetrance)	MNG (~35%) PTC/FTC (~5%)	CMV-PTC (~2-10%)
Morphology	Diffuse nodular hyperplasia with multiple, discrete, well-circumscribed, and occasionally encapsulated nodules with or without atypical nuclear features	Morules and a cribriform growth pattern
IHC	Not specific	β-catenin overexpression
Somatic molecular profile	- Somatic *DICER1* hotspot variants RNase IIIb domain. -Lack well-known oncogenic driver DNA variants and gene rearrangements	Somatic *APC* variants or somatic *CTNNB1* variants, or rarely *RET-PTC gene* fusion.
	Case 2 PDTC + hyperplasia	*Case 5* CVM-PTC

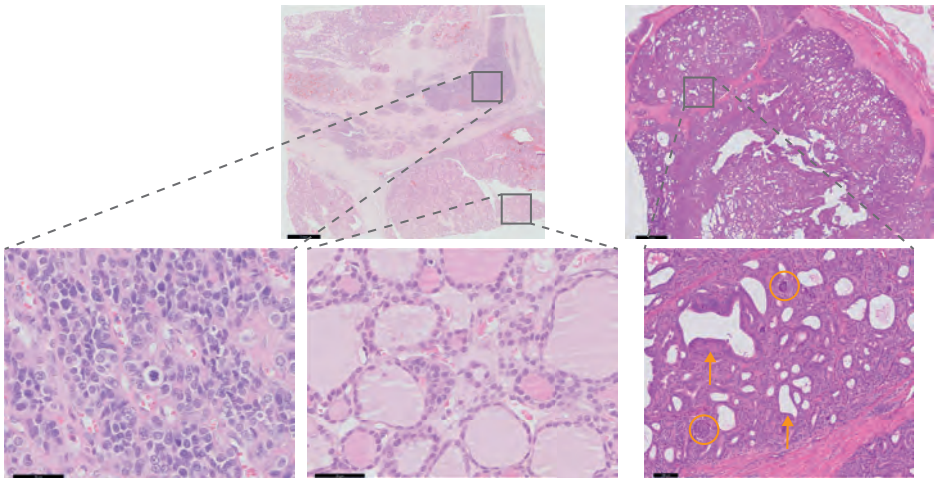


Figure 2. Clinical, histological and molecular features of hereditary syndromes associated with non-medullary thyroid cancer.

MNG; multi nodular goiter, PTC; papillary thyroid carcinoma, FTC; follicular thyroid carcinoma, CMV-; cribriform-morular variant (cribriform growth pattern indicated by arrows, morules indicated by circles), IHC; immunohistochemical staining

atypical nuclear features such as nuclear enlargement and overlap, irregularly shaped follicles and presence of nuclear clearance, however not convincing for the diagnosis of carcinoma. Her family history showed two first degree relatives with NMTC at young age. **Case 4** (*DICER1*, c.3301_3302insA): a 14-year-old female diagnosed with a minimal invasive FTC. Family history was negative for DICER1-related tumors. Tumor tissue was not available for re-evaluation and additional somatic mutation analysis.

APC-related PTC

A pathogenic germline *APC* variant (c.2434_2437del) was identified in one patient. **Case 5:** a 15-year-old female diagnosed with PTC. During re-evaluation the tumor has morules and a cribriform growth pattern, classified as a cribriform-morular variant of papillary thyroid carcinoma (CMV-PTC). CMV-PTC has a distinctive histologic morphology related to germline and/or somatic *APC* variants or somatic *CTNNB1* variants. Immunohistochemical staining showed nuclear β-catenin staining related to the permanent activation of the Wnt pathway. She had no remarkable personal medical history and no family history of FAP (see phenotype description in Figure 2).

Possibly PTEN-related PTC

A novel germline *PTEN* variant of uncertain significance (c.421C>T, p.His141Tyr) was identified in one patient. **Case 6**: a 15-year-old female diagnosed with PTC. Additional immunohistochemical staining showed weak positive PTEN expression. She had no remarkable personal medical history and no clear family history of *PTEN* associated tumors. The identified variant is associated with a highly conserved nucleotide (phyloP: 5.53 [-14.1;6.4]) and moderately conserved amino acid. The physicochemical difference between His and Tyr is moderate (Grantham dist.: 83 [0-215]). Prediction programs showed conflicting results (i.e. SIFT predicts tolerated while mutation taster predicts disease causing). This *PTEN* variant has also not been reported in LOVD (https://www.LOVD.nl/PTEN, accessed on April 15, 2019). Moreover, the histology showed PTC and was not distinctive, i.e. not classic PTEN-associated immunohistochemical negative FTC.[19,20] However, while FTC is one of the major criteria for PHTS, PTC and benign nodules have been frequently described in PHTS. PTEN protein immunostaining seems sensitive and specific of PHTS and therefore staining can aid in the identification of patients with PHTS. However, missense variants (as in *case 6*) may do not lead to the loss of PTEN staining, as these variant might have a relative small effect on the protein structure, however the function can be impaired. Therefore, the pathogenicity of this variant remains so far unclear.

DISCUSSION

The frequency of germline mutations in cancer predisposition genes in children with NMTC is largely unknown. Until recently, genetic testing for NMTC-associated TPS involved sequentially testing single genes, prioritized according to clinical features. Hence we performed whole genome sequencing in unselected pediatric NMTC patients. Our first analysis (e.g. TPS gene group 1-2) showed germline causative pathogenic variants in *DICER1* or *APC* in five out of so far 64 investigated patients (8%). To determine the full contribution of known cancer predisposition genes, gene group 3 need to be analyzed. Moreover, the contribution of novel predisposing genes should be investigated in depth WGS data analysis (see further studies below).

As illustrated by the five described cases with pathogenic germline variants, pathologists may play a crucial role in recognizing features associated with TPS for selecting patients for genetic testing (see Figure 2).

DICER1-related thyroid neoplasia morphologically differ from sporadic disease. DICER1-related thyroid neoplasm are often difficult to classify tumors, characterized by diffuse nodular hyperplasia with multiple, discrete, well-circumscribed, and occasionally encapsulated nodules with atypical nuclear features.[16-18,21] Somatic *DICER1* hotspot variants are present in benign and malignant thyroid nodules from patients with germline pathogenic *DICER1* variants.[16-18] Moreover, these tumors often lacked well-known oncogenic driver DNA variants (e.g. *BRAF*, *RAS*) and gene rearrangements (e.g., *RET/PTC 1-12*, *PPARg-PAX8*, *ALK*, and *NTRK*) that are frequently observed in sporadic TC.16

Cribriform-morular variant of PTC should be a red flag for FAP cause by germline *APC* variants (39-53% of CMV-PTC cases).[22] However, besides the rarely of this subtype, it might be easily overlooked if no special attention is drawn to these often subtle morphological features. Negative family history does not exclude FAP, as *de novo APC* variants are reported in 10–25% of FAP patients.[23-25] Furthermore, TC during childhood might be the first presentation in probands. Germline pathogenic *APC* variants in patients with FAP and CMV-PTC, have been found in in about 85% of the cases exon 15 (as in Case 6).[26] Mutational analysis of the *APC* gene in CMV-PTC should therefore not be restricted to the mutation cluster region (MCR, codons 1286 to 1513). In this study re-evaluation of the histology was done after identification of the germline DNA variant. Knowledge of the identified DNA variant was known to the re-evaluating pathologist. Subtle morphological changes might be easily overlooked by a pathologist without expertise with childhood and hereditary NMTC. Children with TC should be cared for by teams of physicians experienced in the management of TC in children to include, not only high-volume thyroid surgeons, but also experts in (molecular) pathology, nuclear medicine, endocrinology and clinical genetics. Evaluation and care should be organized into a multidisciplinary team that regularly conducts patient review and/or tumor board conferences as has been recommended by the American Thyroid Association (ATA).[27]

In conclusion, our first analysis showed relatively frequent (8%) causative germline pathogenic variants in a subset of known cancer predisposition genes in unselected cases with childhood NMTC. Pathologists may play a crucial role in recognizing features associated with TPS for selecting patients for genetic testing. Extensive analysis is needed to determine the contribution of mutations in all known cancer predisposition genes, and to identify novel thyroid cancer susceptibility genes.

FURTHER STUDIES

Study population

We aim to include another 36 patients in this study, to finally study proximally 100 patients. All Dutch patients with an established diagnosis of NMTC during childhood (<18 years old) until December 2017 were eligible for the WGS study, as soon as they were 18 years old to provide informed consent. The plan of investigation for further studies is summarized in Figure 1 (part 2).

Data analysis

After finishing the analysis of all known TPS genes (group 3) we continue with the second objective, i.e. identifying novel thyroid cancer susceptibility genes based on WGS data. For this purpose we perform pathway analysis combining WGS data with the clinical, pathological and somatic data. For example, in children with intrathyroidal lymphocytic infiltration[28], we focus on human leukocyte antigen (HLA) genes and immune response pathways. In children with somatic chromosomal alterations such as *RET/PTC* 1-12 gene fusions, we focus on so-called caretaker genes that are involved in the maintenance of human genome stability (DNA repair pathways). Somatic DNA variant and gene fusion analysis is performed using respectably a customized Cancer Hotspot Panel (Thermo Fisher Scientific, Waltham, MA) targeting >50 genes (including *BRAF, NRAS, HRAS, KRAS, TP53, PTEN, PIK3CA* and *DICER1*) and/or the FusionPlex comprehensive thyroid and lung kit (ArcherDX, Boulder, CO), which captures relevant exons from >30 genes (including *RET*, *NTRK1-3*, and *ALK*).

Moreover, we look further into genes involved in pathways of thyroid developmental, TSH response and tumorigenesis. For example, genetic alterations related to the mitogen-activated

protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways. Furthermore, we study genes involved in microRNA processing, as for example with the elucidation of the *DICER1* gene involved in thyroid tumorigenesis. Moreover, we combine data of all patients in our cohort to look for genes of which more than one patient has a rare variant.

Variant causality

If applicable additional immunohistochemical staining, loss of heterozygosity, second hit analysis and/or functional analysis will be performed. Novel variants are subsequently selected for replication studies in international databases (e.g. TCGA - The Tumor Cancer Genome Atlas, LOVDplus - Leiden Open Variation Database , COSMIC - Catalogue of Somatic Mutations in Cancer, and ProteinPaint - Pediatric Cancer Genome Project). Furthermore, we collaborate with different (inter)national research groups studying FNMTC and childhood NMTC; candidate genes can thus be replicated in their cohorts.

In conclusion, improving our fundamental understanding of pediatric NMTC pathogenesis and genetic pathways provides a partial answer to questions of patients and parents, namely, *"Why do I have cancer? Are other relatives at risk? And if so, can we prevent cancer?"* Clinical guideline for referral i.e. patient selection, and type of DNA testing i.e. single gene vs gene panel vs whole genome sequencing, should be based on the final results.

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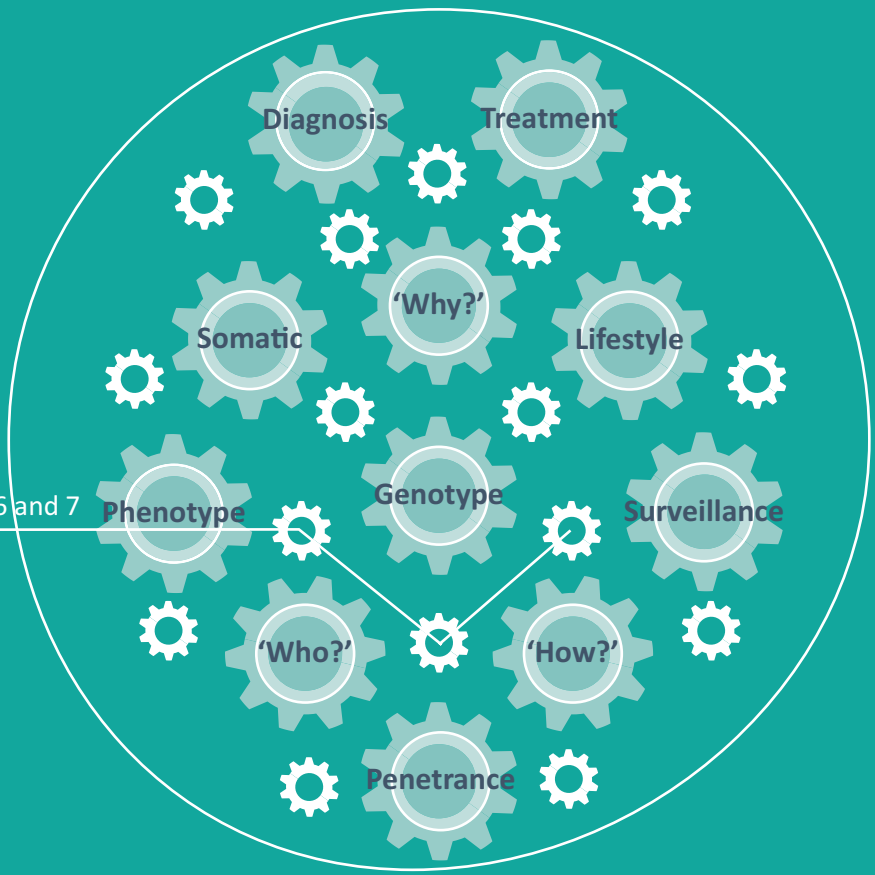

Part III
Chapter 5, 6 and 7

PART III

GENETIC COUNSELING IN ENDOCRINE TUMOR PREDISPOSITION SYNDROMES

This chapter describes the clinical manifestations and penetrance in *CDC73*-related disorders and formulate recommendations to improve case detection in pHPT.

CDC73-Related Disorders: Clinical Manifestations and Case Detection in Primary Hyperparathyroidism

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ABSTRACT

Context

Heterozygous pathogenic germline variants in *CDC73* predispose to the development of primary hyperparathyroidism (pHPT) and, less frequently, ossifying fibroma of the jaw and renal and uterine tumors. Clinical information on *CDC73*-related disorders has so far been limited to small case series.

Objective

To assess the clinical manifestations and penetrance in *CDC73*-related disorders and to improve case detection in pHPT.

Design

Nationwide retrospective Dutch cohort study.

Setting

Tertiary referral center.

Patients

We studied 89 patients with pHPT referred for germline *CDC73* analysis and 43 subsequently tested relatives who proved to be mutation carriers.

Investigation

Germline *CDC73* mutation analysis.

Mean Outcome

CDC73 mutation detection yield, referral rate and *CDC73*-related disease penetrance.

Results

Pathogenic germline *CDC73* variants were identified in 11 of the 89 referred pHPT patients (12.4%), with (suspected) hyperparathyroidism-jaw tumor (HPT-JT) syndrome (n = 3), familial isolated pHPT (n = 5), apparently sporadic parathyroid carcinoma (n = 2), and apparently sporadic parathyroid adenoma (n = 1). The estimated penetrance of *CDC73*-related disorders was 65% at age 50 years (95% confidence interval 48% to 82%) in 43 non-index mutation carriers.

Conclusions

Germline *CDC73* analysis is recommended in individuals with (suspected) HPT-JT syndrome, familial isolated pHPT, atypical or malignant parathyroid histology, and young individuals with pHPT. These criteria would increase germline *CDC73* mutation detection, thus enabling optimal clinical management of pHPT, as well as genetic counseling and surveillance for family members at risk for developing *CDC73*-related disorders. *(J Clin Endocrinol Metab 102: 4534–4540, 2017)*

INTRODUCTION

Primary hyperparathyroidism (pHPT) is a common endocrine disease with a prevalence of 1 to 4 per 1000 persons and with a peak incidence in the sixth decade of life.[1] In the majority of cases, pHPT is caused by a single parathyroid adenoma (PA) and in less than 1% by a parathyroid carcinoma (PC).[2] A genetic predisposition for pHPT can be found in ~10% of pHPT cases. This might be an underestimation because of unavailable, incomplete, or misdiagnosed family history; variable penetrance; or unknown genetic causes. To date, pathogenic variants in at least 11 genes have been found to be associated with hereditary pHPT. The most commonly identified hereditary syndromes associated with pHPT include multiple endocrine neoplasia type 1, 2a, or 4; and *CaSR*-, *GCM2*-, and *CDC73*-related disorders.[3,4] Inactivation of the *CDC73* tumor suppressor gene (formerly known as *HRPT2* and encoding parafibromin) predisposes heterozygous carriers to a spectrum of conditions: hyperparathyroidism-jaw tumor (HPT-JT) syndrome, familial isolated hyperparathyroidism (FIHP), and PC.

The penetrance of pHPT in *CDC73*-related disorders has been reported to be as high as 80% to 95%.[5] The onset is typically in late adolescence or early adulthood, although patients younger than 10 years of age have also been reported.[6,7] PC may be found in >20% of patients with germline pathogenic *CDC73* variants, which is higher than in other hereditary pHPT syndromes.[5] Distinguishing between PA, atypical adenoma (AA) and PC remains a challenge given the lack of specific differentiating clinical, biochemical and histological features among these pathologies. However, the latter is of the utmost importance because it determines the extent and radical nature of initial surgery, which is in turn the major determinant of prognosis.[5]

In addition to pHPT, patients with *CDC73*-related disorders are predisposed to developing ossifying fibromas of the mandible and/or maxilla, uterine tumors (e.g. adenofibromas, leiomyomas, adenomyosis, hyperplasia, and adenosarcomas) and less frequently, a variety of malignant and nonmalignant renal lesions [e.g., Wilms tumor, clear cell renal carcinoma (RCC), papillary renal cell tumor, renal cysts].[5]

In total, about 100 index *CDC73* mutation carriers have been reported to date, with no clearly identified phenotype-genotype relationship.[5] The majority of germline (and somatic) pathogenic *CDC73* variants are frameshift and nonsense variants, although missense variants as well as (small) deletions and insertions have been reported.[7-9]

Limited data are available on the germline *CDC73* mutation detection yield in patients with HPT-JT syndrome, FIHP and PC. In this study, we performed a nationwide evaluation of germline *CDC73* analyses undertaken in pHPT patients in the Netherlands, and characterized the clinical manifestations and penetrance of 12 families with *CDC73*-related disorders.

PATIENTS AND METHODS

Study population and design

All Dutch patients with an established diagnosis of pHPT referred for germline *CDC73* analysis in the Netherlands from February 2004 through July 2016 were included in the study. There were no specific referral criteria for germline CDC73 analysis in the Netherlands during the study period. Data on sex, diagnosis, age at diagnosis, family history, and clinical manifestations were retrieved from DNA request forms.

Referred pHPT patients were grouped in four clinical subgroups based on their personal and/or family history: (1) (suspected) HPT-JT syndrome [pHPT and at least one HPT-JT syndrome-related feature or pHPT and a close relative with (suspected) HPT-JT syndrome], (2) FIHP (pHPT

and at least one first or second degree relative with pHPT), (3) apparently sporadic PC (sPC), and (4) apparently sporadic PA (sPA). HPT-JT related features included pHPT, ossifying fibroma of mandible and/or maxilla, renal lesions and uterine tumors. According to the Dutch genetic testing strategy, before *CDC73* analysis, germline *MEN1* variants had to be excluded in patients with FIHP and sPAs diagnosed before age 35 years.

Index patients with pathogenic *CDC73* variants or variants of uncertain significance (VUS) were evaluated and counseled by a clinical geneticist in their regional university medical center. Written informed consent for collection of clinical, pathological and molecular data was obtained from all index mutation carriers. Relatives were tested for the specific pathogenic *CDC73* variant using cascade screening after counseling. All *CDC73* mutation carriers were referred for surveillance aimed at detecting pHPT or jaw-, renal- and/or uterine abnormalities. We also included in the study an extra family belonging to a Dutch index-patient with *CDC73*-related disorder who underwent genetic testing abroad, wherase genetic testing via cascade screening of relatives was performed at our laboratory.

The study was approved by the local Ethical Committee of the Leiden University Medical Center (P15.016).

DNA sequencing and data analysis

Germline *CDC73* mutation analysis was centralized in the Laboratory for Diagnostic Genome Analysis department of clinical genetics at the Leiden University Medical Center, the Netherlands, during the study period. Germline *CDC73* mutation analysis was performed with Sanger sequencing. *CDC73* deletion/duplication analysis was subsequently performed in 60 patients without pathogenic *CDC73* variant using the MRC Holland P466-A1 kit (MRC Holland, Amsterdam, the Netherlands).

Coding variants were analyzed for their effect on function with Alamut software package v2.7 (Interactive Biosoftware, Rouen, France), which incorporates, for example, Align GVGD, SIFT and PolyPhen2. Variants were annotated to the Genbank reference sequence NM_024529.4. The Leiden Open Variation Database (http://www.lovd.nl/CDC73) was consulted to find variants previously described and classified.

Histological and molecular analysis of parathyroid tumors

The overproducing parathyroid gland(s) were removed in all patients referred for germline *CDC73* mutation analysis and all *CDC73* mutation carriers diagnosed with pHPT as part of standard care. Available tumor tissue was re-examined by a referral pathologist in Leiden (H.M.). Parafibromin immunohistochemistry (IHC), somatic *CDC73* analysis and loss of heterozygosity analysis were performed on formalin-fixed, paraffin-embedded (FFPE) samples as previously described.[10] IHC was scored positive ("normal") if nuclear staining was detected in lesional cells and was only considered negative ("loss") in the presence of positive internal controls.

Statistical analysis

To describe clinical characteristics, the mean ± standard deviation (SD) with range was calculated. Continuous variables were analyzed using an independent sample t-test. Dichotomous variables were compared using the χ^2 test. Age-related penetrance of pHPT was estimated using the Kaplan–Meier method. Statistical significance was set at $P > 0.05$; analyses were conducted using SPSS 23.0 (SPSS, Chicago, IL).

RESULTS

CDC73-related disorders - case detection

Pathogenic germline *CDC73* variants were identified in 11 of 89 (12.4%) clinically heterogeneous pHPT patients referred for mutation analysis. In total, seven different nonsense or frame shift pathogenic variants were identified; two families carried an exon 1 deletion and two families carried a large deletion spanning the entire *CDC73* gene. The clinical characteristics of the study population *(CDC73* vs. non-*CDC73)* are listed in Table 1. Within the clinical subgroups, pathogenic germline *CDC73* variants were identified in 3 of 18 patients with (suspected) HPT-JT (17%), in 5 of 19 patients with FIHP (26%), in 2 of 11 patients with sPC (18%), and in 1 of 41 patients with sPA (2%). The mean age (±SD) at diagnosis of pHPT was 32 ± 15 years (range, 13 to 54 years) in *CDC73* mutation carriers and 42 ± 18 years (range, 10 to 81 years) in those without a detectable mutation (P =0.068). Ten of the 11 *CDC73* mutation carriers were male (91%), as opposed to 41% of non-mutation carriers (P= <0.01). In total, 12 patients were diagnosed with PC (11 apparently sporadic and one in the context of FIHP). Family history was positive for pHPT in 73% of *CDC73* mutation carriers, as opposed to only 24% in non-mutation carriers (P= <0.01). A personal history of Wilms tumor was reported in one *CDC73* mutation carrier and one patient carrying a variant of uncertain significance (VUS, see following section). No other index *CDC73* mutation carrier was diagnosed with renal abnormalities. In total, eight index non-mutation carriers had a personal history of renal abnormities (five with RCC and three with renal cysts).

CDC73 variant of uncertain significance

One *CDC73* variant of uncertain significance [c.14T>G, p.(Leu5Arg)] was further identified in a female aged 37 years with pHPT and a history of a Wilms tumor at age 2 years. IHC showed global loss of parafibromin staining in her PA, and loss of heterozygosity of the wild type *CDC73* allele was also seen. The Wilms tumor sample was not available for further investigation. Family

Table 1. Clinical characteristics of 89 pHPT patients referred for germline *CDC73* analysis

	Pathogenic *CDC73* variant	No pathogenic *CDC73* variant	*P* value	Yield, %
	n=11	n=78		12.4
Age mean ± SD (y)	32.3 ± 14.6	42.6 ± 18	0.068	
Range (y)	13-54	10-81		
Sex, male, n (%)	10 (91)	32 (41)	0.002	
(suspect) HPT-JT syndrome, n (%)	3 (27)	15 (19)		16.7
Familial isolated pHPT, n (%)	5 (45)[a]	14 (18)		26.3
Sporadic parathyroid carcinoma, n (%)	2 (18)	9 (12)		18.0
Sporadic parathyroid adenoma, n (%)	1 (9)	40 (51)		2.4
Familiar pHPT, n (%)	8 (73)	19 (24)	0.003	
Recurrent pHPT or multiple PA	0	12	0.162	
Renal abnormalities	1	9	0.810	
Uterine abnormalities	0	4	0.758	

[a]One of these patients was diagnosed with a PC. In total, 9 of 12 PCs were revised by a referral pathologist.

history showed a maternal cousin with pHPT aged 30 years, whereas the mother and aunt were unaffected. Segregation analysis confirmed the presence of the variant in the affected cousin. However, IHC showed positive parafibromin staining in her PA and no pathogenic somatic *CDC73* variants or loss of heterozygosity of the wild type *CDC73* allele. The c.14T>G variant has not been reported in the Single Nucleotide Polymorphism Database (dbSNP), Exome Sequencing Project (ESP,), Exome Aggregation Consortium (ExAc), Genome of the Netherlands (GoNL), or ClinVar databases and affects an evolutionarily conserved amino acid. The substitution of the leucine residue by an arginine residue results in a relatively large difference in physical and chemical properties (Grantham score 102 [range, 0 to 215])[11]. AGVGD, SIFT and PolyPhen software predicted that this amino acid change will have a major effect on protein function. *In silico* RNA splice prediction software predicted no substantial change compared with the wildtype sequence.

Clinical manifestations in families with *CDC73*-related disorders

The characteristics of the index *CDC73* mutation carriers and their tested relatives are shown in Table 2. Analysis of 77 relatives who were tested via cascade screening for their familial pathogenic

Table 2. Overview of the clinical and molecular characteristics of 12 index CDC73 mutation carriers and their tested relatives

ID	Sex	Tumors Observed (Age at Detection, y)	Family History	Phenotype	Germline *CDC73* variant	Tested Relatives	Non-index carriers (Symptomatic)	Not tested Symptomatic Relatives
A	M	PA (54)	pHPT	FIHP	c.226C>T, p.(Arg76*)	6	2 (1)	2
B	M	PC (54), RCC (57)	Negative	sPC	c.544dup, p.(Ile182Asnfs*11)	3	1 (0)	0
C	F	PA (17)	pHPT	FIHP	c.358C>T, p.(Arg120*)	1	1 (1)	1
D[a]	M	PA (34)	pHPT, Renal cysts	Suspect HPT-JT syndrome	c.687_688dellAG, p.(Arg229Serfs*37)	37	24 (14)	2
E	M	Jaw (15), PA (22)	pHPT, Wilms tumour	HPT-JT syndrome	c.3_15dup, p.(Ser6Glyfs*5)	3	3 (3)	0
F	M	PA (13)	Negative	sPA	Whole gene deletion	4	2 (0)	0
G	M	PC (45)	pHPT	FIHP	Whole gene deletion	9	4 (2)	0
H	M	Wilms tumor (8), PA (33)	pHPT, uterine fibroids	FIHP	c.3_15dup, p.(Ser6Glyfs*5)	3	1 (1)	0
I	M	PC (18)	Negative	sPC	Exon 1 deletion	0		0
J	M	PA (40)	pHPT	FIHP	Exon 1 deletion	2	1 (1)	1
K	M	PA (25)	pHPT	FIHP	c.685_688delAGAG, p.(Arg229Tyrfs*27)	0		2
L	M	PA (40)	pHPT, jaw	HPT-JT syndrome	c.760C>T, p.(Gln254*)	8	4 (1)	0

Abbreviations: M; male, F; female, Jaw; ossifying fibroma jaw, [a] Published before (Haven *et al*; 2000)

CDC73 variant revealed 43 non-index mutation carriers in 10 families. Detailed information on all *CDC73* mutation carriers can be found in Supplemental Table 1 and in pedigrees A though K (Supplemental Fig. 1). The mean age (± SD) at DNA analysis was 42 ± 20 years (range, 10 to 80 years) in the non-index *CDC73* mutation carriers. In total, 24 of 43 (56%) non-index mutation carriers were diagnosed with one or more *CDC73*-related disorder features, including pHPT (n = 20), ossifying fibroma of the jaw (n = 5), renal abnormalities (n = 8) and uterine fibroids (n=1). In non-index mutation carriers, pHPT was associated with a single PA, atypical adenoma and PC in 17 (85%), 1 (5%), and 2 (10%) cases, respectively. In addition, at least eight family members from five different families (families A, C, D, J and K) have been diagnosed with pHPT but have not (yet) been tested for the pathogenic *CDC73* variant in their family. The age-related overall penetrance values for the 43 non-index *CDC73* mutation carriers were 11% at age 25 years [95% confidence interval (CI) 2% to 20%], 65% at age 50 years (95% CI, 48% to 82%), and 83% at age 70 years (95% CI, 57% to 99%) (Fig. 1A). The mean age (± SD) at diagnosis of pHPT was 39 ± 14 years (range, 10 to 67 years) in the affected non-index mutation carriers, compared to 33 ± 15 years (range, 13–54 years) in the index mutation carriers (p=0.32). The age-related pHPT penetrance values for the 43 non-index *CDC73* mutation carriers were 8% at age 25 years (95% CI, 0% to 16), 53% at age 50 years (95% CI, 33% to74) and 75% at age 70 years (95% CI, 54% to 95%) (Fig. 1B).

DISCUSSION

Here, we report the results of a nationwide retrospective *CDC73* survey to investigate *CDC73* mutation detection yield and clinical phenotype in so far genetically unexplained pHPT patients. We identified pathogenic germline *CDC73* variants in 11 of 89 pHPT patients (12.4%). In our study population, mutation detection was associated with younger age, male sex, malignant disease and a positive family history. The estimated penetrance of *CDC73*-related disorders was 83% at

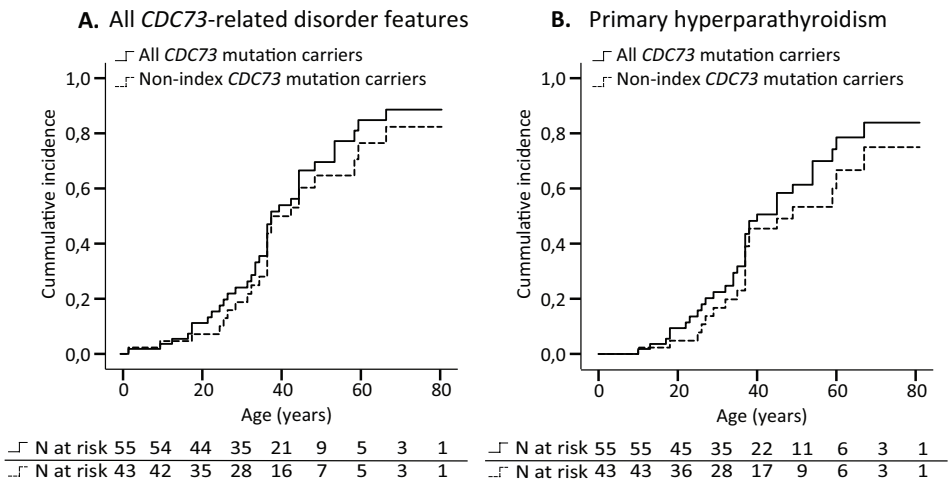

Figure 1. Age-related penetrance of *CDC73*-related disorder features in all *CDC73* mutation carriers (n = 55) vs non-index mutation carriers (n = 43). **A.** Age-related penetrance of all *CDC73*-related disorder features for all *CDC73* mutation carriers (black line) and only non-index CDC73 mutation carriers (dotted line). **B.** Age-related penetrance of pHPT for all *CDC73* mutation carriers (black line) and only non-index *CDC73* mutation carriers (dotted line).

age 70 (95% CI, 57% to 99%) in 43 non-index mutation carriers. Prospective studies in larger series of *CDC73* mutation carriers, including genotype-phenotype relationships, genetic modifiers and/or environmental factors, are required to determine the optimal age at which surveillance should be initiated and the monitoring intervals required to detect the different manifestations of *CDC73*-related disorders as they develop.

Improving future detection of *CDC73*-related disorder cases

In light of the relatively high incidence of pHPT and of the importance of genetic diagnosis, there is an unmet clinical need for development of guidelines for genetic testing. Based on data from our nationwide cohort analysis, we recommend germline *CDC73* analysis in the four clinical subgroups of patients with pHPT listed next, a recommendation that is also in line with the 2015 Consensus Report on hereditary hyperparathyroidism of the European Society of Endocrine Surgeons.[5]

- All patients with HPT-JT syndrome

First, germline *CDC73* analysis is recommended in individuals with (suspected) HPT-JT syndrome. Although the mutation detection yield (3/18, 17%) in our study population was lower than in a previous study (13/24, 54%), the high yield in the initial study might have been an overestimate due to ascertainment and selection bias.[12]

- All patients with familial pHPT (after exclusion of other gene abnormalities)

Second, germline *CDC73* analysis is recommended in patients with FIHP after exclusion of pathogenic germline *MEN1* variants. The mutation detection yield in our study population was 27% in patients with at least one first or second-degree relative with pHPT. Different mutation detection yields ranging from 0% to 28% were found in previous, mostly small, studies[13-17]

- All patients with PC or atypical histology of PA

The third subgroup of patients with pHPT in which germline *CDC73* analysis is recommended includes individuals with apparently sporadic atypical or malignant parathyroid pathology. In our study population, the mutation detection yield in s PC was 17%. The detection yield observed in previous studies varies substantially per study population; ranging from 6%, 17% to 29%, 18%, 20%, and 31% to 38% in patients from Finland[18], Italy[19-21], France[7], United States/Japan[22] and China[23,24], respectively. The study size and patient selection differed between studies and that a unequivocal morphological diagnosis can be challenging. Referral to an experienced parathyroid surgeon and an expert pathologist should be considered in all patients with suspected PC. Subsequent parafibromin IHC and somatic *CDC73* analysis could be considered for diagnostic and prognostic purposes.[25] The frequency of pathogenic germline *CDC73* variants in individuals with atypical adenoma has not been extensively studied and limited data are available on the contribution of IHC in cases with equivocal histology.

- All patients with sporadic pHPT, younger than 35 years

The fourth subgroup of patients with pHPT in which germline *CDC73* analysis is recommended includes young individuals with apparently sporadic benign pHPT, after exclusion of pathogenic germline *MEN1* variants. In our study population, one patient with sPA (a 13-year-old boy) carried a pathogenic germline *CDC73* variant. The yield of germline *CDC73* testing in patients with sPA has barely been studied; therefore, no age-specific criteria can be identified. Dutch guidelines recommend germline *MEN* analysis in patients with pHPT diagnosed before age 35 years.[26] For practical reasons, subsequent germline *CDC73* analysis should also be considered in these patients.

Gene panel testing

To date, genetic testing for germline variants in genes predisposing to hereditary pHPT involved mainly sequential testing of single genes, prioritized according to clinical features. This type of testing protocol is expensive and time-consuming because at least 11 genes are associated with hereditary pHPT. The introduction of gene panel testing using next generation sequencing would improve genetic testing for these rare disorders. However, complete analysis of *CDC73* in next generation sequencing panels will be challenging because of the presence GC-rich regions and frequent germline *CDC73* deletions (4 of 12 in our study cohort).

Limitations and strengths of the study

The main strength of the current study is that all pHPT patients referred for germline *CDC73* analysis in the Netherlands within a defined period (2004 through 2016) were included in the study. A further strength is that a total of 55 *CDC73* mutation carriers from 12 families were clinically investigated, in close collaboration with a number of Dutch University Medical Centers, representing one of the largest *CDC73*-related disorder series to date.

The study also has a number of limitations. The first is that the estimated mutation detection yield in this study was found in a retrospective diagnostic cohort, which despite being one of the largest *CDC73*-related cohorts published, might not be representative of the total patient population. Second, because we were not able to revise the histology of all parathyroid tumors from patients referred for germline *CDC73* analysis, some patients may have been misclassified. And third, a possible explanation for the relatively low penetrance for jaw, uterine and renal lesions could be inadequate surveillance and incomplete follow-up data. Alternatively, the high penetrance observed in prior studies (20% to 60%)[7,9,27,28] is likely due to ascertainment and selection bias.

In conclusion, our data demonstrate that pathogenic germline *CDC73* variants are frequently found in previously genetically-unexplained pHPT patients. Our findings further suggest that genetic testing should be recommended in individuals with pHPT and HPT-JT-syndrome related features, familial isolated pHPT, atypical or malignant parathyroid histology, and in young individuals with pHPT. Gene panel testing or consecutive gene testing, including additional deletion and Sanger sequencing testing, should be considered, depending on the phenotype and available genetic testing options. Clinical use of these criteria will enhance the identification of individuals with *CDC73*-related disorders, thus improving both early detection of tumor development and genetic counseling.

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SUPPLEMENTAL DATA

Supplementary Table 1. Clinical characteristics of all CDC73 mutation carriers in this study

ID	Sex/ Age*	Tumors observed (age at detection, years)	(Re-) examined histology	Parafibromin IHC	Somatic *CDC73* mutation	LOH
Family A; c.226C>T, p.(Arg76*)						
II.1	M/64	PA (59)	N			
II.2	**M/59**	**PA (54)**	**N**			
III.4	M/32					
Family B; c.544dup, p.(Ile182Asnfs*11)						
II.3	**M/61**	**PC (54), RCC (57)**	**Y (PC)**	**Focal loss**	**No**	No
III.1	M/30					
Family C; c.358C>T, p.(Arg120*)						
II.6	F/57	PA (25)	N			
III.7	F/25	PA (17)	N			
Family D§; c.687_688dellAG, p.(Arg229Serfs*37)						
II.1	F/80'	PA	N			
III.1	F/47'	PA (37), renal cysts, Hürthle cell adenoma thyroid, Pancreatic Ductal Adenocarcinoma (47)	N			
III.2	F/80					
III.3	M/79	PA (25), renal cysts, RCC (54)	N			
III.4	F/77	PA (29), renal cysts	N			
III.5	F/38'	PA, renal cysts	N			
III.6	F/53'	PA (32), PC (36), renal cysts, Pancreatic Ductal Adenocarcinoma (36)	Y (PC)	Global loss	No	No
III.7	M/74	PA	N			
III.9	F/69	PA	N			
IV.1	**M/53**	**PA (34)**	**Y**	**Normal**	**NA**	**NA**
IV.2	F/51	PA (18)	N			
IV.3	M/48	mixed germcell testicular tumor (30)	N			
IV.7	M/42					
IV.11	M/56	PA (37)	Y	NA	NA	NA
IV.13	M/51	Jaw* (33)	NA			
IV.14	F/45	PA (26), Jaw* (27)	Y	NA	NA	NA
IV.19	F/48					
IV.20	F/41	PA (38)	N			
IV.21	F/49	PA (49)	N			
IV.22	F/43					

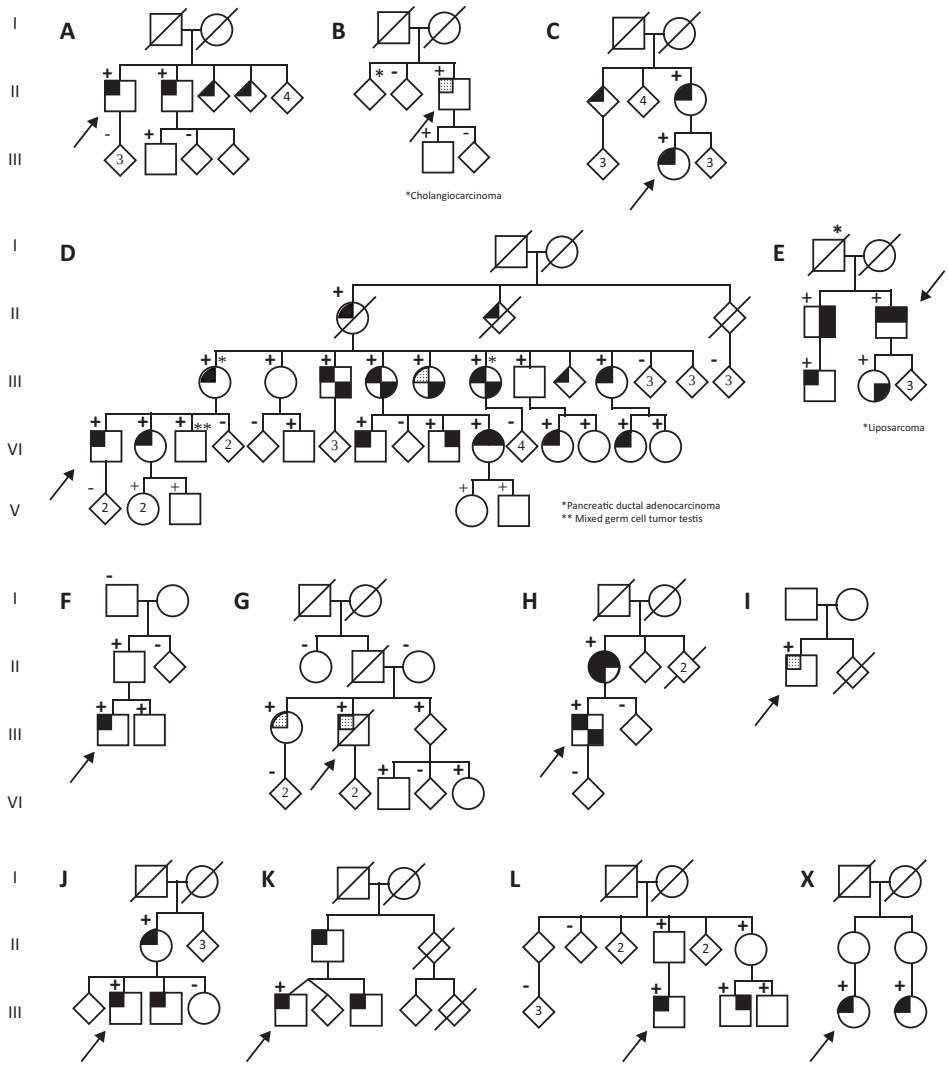
Supplementary Table 1. (continued)

ID	Sex/ Age*	Tumors observed (age at detection, years)	(Re-) examined histology	Parafibromin IHC	Somatic *CDC73* mutation	LOH
V.3	F/23					
V.4	M/21					
V.5	F/17					
V.6	F/17					
V.7	M/12					
Family E; c.3_15dup, p.(Ser6Glyfs*5)						
II.1	M/59	Jaw (43), Renal cyst	NA			
II.2	**M/57**	**Jaw (15), PA (22)**	**N**			
III.1	M/20	AA (10)	Y	Global loss	No	No
III.4	F/21	Congenital urinary tract abnormality, Wilms tumor (2)	N			
Family F; Whole gene deletion						
II.1	M/49					
III.1	**M/17**	**PA (13)**	**N**			
III.2	M/19					
Family G; Whole gene deletion						
III.1	F/54	PC (27)	Y	Normal	NA	NA
III.2	**M***	**PC (45)**	**Y**	**Focal loss**	**p.(Ser31Glyfs*7)**	
III.3	M/51	PA (45)	N			
IV.5	M/21					
IV.7	F/16					
Family H; c.3_15dup, p.(Ser6Glyfs*5)						
II.1	F/70	Uterus fibroids (36), PA (60), Jaw* (69)	Y			
III.1	**M/36**	**Wilms tumor (8), PA (33)**	**Y (PA)**	**Global loss**	**No**	**No**
Family I; Exon 1 deletion						
II.1	**M/30**	**PC (18)**	**Y**	**Global loss**	**p.(Glu29*)**	
Family J; Exon 1 deletion						
II.1	F/69	PA (67)	N			
III.2	**M/41**	**PA (40)**	**N**			
Family K; c.685_688delAGAG, p.(Arg229Tyrfs*27)						
III.1	**M/28**	**PA (25)**	**N**			
Family L; c.760C>T, p.(Gln254*)						
II.4	M/72					
II.6	F/70					
III.4	**M/45**	**PA (40)**	**N**			
III.8	M/49	Jaw	NA			
III.9	M/47					

Supplementary Table 1. (continued)

ID	Sex/ Age*	Tumors observed (age at detection, years)	(Re-) examined histology	Parafibromin IHC	Somatic *CDC73* mutation	LOH
Family X; c.14T>G, p.(Leu5Arg)^						
III.1	**F/44**	**PA (40)**	**Y**	**Global loss**	**No**	**Yes**
III.2	F/41	PA (30)	Y	Normal	No	No

IDs are according to the pedigrees (see suppl. figure 1), index mutation carriers are in bold.
Abbreviations: PA; parathyroid adenoma, PC; parathyroid carcinoma, Jaw; ossifying fibroma jaw, RCC; clear cell renal carcinoma. §Published before *(Haven et al, 2000)*; †deceased, *age last update clinical information or age of death, '= asymptomatic, detection during surveillance, ^Variant of uncertain significance.



Supplemental Figure 1. Pedigrees of 12 families with *CDC73*-related disorders (A-L) and one family (X) with a germline unclassified *CDC73* variant. Please note that the pedigree has been adjusted to protect the identity of the family without a loss of scientific integrity. Circles represent females; squares represent males; diamonds represent undisclosed gender, cross striped individuals are death, parathyroid adenoma (left top, black), parathyroid carcinoma (left top, dotted pattern), ossifying fibroma jaw (right top, black), uterine fibroids (left bottom, black), renal abnormalities (right bottom, black), *CDC73* mutation carrier (+); non-carriers (-).

This chapter describes the contribution of germline pathogenic *SDHA* variants in paraganglioma patients, assess the clinical manifestations and determine the age-related penetrance.

Clinical Aspects of *SDHA*-Related Pheochromocytoma and Paraganglioma: A Nationwide Study

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ABSTRACT

Context

Paraganglioma (PGL) has the highest degree of heritability among human neoplasms. Current clinical understanding of germline *SDHA* mutation carriers is limited.

Objective

To estimate the contribution of *SDHA* mutations in PGL and to assess clinical manifestations and age-related penetrance.

Design

Nationwide retrospective cohort study.

Setting

Tertiary referral centers in the Netherlands (multicenter).

Patients

Germline *SDHA* analysis was performed in 393 patients with genetically unexplained PGL. Subsequently 30 index *SDHA* mutation carriers and 56 non-index carriers were studied.

Mean outcome

SDHA mutation detection yield, clinical manifestations and *SDHA*-related disease penetrance.

Results

Pathogenic germline *SDHA* variants were identified in 30 of the 393 referred patients (7.6%) with PGL, who had head and neck PGL (21 of 174 [12%]), pheochromocytoma (4 of 191 [2%]) or sympathetic PGL (5 of 28 [18%]). The median age at diagnosis was 43 years (range, 17 to 81 years) in index *SDHA* mutation carriers compared with 52 years (range, 7 to 90 years) in nonmutation carriers (P=0.002). The estimated penetrance of any *SDHA*-related manifestation was 10% at age 70 years (95% confidence interval 0% to 21%) in non-index mutation carriers.

Conclusion

Germline *SDHA* mutations are relatively common (7.6%) in patients with genetically unexplained PGL. Most index patients presented with apparently sporadic PGL. In this *SDHA* series, the largest assembled so far, we found the lowest penetrance of all major PGL predisposition genes. This suggests that recommendations for genetic counseling of at-risk relatives and stringency of surveillance for *SDHA* mutation carriers might need to be reassessed. *(J Clin Endocrinol Metab 103: 438–445, 2018)*

INTRODUCTION

Paragangliomas (PGLs) are rare neuroendocrine tumors that carry the highest degree of heritability among human neoplasms.[1,2] PGLs are classified according to their anatomic location (intra-adrenal or extra-adrenal PGL) and whether they are of sympathetic or parasympathetic origin. Head and neck paragangliomas (HNPGLs) emerge from the parasympathetic nervous system and are usually benign slow-growing tumors.[3,4] Common sites include the carotid body, the temporal bone, and the vagal body. Parasympathetic PGLs are most often nonsecreting, although about 30% are associated with elevated levels of the dopamine metabolite 3-methoxytyramine (3-MT).[5] Pheochromocytoma (PHEO) and sympathetic paraganglioma (SPGL) are catecholamine-secreting tumors.[6] PHEOs are derived from the chromaffin cells of the adrenal medulla and SPGLs are found in close relationship to the peripheral sympathetic nervous system from the level of the superior cervical ganglion down the trunk into the pelvis.[7] Metastases are more often present in SPGL than are to PHEO and HNPGL.[3]

About one third of patients with PGL have been reported to carry pathogenic germline variants in a growing list of susceptibility genes.[8] The most described genes are *NF1, RET, VHL, SDHD, SDHC, SDHB, SDHAF2, SDHA, TMEM127 and MAX*. Germline variants in the succinate dehydrogenase (*SDH*) genes are the most common genetic cause of PGLs, occurring in up to 15% of all patients with PGL and half of all familiar cases.[2,9] In 2010, a direct association between germline *SDHA* variants and PGL was reported.[10] *SDH*-associated syndromes are characterized by the development of PGLs, with an additional risk of developing other tumor types [e.g., clear cell renal cancer (RCC), gastrointestinal stromal tumors (GISTs) and more rarely, neuroendocrine tumors and pituitary adenomas].[11-13] So far, information on prevalence, phenotype, penetrance and pathogenicity of *SDHA* variants is limited to one large series[14] and a few small series.[15,16] In this study we performed a nationwide evaluation of germline *SDHA* analyses undertaken in patients with PGL and characterized the clinical manifestations and disease penetrance in 30 index *SDHA* mutation carriers and their relatives.

PATIENTS AND METHODS

Study population and design

All patients with an established diagnosis of PGL who were referred for germline *SDHA* analysis in the Netherlands from February 2011 through July 2016 were included in this study. Referred patients with PGL were grouped into three clinical subgroups - HNPGL, PHEO, or SPGL - on the basis of clinical, biochemical, imaging and/or histological characteristics. Data on sex, diagnosis and age at diagnosis were retrieved from DNA request forms. In accordance with the Dutch national genetic testing strategy, all patients with PGL referred for *SDHA* analysis lacked pathogenic germline variants in *SDHB*, *SDHC*, *SDHD*, and *SDHAF2*. All patients with PHEO and SPGL furthermore lacked pathogenic germline variants in *TMEM127*, *MAX*, *RET* and *VHL* and had no clinical symptoms suggesting neurofibromatosis type 1.

Index patients with (likely) pathogenic *SDHA* variants or variants of uncertain significance (VUS) were evaluated and subsequently counseled by a clinical geneticist in their regional University Medical Center. Patients with pathogenic and likely pathogenic variants are annotated as *SDHA* mutation carriers in this manuscript. Clinical characteristics (e.g. sex, age at diagnosis, tumor location, or locations, presence of metastases, biochemical phenotype and additional non-PGL tumors) and pedigrees were collected.

Genetic counseling and testing for the family-specific (likely) pathogenic *SDHA* variant were offered to relatives via cascade screening. All *SDHA* mutation carriers age ≥18 years were referred to departments of otorhinolaryngology and endocrinology for annual clinical surveillance aimed at detecting PGL. According to national guidelines,[17] surveillance consisted of magnetic resonance imaging of the head and neck region once every 3 years and magnetic resonance imaging or computed tomography of the thorax, abdomen and pelvis once every 2 years. Annual routine biochemical testing included the measurement of (nor)epinephrine, (nor)metanephrine, dopamine and/or 3-MT in 24 hour urine samples and/or plasma, depending on the center. In cases with excessive catecholamine secretion (i.e., any value above the upper reference limit), radiological assessment of the thorax, abdomen, and pelvis was performed to identify potential sources of excessive catecholamine production. The current study was approved by the local medical ethical committee of Leiden University Medical Center (G16.063).

DNA sequencing and data analysis

Germline *SDHA* variant analysis was performed in the d\Department of Human Genetics at the Radboud University Medical Center and the Laboratory for Diagnostic Genome Analysis of the Department of Clinical Genetics at Leiden University Medical Center, the Netherlands. Genomic DNA was extracted from peripheral blood leukocytes according to standard procedures. Germline *SDHA* analysis was performed with Sanger sequencing or next generation (gene panel) sequencing (NGS) depending on the testing period. For the detailed NGS procedure, see the Supplemental Method.

Coding variants were analyzed for their effect on function by using the Alamut software package version 2.7 (Interactive Biosoftware, Rouen, France), which incorporates Align GVGD,[18] polymorphism phenotyping (PolyPhen2),[19] and sorting intolerant from tolerant (SIFT).[20] Variants were annotated to the Genbank reference sequence NM_004168.2. The Leiden Open Variation Database (http://www.lovd.nl/*SDHA*) was consulted to find variants previously described and classified. Variant interpretation was done in line with the recent consensus statement on NGS-based diagnostic testing of PHEO and PGLSs.[21] Variant nomenclature is in accordance with Human Genome Variation Society guidelines version 2.0 (http://www.hgvs.org).

To obtain further support for the pathogenicity of certain *SDHA* variants, SDHA immunohistochemistry and loss-of-heterozygosity (LOH) analysis were performed on formalin-fixed, paraffin-embedded samples, as described elsewhere.[22]

Statistical analysis

Descriptive statistics were used to characterize the study population, to determine the age of PGL onset, and to examine the difference between patients with germline *SDHA* mutation and those without germline *SDHA* mutation. Continuous variables were analyzed by using an independent sample t test. Dichotomous variables were compared using the χ^2 test. Age-related penetrance of PGL was estimated by using the Kaplan-Meier method. Because most of the non-index mutation carriers were recently identified, we used the age at 1 year after DNA analysis (or age at death) for the penetrance estimation. By completion of this manuscript, 80% of the non-index mutation carriers had participated in surveillance at least once. Statistical significance was set at $P < 0.05$, and the analyses were conducted by using SPSS software, version 23.0 (IBM, Armonk, NY).

RESULTS

SDHA case detection in the study population

Pathogenic germline *SDHA* variants were identified in 30 of 393 (7.6%) patients with PGL who were referred for *SDHA* genetic testing. The clinical characteristics of the study population *(SDHA* vs. non-*SDHA)* are listed in Table 1. Within the clinical PGL subgroups, pathogenic *SDHA* variants were identified in 21 of 174 patients with HNPGL (12%), 4 of 191 patients with PHEO (2%), and 5 of 28 patients with SPGL (18%). The median age at diagnosis of PGL was 43 years (range, 17 to 81 years) in *SDHA* mutation carriers and 52 years (range, 7 to 90 years) in those without a detectable mutation (p=0.002). Half of the *SDHA* mutation carriers were males, compared with to 32% of patients without an *SDHA* mutation (P = 0.049).

SDHA variants

Seven different (likely) pathogenic germline *SDHA* variants were identified in 30 patients with PGL (Table 2). Three variants had been reported previously: The common nonsense mutation c.91C>T, (p.Arg31*)[10] was observed in 23 patients, the c.1753C>T, (p.Arg585Trp)[16] missense mutation was observed in two patients and the nonsense c.1534C>T, p.(Arg512*)[22] was observed in one patient. Moreover, four not previously reported and three previously reported *SDHA* VUS were identified (Supplemental Table 1). All VUS were identified in patients with apparently sporadic HNPGL diagnosed between the ages of 28 and 52 years. Additional immunohistochemical staining and LOH analysis were performed in two of the seven VUS-related HNPGLs, but showed no loss of SDHA or SDHB staining or LOH. The other five HNPGLs were not available for further analysis.

Table 1. Clinical Characteristics of Patients With Germline SDHA Mutation and Patients Without Germline *SDHA* Mutation

Characteristic	*SDHA* mutation (n=30)	No *SDHA* mutation (n=363)	*P*-value	Mutation Yield (%)
All patients with PGL				7.6
Age diagnosis (y)	43 (17–81)	52 (7–90)	0.002	
Males, n (%)	15 (50)	117 (32)	0.049	
HNPGL	21	153		12
Age diagnosis (y)	43 (18-81)	54 (19-90)	0.008	
Males, n (%)	10 (48)	33 (22)	0.010	
PHEO	4	186		2
Age diagnosis (y)	35 (17-70)	51 (7-81)	0.14	
Males, n (%)	0	71 (38)	0.118	
SPGL	5	24		18
Age diagnosis (y)	36 (22-60)	53 (23-73)	0.118	
Males, n (%)	5 (100)	13 (54)	0.055	

Data are presented as median (range) or number and percentage. *P* values are derived from χ^2 or independent sample t test.

Table 2. Clinical and Molecular Characteristics of 30 Index SDHA Mutation Carriers and Their Relatives

Patient No.	Family	Sex	Tumors Observed (Age at Detection, y)	Biochemistry Results
1	A	F	GCT-ri (43)	Normal
2	B	M	GVT-le (38)	Normal
3	C	F	GVT-ri (81)	Normal
4	D	F	GCT-le 2x (35)	Normal
5	E	F	GCT-le, GCT-re, GJT-le (48)	Normal
6	F	M	GCT-le (30)	NA
7	G	M	GCT-ri (56), Prolactinoma (58)	Normal
8	H	M	GJT-le (43)	Normal
9	I	F	GCT-ri, GCT-le (26, 49, 50), Prolactinoma (45), Multiple Meningioma (45,62)	Normal
10	J	M	GCT-ri (23)	Normal
11	K	M	GJTT-le (38)	3-MT
12	L	F	GCT-le (40)	Normal
13	M	F	GCT-le (61)	NA
14		F	GJTT-ri (58)	Normal
15		F	GVT-le (53)	Normal
16		F	GVT-ri (42)	Normal
17		F	GVT-ri (53)	Normal
18		M	GJT-ri (18)	NA
19		M	GCT-ri (48), Uveal melanoma (48)	Normal
20		M	GVT-ri (60)	3-MT
21		M	GVT-ri, GVT-le, GCT-ri (49)	Normal
22	N	F	PHEO-ri (17)	NM
23		F	PHEO-ri (20), Wilms tumor (4)	NM
24		F	PHEO-le (50), metastasis (60)	NM
25		F	PHEO-ri (70), RCC (70)	Normal
26	O	M	Para-aortal SPGL (60)	NA
27	P	M	Testis SPGL (23), metastases (26)	NM/3-MT
28	Q	M	Retroperitoneal para-aortal SPGL (50)	NM/3-MT
29	R	M	Malignant retroperitoneal SPGL (36)[f]	NM/3-MT
30	S	M	Para-aortal SPGL (22)	NM

See Supplemental Fig. 2 for the pedigrees of families A through S.
Abbreviations: GCT; glomus carotid body tumor, GJT; glomus jugularis tumor, GJTT; glomus jugulotympanicum tumor, GTT; glomus tympanicum tumor, GVT; glomus vagal tumor, ri; right, le; left, NA; not available, PTC;

Family History	Germline *SDHA* variant	Tested relatives (Carriers[a])	Reference
Negative	c.91C>T, p.(Arg31*)	5 (3)	[10]
Negative	c.91C>T, p.(Arg31*)	2 (1)	[10]
Negative	c.91C>T, p.(Arg31*)	3 (1)	[10]
Negative	c.91C>T, p.(Arg31*)	1 (1)	[10]
Negative	c.91C>T, p.(Arg31*)	6 (4)	[10]
Negative	c.91C>T, p.(Arg31*)	10 (6)	[10]
Negative	c.91C>T, p.(Arg31*)	4 (2)	[10]
Negative	c.1432_1432+1delGG[b]	3 (2)	Novel
Negative	c.91C>T, p.(Arg31*)	8 (2)	[10]
GIST[c]	c.91C>T, p.(Arg31*)	8 (6)	[10]
Negative	c.91C>T, p.(Arg31*)	2 (1)	[10]
RCC[c]	c.985C>T, p.(Arg329*)	1 (1)	Novel
Negative	c.91C>T, p.(Arg31*)	8 (5)	[10]
Negative	c.91C>T, p.(Arg31*)	0	[10]
Negative	c.91C>T, p.(Arg31*)	0	[10]
Negative	c.91C>T, p.(Arg31*)	0	[10]
RCC[d]	c.91C>T, p.(Arg31*)	0	[10]
Negative	c.91C>T, p.(Arg31*)	0	[10]
Negative	c.91C>T, p.(Arg31*)	0	[10]
Negative	c.1795-3C>G[¥]	0	Novel
Negative	c.667delG, p.(Asp223fs)	0	Novel
Negative	c.1753C>T, p.(Arg585Trp)	3 (1)	[16]
Negative	c.1753C>T, (p.Arg585Trp)	0	[16]
Negative	c.91C>T, p.(Arg31*)	0	[10]
Negative	c.91C>T, p.(Arg31*)	0	[10]
RCC[e]	c.91C>T, p.(Arg31*)	10 (8)	[10]
HNPGL[c]	c.1534C>T, p.(Arg512*)	3 (4)	[22]
Negative	c.91C>T, p.(Arg31*)	8 (3)	[10]
Negative	c.91C>T, p.(Arg31*)	8 (2)	[10]
Negative	c.91C>T, p.(Arg31*)	3 (3)	[10]

papillary thyroid carcinoma, [a] Including obligate mutation carriers, [b] Likely pathogenic *SDHA* variant, [c] *SDHA* mutation carrier, [d] *SDHA* mutation status unknown, [e] No *SDHA* mutation carrier, [f] Dead of disease. Reference sequence *SDHA:* NC000005.9, NM004168.3

Clinical manifestations in index *SDHA* mutation carriers

The clinical and molecular characteristics of the 30 index patients with germline pathogenic *SDHA* variants are listed in Table 2. Germline *SDHA* mutations were identified in 21 index patients with HNPGL, 4 with PHEO and 5 with SPGL. Four patients were diagnosed with multiple HNPGLs. The HNPGL anatomic locations were distributed as follows: 15 carotid body, 8 vagal, 3 jugular, and 2 jugular tympanic. Two patients with HNPGL had elevated 3-MT levels. Three patients with PHEO had elevated normetanephrine levels. One patient had developed a metastatic PHEO, but no bilateral PHEOs were detected. Four SPGLs had a retroperitoneal para-aortal location and one SPGL was found in the testis. Four patients with SPGL had elevated normetanephrine levels, three in combination with elevated 3-MT.Two patients developed metastatic SPGL and one of these patients (no. 27) died at age 27 years.

Furthermore, three index mutation carriers (no. 7, no. 9, no. 25) were diagnosed with one other possibly *SDHA*-related feature, including pituitary adenoma (at ages 58 and 45 years, respectively) and RCC (age 70 years), respectively. One pituitary adenoma was immunonegative for both *SDHA* and *SDHB* and contained an additional somatic pathogenic *SDHA* variant (p.Asp38Val), likely resulting in biallelic inactivation of *SDHA* (Supplemental Fig. 1).[23] The other pituitary adenoma was not resected and therefore not analyzed. Conversely, the RCC tissue showed no loss of SDHA immunohistochemical staining, suggesting that it was not *SDHA*-related. Three additional tumor types were reported in index *SDHA* mutation carriers: multiple meningioma (patient no. 9), uveal melanoma (no. 19, *BAP1*-mutation negative) and Wilms tumor (no. 23). However, it is not clear whether these tumors were related to the *SDHA* mutation. Immunohistochemical staining showed no loss of SDHA staining in both meningiomas. The uveal melanoma lesion and Wilms tumor were not available for analysis. Five *SDHA* mutation carriers had a positive family history for *SDHA*-related tumors, including HNPGL (patient no. 27), GIST (no. 10), and RCC (no. 12, no. 17 and no. 26).

Clinical manifestations in *SDHA* families

In total, 94 available relatives were tested via cascade screening for their familial pathogenic *SDHA* variant, revealing 51 non-index carriers and 5 obligate carriers. Pedigrees of the 19 *SDHA* families

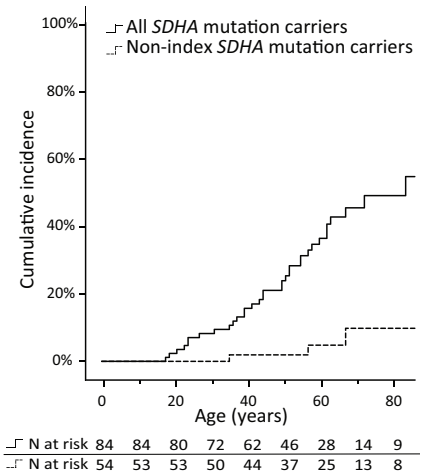

Figure 1. Age related penetrance of any *SDHA*-related manifestations

with at least one non-index mutation carrier are shown in Supplemental Fig. 2. Remarkably, we could confirm in all families, except one (index no. 3 diagnosed at age 81 years), that the mutation was inherited from an unaffected parent. The median age at DNA analysis in the non-index *SDHA* mutation carriers was 58 years (range, 7 to 94 years). In total, 3 of 56 (5%) non-index *SDHA* mutation carriers were diagnosed as having one (possible) *SDHA*-related tumor: HNPGL (n=1), GIST (n=1), and RCC (n=1). Family history did not reveal any not-tested relatives with (possible) *SDHA*-related tumors.

The estimated penetrance of any *SDHA*-related tumor is shown in Fig. 1. The age-related penetrance values for all 86 *SDHA* mutation carriers were 7% at age 25 years [95% confidence interval (CI) 2% to 12%], 26% at age 50 years (95% CI 16% to 36%) and 50% at age 70 years (95% CI 34% to 66%). The age-related penetrance values for the 56 non-index *SDHA* mutation carriers were 0% at age 25 years, 2% at age 50 years (95% CI 0% to 6%) and 10% at age 70 years (95% CI 0% to 21%). By completion of this manuscript, 51 non-index carriers were lacking any identified *SDHA*-related feature, indicating that they could be considered to be healthy mutation carriers.

DISCUSSION

This nationwide retrospective *SDHA* survey investigated *SDHA* mutation detection yield and clinical phenotype in patients with genetically unexplained PGL. We identified pathogenic germline *SDHA* variants in 30 of 393 (7.6%) patients with PGL. Most of our index *SDHA* mutation carriers presented with an apparently sporadic HNPGL. Remarkably, most germline mutations were inherited from an unaffected parent. The clinical phenotype in our *SDHA* families is similar to that seen in previous studies (i.e., with few non-PGL tumors, such as GIST, RCC, and pituitary adenoma).[2,14] This study highlights the low age-related penetrance: 10% at age 70 years in non-index *SDHA* mutation carriers. However, some index mutation carriers presented at very young ages and/or with metastatic disease. These results may give cause to reconsider the current surveillance protocol for *SDHA* mutation carriers. The age at first examination and/or the interval between screenings could possibly be less stringent than for *SDHB/C/D* mutation carriers.

SDHA mutation analysis

On the basis of a detection yield of 7.6% in this nationwide cohort analysis, we recommend germline *SDHA* analysis for all individuals with PGL, preferably by using gene panels. To date, at least 15 genes have been associated with hereditary PGL and it is likely that further rare and low-penetrant genes will be identified. Until recently, a step-wise mutation testing protocol was applied in those suspected of having familial PGL. Multiple algorithms were used, including age at presentation, location of tumor, multifocal or metastatic disease, presence of syndromic features and family history.[1] This type of testing protocol is expensive and time-consuming. Nowadays, gene panel testing using NGS is fast and cost-effective for germline genetic testing of patients with PGL.[24] However, molecular analysis of *SDHA* in NGS panels could be challenging because of the presence of four pseudogenes that are highly homologous to both the coding regions of *SDHA* and the intronic regions of the gene. According to our data, additional *SDHA* Sanger sequencing should be considered in patients with HNPGL and SPGL without detectable mutations following NGS. The *SDHA* mutation detection yield in patients with apparently sporadic HNPGL in our study population (12%) was higher than in a previous study (6%), whereas the detection yield in patients with PHEO in our study population (2%) was similar to that of patients without HNPGL in that study (2%).[14] Although no specific data are available on *SDHA* mutation detection yields in SPGLs, our detection yield was high (18%); however, it was seen against a background of a small sample size. We identified eight *SDHA* variants not previously reported, including four pathogenic

variants and four VUS. More than 60 unique *SDHA* nonsense and missense variants have been reported in the Leiden Open Variation Database, and they are evenly distributed across coding exons. No *SDHA* genotype-phenotype relationship has yet been established. Among Dutch *SDHA* index mutation carriers, the pathogenic variant c.91C>T (p.Arg31*) was most frequent (21 of 30), in contrast to 5 of 29 in a previous study of non-Dutch index patients, an over four-fold difference in frequency.[14] This variant has an allele frequency of 0.027% in the Exome Aggregation Consortium database and 0.039% (6:16000) in our in-house whole exome sequencing database (unpublished data). Together, these data suggest that *SDHA* p.Arg31* is a Dutch founder mutation, in the same vein as the very common *SDHB* and *SDHD* founder mutations reported in the Netherlands.[25]

Genetic counseling

Exploring the genetic basis of hereditary PGL after appropriate counseling provides opportunities for early detection of PGL in patients and relatives. Early removal of tumors may prevent or minimize complications related to mass effects, catecholamine hypersecretion, and metastatic transformation. However, this is counterbalanced by the need for lifelong surveillance starting at an early age and the possible psychological burden of not knowing whether, when and how (benign or metastatic) these tumors will develop. This is a particular challenge in the case of *SDHA*, for which penetrance appears to be much lower than for *SDHD* and somewhat lower than for *SDHB*.[26] Prospective studies in *SDHA* mutation carriers - including genotype-phenotype relationships, genetic modifiers and/or environmental factors - are required to determine the optimal age at which surveillance should be initiated and the best monitoring intervals to capture the different *SDHA*-related manifestations as they develop.

Strengths and limitations of the study

The current study has several strengths as well as some limitations. Its main strength is the size of the cohort investigated, representing the largest *SDHA* series to date (n = 84 carriers). This was possible because of the close collaboration of several of Dutch university medical centers. A further strength is that all patients with PGL referred for germline *SDHA* analysis in the Netherlands within a defined period (2011 to 2016) were included in the study. Finally, the study was initiated at the Center for Endocrine Tumors Leiden and the Radboud Adrenal Center, both tertiary referral centers recognized as national and European centers-of-excellence for rare endocrine tumors, including PGL. The study also has a number of limitations. First, the estimated mutation detection yield in this study was found in a retrospective diagnostic cohort and therefore might not be representative of the total patient population. However, a large proportion of the study population was systematically referred within a defined period and these patients did not differ in age, sex and diagnosis from other patients (unpublished data). Second, a possible explanation for the relatively low penetrance in our *SDHA* families could be inadequate surveillance and incomplete follow-up data. On the other hand, the over-representation of index patients (29 of 37), in a previous study leads to an overestimation of penetrance.[14]

Conclusion

Germline *SDHA* mutations are relatively frequent (7.6%) in patients with genetically unexplained PGL, even in the absence of familial or clinical indications for inherited PGL. Mutation analysis of *SDHA* should therefore be included in the genetic testing of all patients with PGL, preferably by using gene panels. This study confirms the long-suspected low penetrance of disease in *SDHA* mutation carriers and suggests that recommended guidelines for genetic counseling of at-risk relatives and surveillance in mutation carriers might need to be revised.

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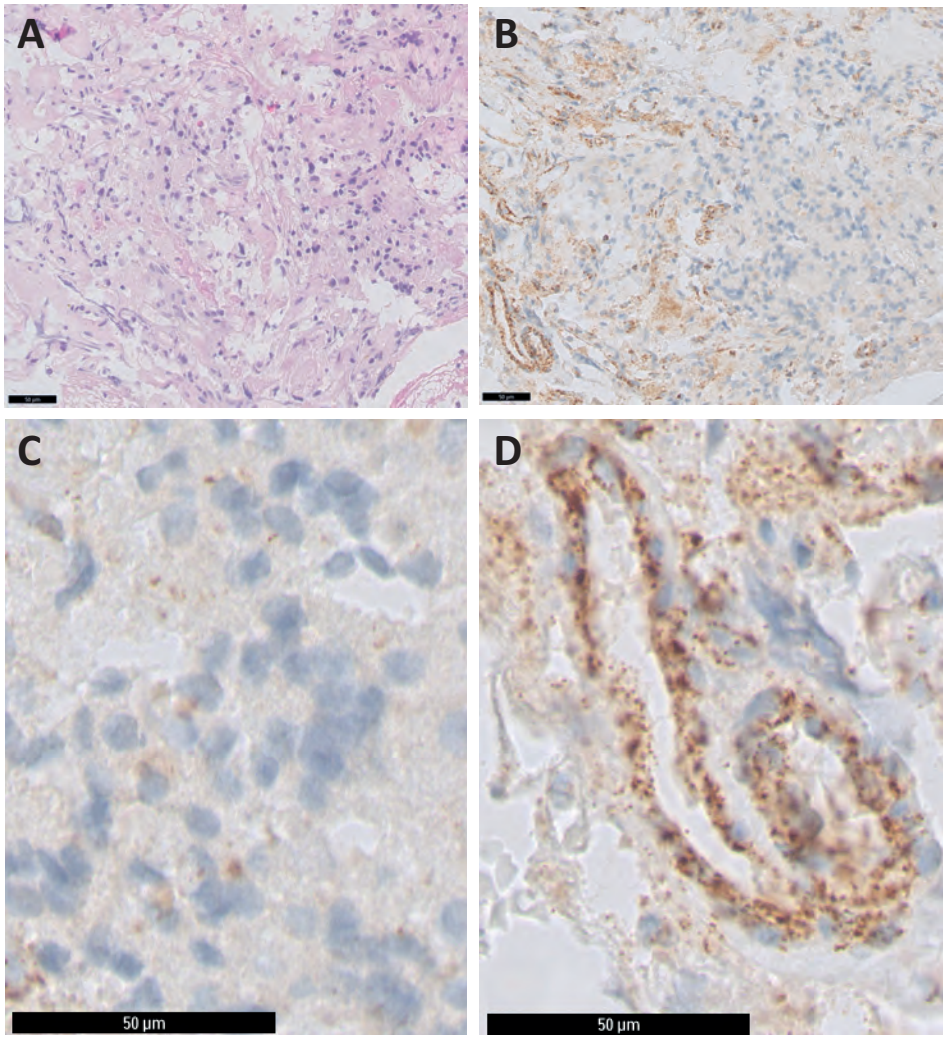
SUPPLEMENTAL DATA

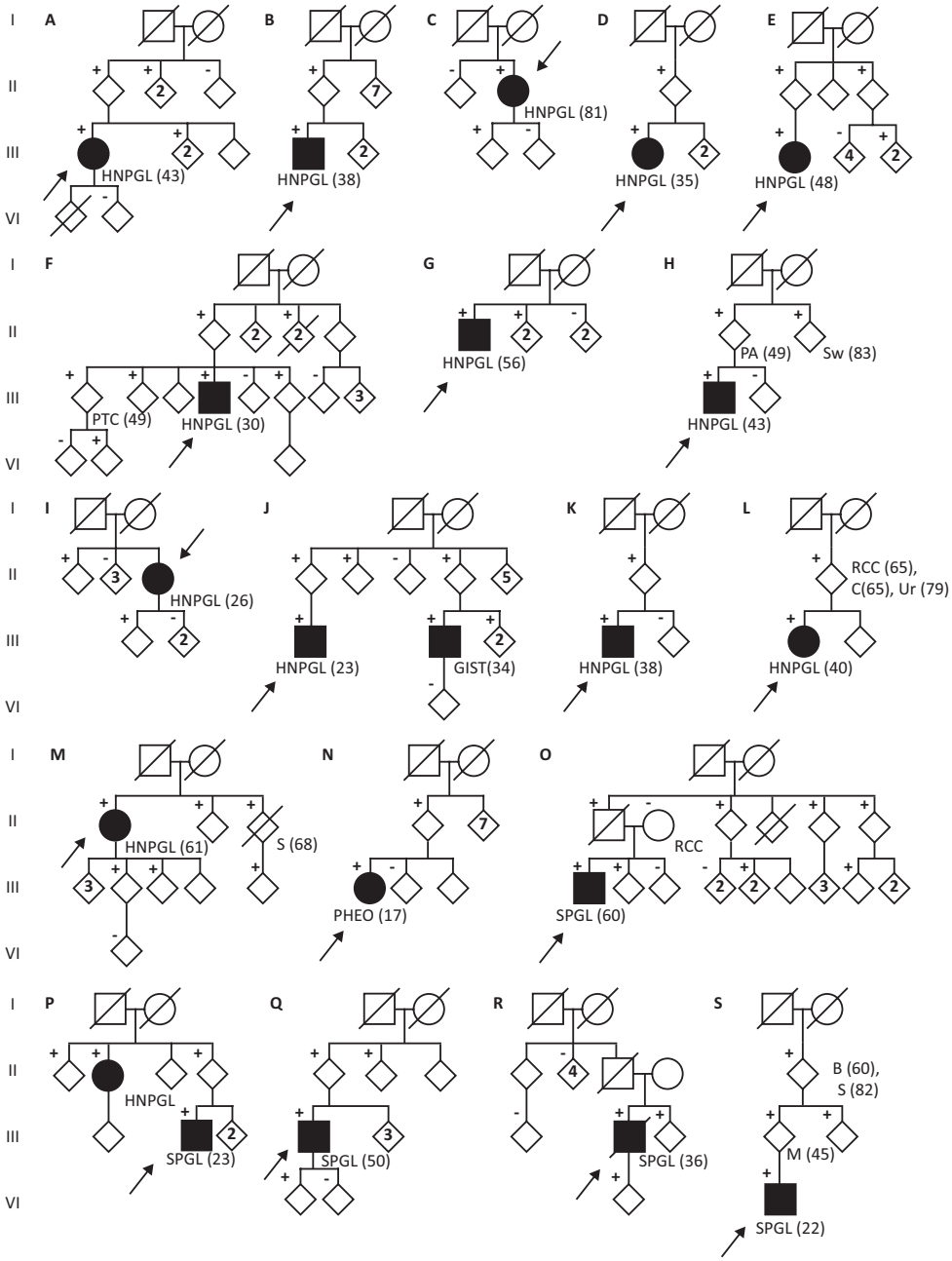
Supplemental Table 1. Overview of the clinical and molecular characteristics of 7 patients with *SDHA* variants of uncertain significance

ID #	Tumors observed (age at detection,y)	Gender	Location tumor	SDHA IHC	Family history	*SDHA* variant	Reference
31	HNPGL (52)	Female	GJT-right	Normal	Negative	c.778G>A, p.(Gly260Arg)	Bausch *et al*. 2017 (class 4), Bannon *et al*. 2017 (loss-of-function)
32	HNPGL (42)	Female	Unknown	Normal	Negative	c.889C>T, p.(Pro297Ser)	novel
33	HNPGL (42)	Male	GCT-left	NA	Negative	c.1115C>G, p.(Pro372Arg)	Bausch *et al*. 2017 (class 3)
34	HNPGL (28)	Female	GJTT-left	NA	Negative	c.1260+1G>A	novel
35	HNPGL (29)	Female	Unknown	NA	NA	c.969C>T, p.(Gly323Gly)	novel
37	HNPGL (33)	Male	GVT-right	NA	Negative	c.1352G>A, p.Arg451His	novel
38	HNPGL (48)	Female	GJT-right	NA	Negative	c.622-8T>C, (p.?)	novel

Abbreviations: HNPGL; head neck paraganglioma, GJT; glomus jugulars tumor, GTT; glomus tympanicum tumor, GJTT; glomus jugulotympanicum tumor, GVT; glomus vagal tumor, GCT; glomus carotid body tumor, IHC; immunohistochemical staining, LOH; loss of heterozygosity wild type *SDHA* allel, NA; not available



Supplemental Figure 1. Immunohistochemical staining SDHA related tumor. ID#9, carriers the germline pathogenic c.91C>T, p.(Arg31*) *SDHA* variant. **A.** Hematoxylin & Eosin (HE) staining prolactinoma, somatic pathogenic p.D38V *SDHA* variant (see Niemeijer *et al*. 2015) **B.** immunohistochemical staining displaying loss of SDHA expression in the **C.** neoplastic cells, with **D.** normal (endothelial) cells serving as positive internal controls. SDHA mouse monoclonal Ab14715 antibody (1:500 dilution; Mitosciences; Abcam)



Supplemental Figure 2. Pedigrees of 19 *SDHA* families. Please note that the pedigree has been adjusted to protect the identity of the family without a loss of scientific integrity. Circles represent females, squares represent males, diamonds represent undisclosed gender, cross striped individuals are deceased, age at diagnosis between brackets, HNPGL; head- neck paraganglioma, PHEO; pheochromocytoma, SPGL; sympathetic paraganglioma, GIST; gastrointestinal stromal tumor, RCC; renal clear cell tumor, B; breast cancer, C; colon cancer, Ut; urothelial cancer, M; melanoma, S; sarcoma, Sw; schwanoma, *SDHA* mutation carrier (+), non-carriers (-).

Supplemental Method

DNA sequencing

Mutation analysis was performed at the Radboud University Medical Center or the Leiden University Medical Center.

For Sanger sequencing, all exons and intron-exon boundaries of *SDHA* were sequenced with M13-labeled primers specifically targeting *SDHA* (NM_004168.2), but not the 4 pseudogenes: *SDHAP1* (ENSG00000185485) LOC220729 (ENSG00000214135) and *SDHAP2* (ENSG00000215837) on chromosome 3q29, and *SDHAP3* (ENSG00000185986) on chromosome 5p15. Primer sequences are available on request.

Next generation panel sequencing included all exons and intron-exon boundaries of *SDHA* (NM_004168.2), *SDHB* (NM_003000.2), *SDHC* (NM_003001.3), *SDHD* (NM_003002.3), *SDHAF2* (NM_017841.2), *VHL (*NM_000551.3), *MAX* (NM_002382.4) and *TMEM127* (NM_017849.3), and exons 10, 11, and 13 to 16 of the *RET* proto-onco gene (NM_020975.4). Ampliseq primer pools were designed using the online designer tool from Life Technologies (http://www.ampliseq.com). No SNPs with a frequency of over 0.5% were allowed in the five most 3' nucleotides, which was confirmed by SNPCheck v3 analysis (https://ngrl.manchester.ac.uk/SNPCheckV3/snpcheck.htm). At most four low-frequency SNPs were allowed at other positions. The coding sequence and at least 10 bp of padding sequence including the intron-exon boundaries was fully covered. In cases where exons were covered by multiple amplicons overlap was ensured, without overlap of primer sequence. The full coding sequences were covered in two multiplex pools consisting of 34 and 28 amplicons, respectively. Primer sequences are available on request.

Ampliseq enrichment and Ion Torrent PGM sequencing

Libraries were generated according to the manufacturer's protocol, using at least 10 ng of DNA per pool and amplification in 19 cycles using the Ion Ampliseq mastermix (Life Technologies). After barcode and adapter ligation DNA was purified with Agencourt AMPure XP beads (Beckman Coulter Genomics, High Wycombe, UK). The library was diluted to 12 pM. Emulsion PCR was performed using the Ion OneTouch 200 Template kit on the Ion OneTouch System (Life Technologies). Next, Ion Sphere Particles (ISPs) were recovered and enriched for template positive ISPs using Dynabeads MyOne Streptavidin C1 beads (Life Technologies) in the Ion OneTouch ES instrument (Life Technologies). ISP enrichment was quantified using a Qubit 2.0 fluorometer (Life Technologies). Sequencing primer and polymerase were added to the final enriched spheres before loading onto a 316 chip using the Ion PGM 200 sequencing kit (Life Technologies). Chips were run on a Ion Torrent Personal Genome Machine (PGM) (Life Technologies).

NGS sequencing data analysis

Data from the PGM runs were processed using Ion Torrent Suite v3.1 to generate sequence reads, trim adapter sequences, filter and split the reads according to the barcode in succession. FastQ files were loaded into JSI Seqpilot module SeqNext v4.0 (JSI Medical Systems, Kippenheim, Germany) for mapping and analysis of the data. Pseudogene reads of *SDHA* and *SDHD* were filtered out by skipping the reads containing at least two pseudogene variants.

All nucleotides were covered at least 40X, or Sanger sequenced if this depth could not be reached (especially exon 1 and 14 of *SDHA*).

Variants were called if the variant frequency was >15% of all reads in both the forward and the reverse direction. All identified (likely) pathogenic variants were confirmed using Sanger sequencing.

This chapter describes an unusual case of apparent non-penetrance in a family with Multiple Endocrine Neoplasia syndrome type 2.

A 93-year-old MEN2a Mutation Carrier Without Medullary Thyroid Carcinoma: A Case Report and Overview of the Literature

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Cancer Research Frontiers. 2016 Feb; 2(1): 60-66

ABSTRACT

Background

Multiple Endocrine Neoplasia (MEN) type 2 is a neuroendocrine neoplasia predisposition syndrome caused by a heterozygous germline mutation in the *RET* proto-oncogene. Mutation carriers have a lifetime risk of nearly 100% of developing medullary thyroid carcinoma. Approximately 40-50% of patients with MEN2A develop a pheochromocytoma and 20-30% develop primary hyperparathyroidism.

Case report

We describe an unusual case of apparent non-penetrance in a 93-year-old carrier of an apparent *de novo RET* germline mutation (c.1858T>C, p.C620R) without clinical symptoms of MEN2A. Different tissue types were tested for this mutation, making mosaicism less likely. His son was diagnosed at the age of 50 years old with metastasized medullary thyroid carcinoma. Pathological examination of material of a prophylactic thyroidectomy from his 19-year-old grandson showed multiple micro-carcinomas.

Discussion and conclusion

This unusual case of a 93-year-old carrier without apparent medullary thyroid carcinoma questions the full penetrance of a germline mutation in *RET* codon 620. Further investigation of genetic modifiers is warranted, to further explore phenotypic risk profiles of specific *RET* genotypes. *(Cancer Research Frontiers.2: 60-66 2016)*

INTRODUCTION

Medullary thyroid cancer (MTC) originates from the calcitonin-producing thyroid C-cells derived from the neural crest, and represents approximately 5-10% of all thyroid tumours, and about 15% of all thyroid cancer-related deaths. MTC may occur sporadically (75%) or as part of an autosomal dominantly inherited cancer syndrome (25%); multiple endocrine neoplasia (MEN) type 2. MEN2 is a neuroendocrine neoplasia predisposition syndrome characterized by variable penetrance of MTC and other endocrinopathies.[1, 2] MEN2 includes two phenotypes; MEN2A (95%), subdivide in four variants (Classical MEN2A, MEN2A with cutaneous lichen amyloidosis, MEN2A with Hirschsprung's Disease and familial MTC) and MEN2B (5%). In all subtypes of MEN2, MTC is generally the first neoplastic manifestation because of its earlier manifestation and higher penetrance compared with other endocrinopathies.

MEN2 is caused by a heterozygous germline mutation in the *RET* proto-oncogene that is located on chromosome 10 and encodes a membrane-bound tyrosine kinase receptor with extracellular and cytoplasmic domains. Receptor tyrosine kinases transduce the extracellular signals for processes such as diverse as cell growth, differentiation, survival and programmed cell death. The majority of MEN2A and FMTC cases are caused by activating missense mutations in the extracellular cysteine codons in exon 10 and exon 11. Mutations in this extracellular domain lead to ligand-independent homodimerization of the receptor with constitutive activation and downstream signalling of the mitogen-activated protein [MAP] kinase pathway.

The estimated prevalence of MEN2A is 1-2 per 200,000 persons in the general population[3, 4] and males and females are equally affected. In general, *RET* germline mutation carriers are reported to have a lifetime risk of nearly 100% of developing MTC. Approximately 40-50% of patients with MEN2A develop a pheochromocytoma and 20-30 develop primary hyperparathyroidism.[5-8] In the majority of MEN2 families, associations between specific *RET* mutations (genotype) and aggressiveness of MTC and presence of their endocrine tumour (phenotype) are well documented.[8-10] As mentioned, the penetrance of MTC in MEN2A is reported high, i.e. almost complete at advanced age.[6] Here, we describe an unusual case of non-penetrance in a 93-year-old carrier of an *de novo RET* germline mutation without clinical symptoms of MEN2A.

CASE PRESENTATION

A 50-year-old male with no family history of endocrine malignancy presented with swelling in the neck and vocal cord paralysis. Physical examination showed enlarged lymph nodes in the neck and blood investigation revealed elevated serum levels of calcitonin (Ctn) and carcinoembryonic antigen (CEA). Neck ultrasound imaging showed a solitary lesion in the left thyroid lobe of 2.3 cm in diameter with calcification and pathologic lymphadenopathy on both sides, mainly in the mid and inferior jugular region. No pheochromocytoma screening was performed at that time. After surgery the diagnosis of metastasized medullary thyroid carcinoma was histopathologically confirmed. Molecular genetic analysis revealed the presence of a germline mutation in exon 10 of the *RET* proto-oncogene (NM_020975.4: c.1858T>C, p.C620R). In addition, DNA-testing for the *RET* mutation was recommended for his family members. The family history showed no signs of Hirschsprung's disease. Subsequently, investigation of other potentially affected endocrine organ systems was performed, there were no signs of pheochromocytoma or hyperparathyroidism. Annually clinical and biochemical screening was done. Five years later, he underwent adrenalectomy because of clinical symptoms (vertigo and palpitations), chemically suspected pheochromocytoma (increased catecholamine excess in urine) and growth of a lesion

in the left adrenal (12mm within one year). Histological examination showed adrenal medullary hyperplasia, but there was insufficient evidence for the definitive diagnosis pheochromocytoma.

His 19-year-old son, was identified as a pre-symptomatic mutation carrier and underwent prophylactic total thyroidectomy. Blood investigation showed no increased calcitonin or CEA levels and catecholamine excess in urine was not found. Histopathological analysis showed 3 foci of medullar micro-carcinoma, 1mm, 2mm and 3mm respectively, with some surrounding C-cell hyperplasia.

The father of the proband was also identified as a carrier of the *RET* mutation at an age of 91 years old without thyroid surgery or any symptoms of the thyroid or other health problems. However, he did not want any further clinical evaluation for MEN2A. We confirmed the presence for the *RET* mutation in colon, spleen and nasal polyps, making germline mosaicism very unlikely. Additional sequencing of the whole coding sequence of the *RET* gene was performed to exclude other mutations in the *RET* gene in the 91-year-old patient which could conceal the effects of the c.1858T>C mutation. He died at age 93 because of respiratory insufficiency with a recently diagnosed prostate carcinoma with possibly bone metastasis. No clinical or laboratory diagnostic evaluation was performed during hospitalisation that would have indicated the presence of MTC, pheochromocytoma or primary hyperparathyroidism. In particular no blood test for Ctn, calcium, catecholamine or PTH levels and no thyroid ultrasound or CT-scan of the head neck region. No autopsy was conducted.

Furthermore, no other paternal family members were identified as mutation carriers (see pedigree Figure 1). The first generation was not available for DNA testing, they had no symptoms of MTC, pheochromocytoma or primary hyperparathyroidism, although no specific diagnostic evaluation was performed. In the second generation, 11 siblings did not carry the *RET* germline mutation, while three siblings were not available for DNA testing. These three siblings deceased after the age of 50 years old and had no symptoms nor diagnostic evaluation. Subsequently, their offspring was offered testing for the *RET* mutation. In total, 10 children of these three siblings were tested and all did not carry the *RET* mutation, while one sibling was not available for DNA testing. This strongly suggests a *de novo* origin of the *RET* mutation in the father of the proband. Written informed consent was obtained from the patients for publication of this case report and any accompanying images.

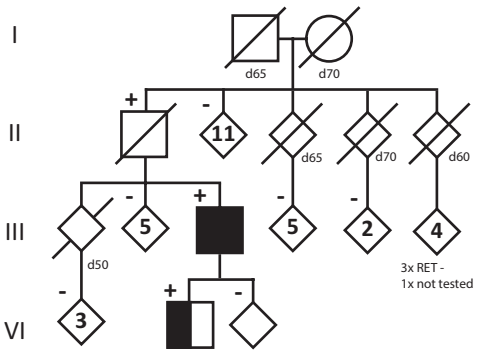

Figure 1. Multiple endocrine neoplasia type 2A pedigree showing *RET* mutation carriers (*RET*+) and non-carriers (*RET*-). Please note that the pedigree has been adjusted to protect the identity of the family without a loss of scientific integrity. Circles represent females; squares represent males; diamonds represent undisclosed gender. Ages of death (d) are given in the nearest 5-year tier, medullar thyroid carcinoma and adrenal hyperplasia (fully shaded), medullar thyroid carcinoma (half shaded), *RET* mutation carriers (*RET*+) and non-carriers (*RET*-).

DISCUSSION

In order to appreciate the relevance of apparent non-penetrance in a 93-year-old carrier of a *RET* germline mutation, we must first examine the reported genotype-phenotype correlations of the *RET* proto-oncogene codon 620. In 2011, the International RET Exon 10 Consortium, comprising 27 centres from 15 countries obtained molecular based neoplastic risk profiles and codon-specific age related penetrance.[6] Fifty percent penetrance for MTC was achieved by the age of 31 years for mutations in codon 620, reaching 80% penetrance by age 50 and almost 100% by age 70. Given these percentages, survival beyond middle age was thought unlikely without the intervention of thyroidectomy. There was no significant difference in age at diagnosis between symptomatic and screened ascertainment for MTC. Age related penetrance in patients in whom pheochromocytomas were assessed was 23% at 50 years. Penetrance was significantly different between pheochromocytomas ascertained from symptomatic assessment and those detected under surveillance. This underscores the great importance of clinical surveillance. The International RET Exon 10 Consortium, examined 23 families with a total of 101 mutation carriers with the same mutation as our family (codon 620 c.1858T>C). The median age of MTC was 29 years, ranges 6-73 years. About 15% of these patients also had a pheochromocytoma, and 3% had hyperparathyroidism. Our case, an asymptomatic carrier of the codon 620 *RET* mutation at the age of 93 years old, albeit without any prior intervention or treatment, questions the full penetrance of this *RET* mutation. Notably, the occurrence of MTC in a 87-year-old patient was described, also with a codon 620 mutation.[11] Moreover, no further cases of MTC have been reported in MEN2A patients older than 75 years old, although this information may not have been the focus of reporting.

In 2015, the International Workshop on MEN published a consensus statement on the management of hereditary medullary thyroid carcinoma.[12] Carriers of the *RET* codon 620 mutation, characterised as moderate risk, are recommended to undergo prophylactic thyroidectomy during childhood, or young adulthood, the timing and extent of surgery will be guided by the serum Ctn levels (Table 1). One concern is whether it is justifiable to put children and young adults at risk for permanent recurrent laryngeal nerve palsies and permanent hypoparathyroidism.[9] Prospective surveillance and early treatment of other manifestations of MEN2A, like pheochromocytoma and hyperparathyroidism, can reduce the morbidity and mortality.[13]

About 90-95% of individuals with MEN2A have an affected parent, and 5-10% are *de novo* cases.[14, 15] Our 93-year-old *RET* mutation carrier is most likely one of these rare *de novo* cases, because none of his 14 siblings (or their tested offspring) were *RET* mutation carriers. Bayesian statistics lower the *a prior* chance of inherited disease from 95% to a *post prior* chance of 0.05%.

Because of the age-dependent risk ratio and the extremely rare incomplete penetrance of the mutant allele, testing for this specific mutation in asymptomatic parents of MEN2A-affected children carrying the RET codon 620 mutation is important. [16, 17] Our 93-year-old patient is most likely an example of this rare incomplete penetrance. Alternatively, MTC in an apparently *de novo* patient may arise from paternal mosaicism in one or more germ layers or organ systems without *RET* mutation in peripheral blood. In our 93-year-old apparent asymptomatic person, the *RET* mutation was detected in three different germ layers; peripheral blood, colon, spleen, nasal polyps and obviously affected germ cells, making mosaicism less likely. In addition, no cases of mosaic *RET* mutations have been reported.

Inter- and intra-familial phenotypic variability is described among the MEN2 families, also when the disease is caused by the same *RET* mutation.[18] At present, knowledge of possible genetic modifiers that may affect the clinical course of this disease is still limited. Some research projects suggest a role for genetic modifiers. Examples of these are additional somatic mutations[19] or specific polymorphisms, such as over-representation of the G12S polymorphism of the *SDHD*

Table 1. Summary of the American Thyroid Association [2015] recommended genotype-bases management of *RET* mutation carriers[12]

	RET genotype*			Recommended Start Interventions			
ATA Group	Exon	Codon Mutation	MEN2 classification	PE/US/ Ctn	TTX	Screening PHEO	Screening HPT
HST	16	M918T	MEN2B	1 month	before the first year of life †	11 years	NA
H	11	C634F/G/R/S/W/Y	Classical MEN2A / MEN2A with CL	3 years	at or before 5 years of age†	11 years	11 years
	15	A883F	MEN2B				NA
MOD	10	C609F/G/R/S/W/Y C611R/S/W/Y C618F/G/R/S/W/Y C620F/G/R/S/W/Y	Classical MEN2A	5 years	Childhood, or young adulthood†	16 years	16 years
		C611F	FMTC			NA	NA
		C609G C611S C618/R/S C620/R/S/W	MEN2A with HD				
	11	C630R/Y D631Y S649L K666E	Classical MEN2A			16 years	16 y
	13	E768D	FMTC			NA	NA
		L790F Y791F	Classical MEN2A			16 years	16 years
	14	V804L/M	Classical MEN2A / MEN2A with CL			16 years / NA	16 years / NA
	15	S891A	Classical MEN2A			16 years	16 years
	16	R912P	FMTC			NA	NA

ATA=American Thyroid Association risk categories (HST=highest risk, H=high risk, MOD=moderate), *RET*=*RE*arranged during *T*ransfection, PE=Annual physical examination, US=cervical ultrasound, Ctn= serum Calcitonin levels, TTX=Total thyroidectomy, PHEO=Pheochromocytoma, HPT=hyperparathyroidism, CL=cutaneous lichen amyloidosis, HD=Hirschsprung's Disease, FMTC=familial medullar thyroid carcinoma, NA= not applicable.*Include most common mutation and no chromosomal alterations activating *RET* such as deletions, insertions, duplications, multiple mutations, and homozygous mutations. † the timing and extent of surgery guided by serum Ctn levels.

gene in patients with MEN2A syndrome.[20] The high prevalence of the G12S variant in these patients supports its genetic modifier role, but this association remains to be established. Besides polymorphisms, mitochondrial DNA mutations are suggested to be involved in medullary thyroid carcinoma tumorgenesis and/or progression.[21] MTC could harbor imbalance between mutant and wild type *RET* alleles and in addition, *RET* copy number alterations, either *RET* gene amplification or chromosome 10 aneuploidy are described.[22] Less is known about protective genetic modifiers.

CONCLUSION

In the current report, we described an unusual case of apparently non-penetrance, in a 93-year-old carrier of an apparent *de novo RET* germline mutation. Firstly, this observation questions

the full penetrance of this *RET* germline mutation. Secondly, this case-report sheds new light on the carefully weighing of the benefit of potential cure by prophylactic thyroidectomy in older asymptomatic MEN2A carriers against over- treating in these carriers. Further investigation of genetic modifiers is warranted, to explore nucleotide specific genotype-phenotype correlations in MEN2A.

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PART IV

GENERAL DISCUSSION

This chapter summarizes the main findings this thesis in the context of the current literature. Moreover, future perspectives for genetic testing will be discussed in a broader context.

Discussion and Further Perspectives

This thesis began with the questions of a 12-year-old girl diagnosed with thyroid cancer: *"Why do I have cancer? Are other relatives at risk? And if so, can we prevent cancer?"* In the included studies we have attempted to address these questions, and they contributed to the formulation of the general objectives of this thesis, which were 1) to investigate the role of molecular testing in thyroid cancer (TC) diagnostics and in treatment decision making, 2) to improve knowledge of the genetic background of pediatric non-medullary TC, and 3) to further delineate the genotype and phenotype of DICER1 syndrome, MEN2a syndrome, *CDC73*-related disorder and *SDHA*-associated paraganglioma. The current chapter summarizes the main findings of the studies described in the six previous chapters in the context of the current literature. Moreover, future perspectives for genetic testing will be discussed in a broader context.

PART I. THE ROLE OF MOLECULAR TESTING IN ENDOCRINE CANCER DIAGNOSTICS AND TREATMENT DECISION MAKING

Over the last decade, the study of genetic alternations contributing to tumorigenesis has improved tumor classification, prognostic forecasting and the development of personalized treatment. Emerging evidence from clinical practice indicated that molecular tumor analysis could guide treatment choice, thus optimizing the selection of effective targeted treatments and reducing side effects and treatment costs.

In **Part I** of this thesis we explored the germline and somatic DNA/RNA variants in TC with the dual aims of improving diagnostics and guiding treatment decisions.

In **chapter 2** we described the genetic characterization of ten DICER1-related young onset cases of differentiated thyroid carcinoma (DTC) and reported on follow-up of the affected persons.[1] The identification of distinct somatic *DICER1* RNase IIIb hotspot variants in separate presumed-malignant and benign nodules sampled from individual patient lesions indicated that the tumors were polyclonal lesions. Furthermore, nine of the ten DICER1-related DTC lacked the well-known oncogenic driver DNA variants (e.g. *BRAF*, *RAS*) and gene rearrangements (e.g. *RET/PTC1-12*, *PPARg-PAX8*, *ALK*-, and *NTRK*-) that are frequently observed in sporadic TC. In addition to these molecular findings, occasional ambiguous histological features and lack of extrathyroidal extension, infiltrative growth, vascular invasion, or lymph node or distant metastasis (at a mean follow-up of 8 years), suggests that most DICER1-related young onset DTCs form a low-risk subgroup within the DTC spectrum. Which is in contrast to children with sporadic DTC whom frequently present with advanced disease (e.g. lymph node involvement at diagnosis, distant metastases, and multifocal disease).[2] Despite the excellent prognosis for pediatric DTC patients (30-year mortality <5%), morbidity caused by the treatment (surgery and radioactive iodine) remains considerable.[3] Based on our findings, DICER1-related thyroid neoplasia might often require hemithyroidectomy or total thyroidectomy due to the extent of nodules, but radioiodine treatment may be unnecessary given the patients' age and the tumors' low propensity for metastases (based on reports published thus far).[4-13]

While TC typically has a good prognosis following standard treatments, advanced cases of radioactive iodine refractory (RAI-R) DTC, anaplastic thyroid carcinoma and medullary thyroid carcinoma (MTC) have a poorer prognosis.[14-17] Management options in these patients include active surveillance, local therapy for metastatic sites (e.g. surgery or external beam radiation), or multi-kinase inhibitor therapy for rapidly progressing, symptomatic, or life-threatening disease.[17,18] Our improved understanding of the molecular alterations underlying TC has allowed researchers to develop targeted drugs and strategies.[19]

Our study, described in **chapter 3**, shows that mitogen-activated protein kinase (MAPK)-related gene fusions are relatively frequently found in recurrent RAI-R TC. For technical reasons, gene fusion analysis in RNA isolated from formalin-fixed tumor tissues has been limited until recently. We now show that extensive gene fusion analysis on formalin-fixed samples is feasible and effective. This is important because patients with TC and other tumor types are often treated in hospitals where tumors are solely processed using formalin fixation and paraffin embedding. The identified gene fusions in RAI-R TC provide a rationale for the incorporation of specific kinase inhibitors in the treatment regimen for these patients, with the intention to restore iodine transport and/or take advantage of a direct effect on tumor cell viability. However, the identification of variants in actionable genes does not necessarily mean that an associated targeted treatment will be effective in clinical practice.

Not unlike RAI-R TC, treatment options for metastatic parathyroid carcinoma (PC), paraganglioma (PGL) and pheochromocytoma (PHEO) are limited. The following paragraphs will discuss the future perspectives for metastatic PC, PGL and PHEO treatment, respectively, in relation to recently published integrated molecular data.

With multiple surgical resections of recurrent or metastatic disease, most patients with PC show long-term survival (5-year mortality ~10%). However, systemic therapy may be required over the long term. Cytotoxic regimes have not proven effective and current treatment focuses on controlling hypercalcemia. Previous studies reported often mutually exclusive somatic genetic alterations in *MEN1* and *CDC73*, which are currently not amenable to targeted therapy. Recent comprehensive genetic profiling studies have reported additional genetic alterations (e.g. *PTEN, PIK3CA, NF1, KDR*), in the presence of *CDC73* mutations, with rationally matched targeted agents (e.g. buparlisib). [20,21] The PI3K/Akt/mTOR pathway was the most frequently altered pathway and clinical trials are currently underway that target the PI3K/Akt/mTOR pathway in solid tumors. Single PC cases have shown clinical benefit from tyrosine kinase inhibitors.[20] As PC is one of the rarest of all human cancers (<0.005% of all cases), patients with unresectable metastatic disease should be enrolled in so called bucket (or basket) trials, i.e. one molecular abnormality targeted across multiple tumor types. Furthermore, immunotherapy that includes immune checkpoint inhibitors (i.e. targeting and disrupting PD-1/PD-L1 and CTLA-4/B7 interactions to boost the immune response against cancer cells) has transformed the treatment approaches for solid tumors in recent years.[22,23] Tumor mutational burden (TMB) serves as one of the biomarkers for response to checkpoint inhibitor treatment. [24] High TMB typically translates into a higher neo-antigen load, and therefore a greater chance that an antigen capable of stimulating an immune reaction will be expressed on the tumor cell surface and recognizable to a cytotoxic T-cell.[24,25] Although the median TMB in PC is relatively low (1.7 mutations per megabase (m/Mb) compared to 3.6m/Mb for all human cancers), about 20% show a high mutational burden (>20m/Mb).[20] These patients might benefit from immunotherapy, including checkpoint inhibitors.

The molecular understanding of PGL and PHEO has recently advanced due to comprehensive characterization of these rare tumors.[26] PGLs/PHEOs are driven by diverse alterations affecting multiple genes and pathways. Several molecular markers (e.g. *MAML3* fusion gene, *SDHB* germline mutations and somatic mutation in *SETD2* or *ATRX*) were associated with increased risk of metastatic disease.[26] Current therapeutic options rely on classic chemotherapy regimens, conventional external beam radiation, radiopharmaceuticals (Iodine-131 metaiodobenzylguanidine, ^{131}I-MIBG) or Peptide Receptor Radionuclide Treatment (PRRT e.g. ^{177}Lu-DOTATATE).[27,28] The described genetic alterations may also serve as potential drug targets in metastatic disease for which treatment options are limited. *SDH*-mutated tumors, associated with high levels of glutamine, might benefit

from the glutaminase inhibitor CB-839, as currently evaluated in a clinical trial (NCT 02071862). PHEOs with an altered Wnt pathway could potentially benefit from downstream inhibitors such as β-catenin and STAT3 antagonists.[29,30] Furthermore, FDA-approved targeted (indirect) therapies are available for specific tumors carrying *VHL, RET, BRAF, EPAS1* or *FGFR1* mutations. Despite the recent progress in this area, none of the therapy options mentioned has been approved for metastatic PGL/PHEO due to the rarity of the disease and lack of prospective studies.

To summarize Part I, recent molecular analysis of advanced endocrine tumors (e.g. RAI-R TC, metastatic PC, PGL and PHEO) has improved both our fundamental understanding of these rare neoplasms and has provided further possibilities for novel targeted therapies. Besides functional analysis of the detected variants, clinical trials are needed to determine the feasibly of these treatments. Furthermore, about half of the advanced endocrine cancer cases do not harbor genetic alterations in known cancer-associated genes. Integration of different 'omics' data (e.g. genomics, epigenomics, transcriptomics, proteomics and metabolomics) will be an important challenge in the near future.[31]

PART II: IDENTIFICATION OF GENETIC PREDISPOSITION IN PEDIATRIC NON-MEDULLARY THYROID CARCINOMA

With the introduction of next-generation sequencing (NGS), the last decades have seen remarkable advances in our understanding of the genetic contribution to disease. A growing list of genes has been associated with hereditary endocrine tumors. Nevertheless, most children who develop DTC are still genetically unexplained. To the best of our knowledge, the frequency of various germline mutations in cancer predisposition genes has not been systematically studied in a large cohort of unselected children with DTC. Earlier studies mainly relied on a candidate-gene approach in selected patients, an approach that is limited by design.

The aim of **Part II** was to improve knowledge of the genetic background of pediatric DTC by 1) determining the contribution of mutations in known cancer predisposition genes, and 2) identifying novel DTC susceptibility DNA variants.

Chapter 4 describes the first results of our study investigating the Genetic background of Non-medullary Thyroid cancer in Pediatrics (GeNoThyPe) using whole genome sequencing. So far 33 genes are analyzed in 64 out of 100 pediatric thyroid cancer patients. Causative germline mutations were relatively frequent (8%) in a subset of known cancer predisposition genes, including *DICER1* and *APC*. Based on distinct thyroid histology, pathologists may play a crucial role in recognizing features for selecting patients for genetic testing. Future stepwise analysis of whole genome data might result in the identification of novel DTC susceptibility DNA variants. Combining pathway analysis with the use of somatic molecular profiles seems a good strategy. For example, in children with somatic chromosomal alterations such as *RET/PTC*1-12 fusions, we will focus on so-called caretaker genes that are involved in the maintenance of human genome stability (DNA repair pathways). Moreover, the co-occurrence of thyroiditis and DTC is well recognized. However, it remains unclear whether thyroid inflammation is instrumental in causing the cancer or whether inflammation is a result of the cancer. Intrathyroidal lymphocytic infiltration was frequently seen in our series. Focusing on, for example, human leukocyte antigen (HLA) genes and immune response pathways in these cases may result in the detection of susceptibility alleles. Alongside the acquisition of whole genome data, several challenges came to light, including storage, distribution and analysis of large datasets. New bioinformatic strategies in the coming years, may lead to improved interpretation of whole genome data, making these unique data very valuable.

Likewise, in clinical diagnostics traditional exon-by-exon Sanger sequencing of each candidate gene has been (partly) replaced by high throughput NGS that involves the parallel sequencing of millions of short DNA fragments. The advantages and limitations of single gene testing, panel testing and whole exome sequencing are summarized in Table 1.

In general, comprehensive testing (targeted panel and whole exome sequencing) increases in efficiency both in terms of time and costs if more than one gene is related to a certain disease.[32,33] This major improvement has provided the opportunity to incorporate genetic results into treatment decisions, i.e. surgical management and tailored (neo) adjuvant treatment. Whole exome sequencing allows a syndrome to be identified that was not in the initial differential diagnosis (perhaps due to the clarity or lack thereof of a patient's personal or familial characteristics) and therefore would have been missed with single gene/limited gene testing. Why are we therefore not performing whole exome sequencing for each patient? The drawback of testing many genes is the complex interpretation of the results. The probability of 1) 'secondary' findings, i.e. genetic variants not related to the phenotype, and 2) variants of uncertain significant (VUS) increases with the number of tested genes. Clinical geneticists are currently confronted with the question of whether these incidental findings should be shared with patients. The American College of Medical Genetics and Genomics (ACMG) recommends reporting of germline mutations from a specific set of 59 genes when clinical diagnostic sequencing was ordered, even when unrelated to the primary medical reason for testing.[34] Pathogenic variants in these selected genes may require medical intervention aimed at preventing or significantly reducing morbidity and mortality. In contract to the ACMG listed genes, for other conditions such as hereditary neurological diseases, no treatment or prevention is available. Sharing these findings will not influence morbidity or mortality but this knowledge might potentially influence important life decisions and/or reproductive choices. On the other hand this knowledge might represent a psychological burden (e.g. distress and anxiety). Furthermore, identification of a VUS might require additional testing e.g. immunohistochemical staining, somatic mutation analysis and/or functional assays. Pre-test genetic counseling and tiered informed consent is therefore of utmost importance, as shown in recent studies.[35,36] The patient's preference, and tolerance regarding the possibility of ambiguous or secondary findings, plays a crucial role in establishing the preferred sequence modality. Even for highly educated patients this topic can be difficult to understand.[35,37] Furthermore, on some points our present technical capabilities might have outreached our medical knowledge, e.g. specific phenotypes, estimated

Table 1. Current advantages and limitation of single gene-, panel- and whole exome testing

Characteristics	Single gene testing	Targeted panel	Whole exome sequencing
Comprehensive testing	Low	Moderate	High
Time effective	Low-moderate*	High	Moderate
Phenotypically driven	High	Moderate	Low
Medical management guideline	High	Moderate	Low
Chance of variants of uncertain significance	Low	Moderate	High
Chance of secondary findings	No	Low	Moderate
Costs	Low	Moderate	High

* depending on number of disease-associated genes

cancer risks, lack of surveillance guidelines. Therefore, in many cases, limited phenotype-driven gene testing in clinical diagnostics remains the appropriate testing option.

Given the increasing number of associated genes, in **chapter 5 and 6** we recommend gene panel testing for primary hyperparathyroidism (pHPT)[38] and hereditary PGL, respectively.[39] While NGS technologies are improving rapidly, analysis of some genes with NGS, including *SDHA* and *CDC73*, is still challenging for technical reasons (e.g. presence of pseudogenes, GC-rich regions and/or common deletions/duplication). To obtain comparable sensitivity, additional Sanger sequencing of 'core genes', i.e. genes that are responsible for a significant proportion of the defects, should be considered in cases with a high suspicion of hereditary disease (see below) without detectable mutations following NGS.[40]

To summarize Part II, the introduction of whole exome and whole genome sequencing for diagnostic and research purposes may lead to identification of a syndrome that was not in the initial differential diagnosis. The drawback of testing many genes is the complex interpretation of the results. Pre-test genetic counseling to establish the preferred sequence modality and tiered informed consent is therefore of utmost importance. Genetic testing in a broader context i.e. commercially available DNA tests, is discussed in the end of this chapter.

PART III. GENETIC COUNSELING IN ENDOCRINE TUMOR PREDISPOSITION SYNDROMES

In **Part III** of this thesis we explored the phenotype and penetrance in *CDC73*-related disorder and *SDHA*-related paraganglioma.[38,39] The main objective was to improve identification of individuals with these endocrine tumor predisposition syndromes, thus improving both genetic counseling as well as early detection of tumor development. In addition, we described an unusual case of apparent non-penetrance in MEN2a syndrome.

Chapter 5 demonstrates that germline *CDC73* mutations are frequently (12.4%) found in previously genetically-unexplained pHPT patients. Our finding further suggests that genetic testing should be recommended in four clinical subgroups: 1) individuals with pHPT and HPT-JT syndrome- related features, 2) familiar isolated pHPT, 3) atypical or malignant parathyroid histology, and 4) young individuals with pHPT. The listed clinical subgroups of patients with pHPT are also in line with the 2015 Consensus Report on hereditary hyperparathyroidism of the European Society of Endocrine Surgeons.[41] Clinical use of these criteria could increase mutation carrier detection, thus enabling optimal clinical management of pHPT patients, as well as genetic counseling and surveillance for family members at-risk for developing related disorders. The estimated penetrance of *CDC73*-related disorders in our nationwide study was 83% at age 70 years (95% confidence interval 57-99%) in 43 non-index mutation carriers, comparable with previous studies.[42,43] The relatively low prevalences of ossifying fibroma of the jaw (5/43), renal abnormalities (8/43), and uterine fibroids (n=1/25) in our series could be due to inadequate surveillance and incomplete follow-up data. Alternatively, the high penetrance observed in prior studies (20-60%)[44-47] may be due to ascertainment bias (see below). There are currently no well-established surveillance guidelines for individuals with a *CDC73*-related disorder. Based on our data and literature review we proposed surveillance for *CDC73*-related features as described in Table 2.

Chapter 6 demonstrates that germline *SDHA* mutations are relatively frequent (7.6%) in genetically unexplained PGL patients, even in the absence of familial or clinical indications for inherited PGL. Mutation analysis of *SDHA* should therefore be included in the genetic testing of all patients with PGL. The clinical phenotype in the investigated *SDHA* families is similar to previous studies (i.e. with a few non-PGL tumors such as gastrointestinal stromal tumors, clear cell renal

Table 2. Concept Dutch expert opinion surveillance guidelines for *CDC73*-related disorder

CDC73-related feature	Frequency / starting age	Surveillance modality
pHPT	Annual from age 5-10y old	Clinical, biochemical (Calcium, PTH, vitamin D)*
Jaw	Ones per 5 years from age 18y old	Orthopantomogram
Renal	Ones per 5 years from age 18y old	Ultrasound
Uterine (female)	On indication	Ultrasound

pHPT; primary hyperparathyroidism, y; years
*consider radiology for non-producing parathyroid carcinoma (rare)

cancer and pituitary adenoma).[48,49] Our study confirms the suspected low penetrance of disease in *SDHA* mutation carriers (10% at age 70 years in non-index *SDHA* mutation carriers). This suggests that recommendations for genetic counseling of at-risk relatives and stringency of surveillance for *SDHA* mutation carriers might need to be reassessed. Current Dutch surveillance guidelines for *SDHA*-related PGL starting at age 18 years old are presented in Table 3. Despite very low disease penetrance at young ages, others have proposed surveillance in childhood.[50]

Accurate penetrance estimation is of utmost importance for the development of reliable surveillance guidelines. Current genetic surveillance guidelines and programs are potentially influenced by several forms of bias. The following paragraphs discuss: 1) the different forms of bias, and 2) the phenotypic variability between and within families *(in green)*, as illustrated by the pedigree in Figure 1. These kind of biases, including ascertainment bias (*in blue*), testing bias (*in orange*) and surveillance bias (*in pink*), are often related to a retrospective study design. Likewise, the two nationwide retrospective studies described in **chapters 5 and 6** may have been influenced by these factors.

Ascertainment bias. Disease penetrance can be overestimated due to ascertainment bias *(in blue)*. The clinically ascertained family in Figure 1 was selected due to their compliance with genetic testing and/or referral criteria. Reported families are often severely affected, i.e. an unusually low age at diagnoses (IV-1, 15 years old) and/or many affected family members (e.g. III-1, IV-2). Due to stringent referral criteria for genetic testing (in the past), less severely affected families may not have been referred for genetic testing. Until recently, a step-wise mutation testing protocol was applied in suspected familiar PGL and pHPT. Multiple algorithms were used, including age at presentation, location of tumor, multifocality or metastatic disease, presence of syndromic features and family history.[41,51] This type of testing protocol is relatively expensive,

Table 3. Dutch expert opinion surveillance guidelines for *SDHA*-related paraganglioma

SDHA- related feature	Frequency /starting age	Surveillance modality
HNPGL	Annual control otolaryngology from age 18y old*	Clinical and physical examination MRI head neck 1x/ 3 year
PHEO/SPGL	Annual control endocrinology from age 18y old*	Clinical and physical examination, blood pressure and metanephrines. MRI on indication

HNPGL; head and neck paragangliomas, SPGL; sympathetic paraganglioma, PHEO; pheochromocytoma, y; years
* Consider 5 years before earliest presentation within a family

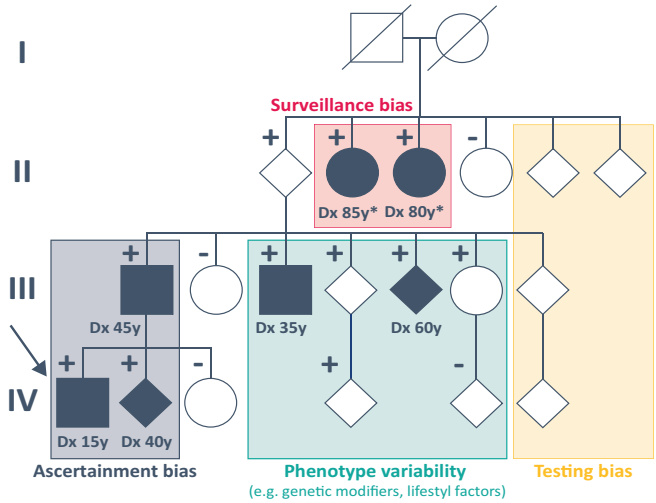

Figure 1. Pedigree illustrating several forms of bias that might occur in retrospective family studies. ircles represent females; squares represent males; diamonds represent undisclosed gender, disease affected (fully shaded), mutation carriers (+) and non-carriers (-), proband indicated with an arrow, Dx; age at diagnose, y; years, *diagnosis after genetic predisposition was confirmed. Patients are in the text referred to their position in the pedigree: generation (I-IV) and number from left to right.

time-consuming and highly dependent on a comprehensive personal and family history, which might result in a lower diagnostic yield. Nowadays, gene panel testing is fast and cost-effective for germline genetic testing of PGL patients.[32] With more widespread access to genetic testing, an increasing number of apparently sporadic PGLs are now being identified as hereditary, as shown in **Chapter 6**. As expected, following the inclusion of these families in recent studies, *SDH*-related paragangliomas showed lower disease penetrance compared to earlier reports. For example, *SDHB* penetrance estimates have fallen over time from ~55% to ~20% by age 50 years.[52-55] Going one step further, mutation carriers detected as a secondary finding of (large) gene panels or whole exome sequencing for another indication differ phenotypically from patients referred for single gene testing, who generally have typical features. A question worthy of further exploration is whether these patients should be treated and counseled differently. The clinical imaging discussion has focused on balancing the benefits of radiological surveillance versus negative effects in terms of patient anxiety, resource allocation and ionizing radiation. Early removal of tumors may prevent or minimize complications. However, this is counterbalanced by the need for lifelong surveillance starting at an early age and the possible psychological burden of not knowing whether, when, or how (benign or metastatic) tumors will develop. Furthermore, surveillance clearly does not guarantee early detection. Interval carcinomas, i.e. tumors which appear after a negative screening test or examination, may occur for technical or biological reasons (e.g. tumor aggressiveness and surveillance interval).

Testing bias. Penetrance estimates are often influenced by testing bias (*in orange*). Affected family members (III-1, III-3, III-5 and IV-2) were referred for genetic testing, whereas II-2, II-3 and III-4 were not tested for the germline variant, possibly because they didn't show a phenotype. Notably, probands (i.e. in this case the first person in the family with a confirmed germline mutation, indicated with an arrow in Figure 1) are the most obvious example of testing bias and excluding them from penetrance estimations should be considered carefully.

Surveillance bias. As part of the genetic diagnostic process, mutation carriers will undergo surveillance for tumors associated with a germline variant. Which in turn might lead to over diagnosis of small indolent lesions which otherwise would not have been diagnosed (II-7, II-8; surveillance bias, *in pink*).

Nowadays, statistical models are available (e.g. modified segregation analysis) to correct for these types of bias and have been used in large studies of hereditary breast and colon carcinoma.[56,57] However, one disadvantage is possible overcorrection of risk estimates and therefore underestimation of the risk in clinic-based families. Besides using all the available retrospective data, researchers should therefore concentrate on building (international) prospective cohorts to provide more reliable data on gene-stratified disease penetrance. This in turn could lead to gene-specific (personalized) surveillance guidelines with, if applicable, integration of *genotype-phenotype* relationships, *polygenic* risk models and lifestyle factors. Another approach is to use data from publicly available *genomic databases*, such as the Exome Aggregate Consortium (ExAC), to calculate penetrance via Bayesian statistics, i.e. the population frequencies of pathogenic germline variants should be inversely proportional to their penetrance for disease.[58] Using this approach, Maniam *et al*. recently provided support for the etiological role of *SDHA* in PGL formation, while simultaneously suggesting that most germline *SDHA* mutations are associated with very low disease penetrance and that therefore carriers might not benefit from periodic surveillance screening.[59]

Furthermore, additional challenges in the development of tailored surveillance guidelines include the commonly reported phenotypic variability between and within families (*in green*). A range of theories have been proposed to explain this observation: e.g. genotype-phenotype correlations, additional genetic susceptibility loci, parent-of-origin effects and lifestyle factors. A polygenic risk score, i.e. multiple genetic loci with associated weighting, might in the future provide better risk prediction. However, data on endocrine tumor predisposition syndromes are currently very limited.

One genotype-phenotype association (i.e. the association between an individual's genotype and the resulting pattern of clinical findings) that represents an exception is the comprehensively studied *RET* gene. Various consortia have defined the risk profiles of common *RET* mutations associated with Multiple Endocrine Neoplasia (MEN) syndrome type 2, including tailored guidelines for surveillance and prophylactic thyroidectomy.[17,60] Although virtually all patients develop MTC, the age of onset and aggressiveness of MTC, and the incidence of PHEO and pHPT in MEN2 (10-50%), is highly dependent on the specific *RET* mutation.[17] In **chapter 7** we describe an unusual case of apparent non-penetrance in a 93-year-old carrier of an ostensibly *de novo* non-mosaic *RET* germline mutation (codon 620). His affected son (MTC at age 50 and PHEO at age 55) and grandson (MTC at age 19) illustrate that disease penetrance still varies among carriers of an identical *RET* gene mutation. *RET* gene polymorphisms, single nucleotide polymorphisms in other genes,[61,62] mitochondrial DNA mutations,[63] copy number alterations[64] and post transcriptional modifications[65] have been suggested as potential genetic modifiers.

In hereditary PGL, gene-phenotype correlations have been used to guide genetic testing, surveillance, and in some cases, to recommend treatment.[51,66,67] These gene-related phenotypes include tumor location, presence of metastases, biochemical profile and aspects on nuclear imaging.[68,69] Genotype-phenotype correlations in *SDHB*[70] and *SDHD*[53] have been suggested and if confirmed, these findings could be used to stratify tumor surveillance programs according to individual mutation risks.

To summarized Part III, the identification of endocrine predisposition syndromes, i.e. Are other relatives at risk?, cannot be seen separately from the question "Do they need to undergo surveillance?" Our studies contributed to the debate on accurate estimation of disease penetrance. Prospective studies, including genotype-phenotype relationships, genetic modifiers and/or environmental factors, are required to determine the optimal age at which surveillance should be initiated, and the monitoring intervals that best capture the different related manifestations as they develop.

FUTURE GENETICS IN A BROADER CONTEXT

In addition to the questions of the 12-year-old girl with TC, many other questions in which genetics plays a major roll have been and will be asked by our society. The Dutch National Research Agenda for example, driven by the Dutch general public, contains over 150 questions related to DNA. In addition to the research projects described in the previous six chapters, I contributed, in collaboration with others, to different projects that focused on communicating genetics to a broader (non-scientific) audience (e.g. Lowland Science, Science Battle and public lectures - see PhD portfolio). The following paragraphs will give an overview of the fast changing field of genetics and discusses the future of genetics in a broader context, focusing on genetic testing and genome editing.

Genetic testing

History was made in 2003 when the Human Genome Project was completed. Sequencing the first complete human genome took about 13 years and cost more than three billion dollars. Today sequencing takes one to two days and costs less than 1000 dollars. We cannot imagine what the situation will be in 15 years. Is the 100 dollar genome really possible?, as suggested by the largest maker of DNA sequencers (Illumina). Questions worth asking include: In time, will the DNA code of every human be known? Will it become normal to receive the DNA code of your newborn baby on a flash drive directly after birth? And who else will have access to these data? Health insurance companies? Your employer? Facebook or Google? Is it reasonable to 'open' specific parts of the DNA code at different stages of life? Can we subsequently prevent or treat major diseases? Will the potentially expensive medical treatments resulting from this new area of research be covered by health insurance companies?

Currently, most genetic testing goes through the clinical geneticists and/or increasingly through treating physicians. However, a growing number of companies (e.g. 23 and me, My heritage) offer direct-to-consumer genetic tests for a variety of purposes (e.g. heritage, fun DNA facts and health-related features). Direct-to-consumer genetic testing has both benefits and limitations, although they are somewhat different to the genetic tests ordered by a healthcare provider. Customers send the company a saliva (DNA) sample and receive their results directly from a secure website or in a written report without necessarily involving a healthcare provider or health insurance company in the process. Consumers are able to learn about their ancestry and health risks at the cost of just $99 to a few hundred dollars. These companies make DNA testing more widely available, which may lead to increased awareness of genetic disease and might help some people to be more proactive about their health. However, since there is currently little regulation of direct-to-consumer genetic testing services, this genetic information might be inaccurate, incomplete or misunderstood. People may subsequently make important decisions regarding disease treatments or prevention based on incorrect results. Furthermore, genetic

testing for cancer risk can be stressful, provides incomplete information regarding your health and cannot definitively determine whether you will or will not develop cancer. The involvement of other genetic factors, family history and/or environmental factors is discussed during a medical consultation, but is often inadequately addressed in direct-to-consumer tests. People who use direct-to-consumer DNA testing are often apparently healthy, which makes the interpretation of (likely) pathogenic variants, as well as VUS, even more challenging than in patients with a specific disease. Moreover, direct-to-consumer DNA testing raises concerns around the privacy of genetic data. Consumers might not realize that these companies retain data (and DNA samples) and that this information might be accessed by third parties without consumer consent through, for example, sale to other companies, hacking, law enforcement and/or the government. Given the growing fascination with genetic testing due to curiosity, ancestry, or recreational motivations, the government and comparable authorities should facilitate consumer education and the regulation and quality control of providers. However, the combination of internet-based genetic testing, different nations and local authorities remains challenging.

Genome editing

In late 2018 the world was shocked by the news that the first genetically modified twins were born. Jiankui He, a Chinese researcher, claimed via YouTube that he had successfully used CRISPR/Cas9 to disable the *CCR5* gene so that the twin girls might be resistant to potential infection with HIV. Although DNA experts knew that this development - introducing alterations into the human germline that can be passed to offspring - was inevitable, it had been considered off-limits. CRISPR/Cas9 is a technique that permits the highly specific and rapid modification of DNA in any genome. Briefly, the Cas9 protein cuts both DNA strands at the place where a single-guide RNA binds. The double strand break will be repaired by non-homologous end joining which often results in mutation of the target gene, or 'correction' when a replacement DNA segment is supplied. Human-related applications might include curing inherited genetic disorders, treating infectious diseases, and advancing cancer treatment.

There is no doubt that CRISPR will be important in the coming years but this technology nowadays faces two major issues. The first issue is one of safety. While CRISPR is a relatively simple and powerful technique, it isn't flawless. A major concern is that the CRISPR technique might introduce (unpredictable) off-target mutations into the genome.[71] The second issue, particularly when using CRIPR to alter 'germline' cells, is a medical ethical dilemma. Most scientists agree that gene editing should be restricted to medical conditions. However, where does one draw the line between treating serious disease and 'enhancing' humans beyond what the society considers 'normal'? And who should determine these boundaries? Medical doctors/scientists? The government? Health incurrence companies? A dedicated committee? Furthermore, genome editing might need to be restricted to conditions where no alternative is available. In many cases, pre-implantation genetic diagnosis (PGD) i.e. embryo selection, might be an appropriate alternative.[72]

The rapidly expanding possibilities of DNA testing and the concept of editing the human genome both raise questions that science alone cannot answer. While physicians and scientist might can determine what will be possible in DNA testing and editing in near future, the broader public should decide if everything that is possible, retains also desirable. "Just because we could, does not mean we should". The relationship between science and the media has in the past been

characterized by terms such as *distance, gap* and *barrier,* this is the moment for the scientific community to break out of their 'ivory tower' and to discuss these topics with a broader public. In other words, while accurately presenting the facts, scientists could actively initiate a public debate about the science and about societal consequences and implications that may arise from potential new applications.

CONCLUDING REMARKS

Within the broad theme of endocrine cancer genetics, there are a few aspects we would like to highlight:

Clinical implications

- Gene fusion analysis in selected patients is effective and feasible for TC classification and stratification for targeted treatment.
- TC patients with DICER1-syndrome may form an low risk subgroup and should be treated in a center of expertise.
- Mutation analysis of *SDHA* should be included in the genetic testing strategy of all patients with PGL, preferably using gene panels.
- Germline *CDC73* mutation detection using clinical testing criteria enables optimal clinical management of pHPT as well as genetic counselling and surveillance

Implications for further studies and clinical practice

- International prospective studies in DICER1 syndrome, *CDC73*-related disorder and *SDHA*-related paraganglioma are needed to determine the optimal age at which surveillance should be initiated and the best monitoring intervals to capture the different related manifestations as they develop. With, if applicable, integration of genotype-phenotype relationships, polygenic risk models and lifestyle factors.
- Patients with advanced endocrine cancers (e.g. RAI-R TC, PC and PGL) should be enrolled in bucket or basket trials to investigated the potential of targeted treatment and/or immunotherapy.
- Exploring the possibility of (partly) mainstreaming genetic testing i.e. gene testing via attending physicians in patients with a high suspicion for an endocrine tumor predisposition syndrome.

Implications for future genetics in a broader context

- Improve information and regulation of direct-to-consumer genetic testing services.
- Determine CRISPR/Cas9 safety and discuss related medical ethical dilemmas.
- Strengthen the responsibility of the scientific community in the public debate about genetic testing and genome editing.

In order to provide answers to the questions Why?, Who? and How? for every patient, endocrine cancer care encourages active collaboration between among others the departments of endocrinology, oncology, surgery, pathology, chemistry, radiology, nuclear medicine and clinical genetics. Moreover, local- national- and international collaborations between basic- and clinical researchers should take research from bench to beside and back again.

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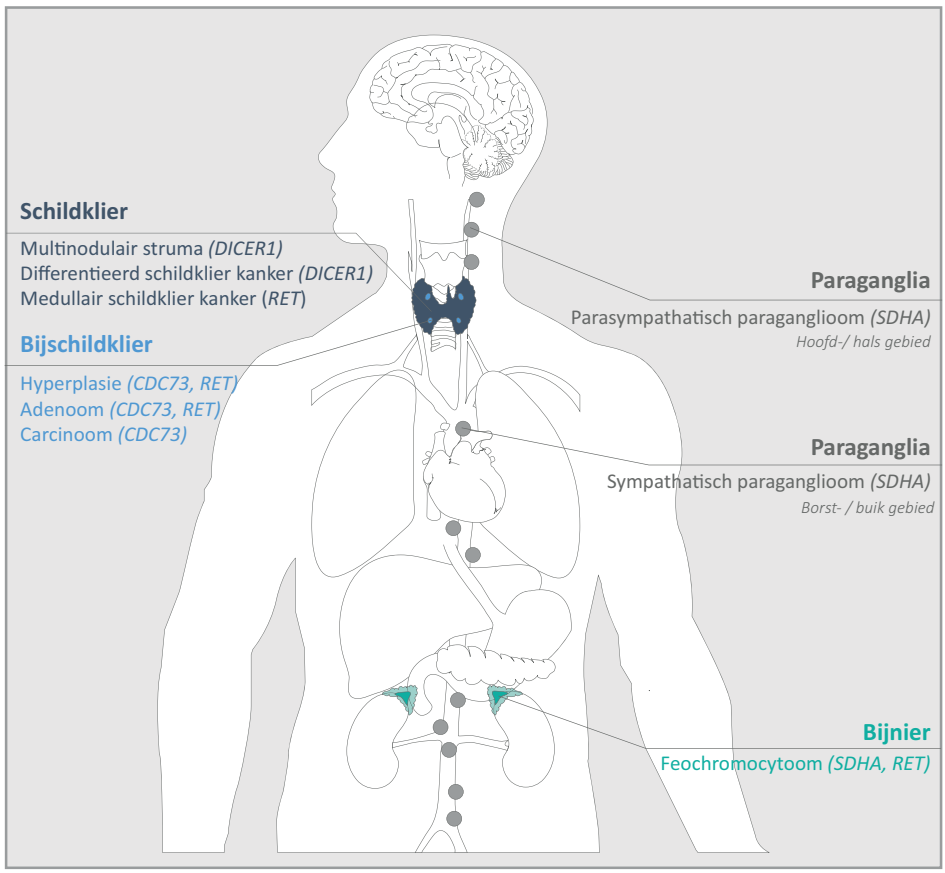
Dit hoofdstuk vat op toegankelijke wijze de verschillende wetenschappelijke hoofdstukken van dit proefschrift samen. Tevens blikt dit hoofdstuk vooruit op de toekomst van DNA onderzoek in een bredere context.

Nederlandse Samenvatting

Een aantal jaren geleden ontmoette ik Julia[1] op de poli voor vragen over erfelijkheid (Klinische Genetica) in het Leids Universitair Medisch Centrum (LUMC). Ik, net afgestudeerd, nog wat onwennig in mijn witte jas en zij in een fel gekleurde Disney trui. Julia is een meisje van net 12 jaar oud, waarbij schildklierkanker werd vastgesteld. Zij en haar ouders hadden drie belangrijke vragen voor mij. Julia vroeg: "Waarom heb ik schildklierkanker? Haar ouders vroegen: "Kunnen haar broer Jan, en zusje Anne dat ook krijgen? en zo ja, kunnen we dat dan voorkomen?" Deze vragen zijn de motivatie voor de onderzoeken beschreven in dit proefschrift (*hoofdstuk 2 t/m 7*) naar de erfelijke aanleg voor zeldzame tumoren van hormoon producerende organen (*endocriene tumoren*; o.a. schildklier, bijschildklier, bijnier en paraganglia zoals weergegeven in figuur 1).

Hoofdstuk 1 van dit proefschrift is een inleiding en beschrijft wat er al bekend is over de diagnostiek, behandeling en erfelijke aanleg voor zeldzame endocriene tumoren.

Om antwoord te geven op bovenstaande drie vragen moeten we eerst iets meer leren over het ontstaan van kanker en welke rol ons DNA daarbij speelt. Onze DNA-code bestaat uit 6 miljard



Figuur 1. Endocriene tumoren en erfelijke aanleg beschreven in dit proefschrift.

[1] Alle namen zijn fictief.

letters die er samen gedeeltelijk voor zorgen dat jij bent wie je bent, en ik ben wie ik ben. Je kunt onze DNA-code zien als een boek, waarin al onze erfelijke eigenschappen zijn beschreven. Dit boek heeft ruim 20.000 hoofdstukken (*genen*). De gehele DNA-code (2 meter) zit in alle cellen van ons lichaam. We hebben de helft van ons DNA van onze vader (zaadcel) en de andere helft van onze moeder (eicel). Daardoor hebben we alle erfelijke eigenschappen dubbel. Op een kritieke plaats in de DNA-code kan 1 letterverandering het verschil betekenen tussen jou en iemand met een ernstige lichamelijke en/of verstandelijke beperking. Terwijl je op een andere plaats wel een hele bladzijde of zelfs meerdere hoofdstukken (*genen*) kunt missen zonder dat je daar direct problemen van ondervindt.

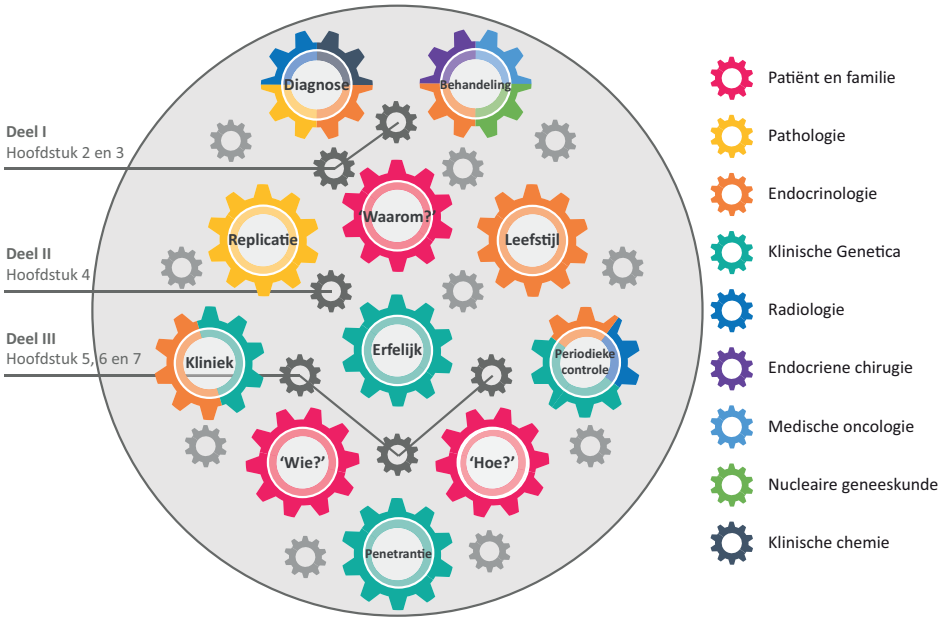
Kanker ontstaat doordat een aantal cellen zich ongecontroleerd gaan vermenigvuldigen. Dat komt omdat er 'foutjes' (*varianten*) in de DNA-code zijn ontstaan. Er zijn meerdere opeenvolgende DNA-fouten in één cel nodig voordat er kanker ontstaat. DNA varianten kun je overgeërfd hebben van je ouders maar ze kunnen ook gedurende het leven ontstaan (*somatisch*). Deze 'nieuwe' DNA varianten ontstaan door pech (*replicatiefouten*) of omgevingsfactoren.

Iedere keer wanneer een cel zich vermenigvuldigt moet de gehele DNA-code gekopieerd worden, zodat het DNA daarna opgesplitst kan worden in twee dochtercellen. Je kunt je voorstellen dat als je de gehele DNA-code (6 miljard letters) moet overschrijven, dat je dan weleens een foutje maakt. Dit soort fouten zijn pech en noemen we replicatiefouten. Hoe ouder je bent, hoe vaker je cellen vermenigvuldigd zijn, dus hoe meer DNA-fouten er zijn ontstaan en hoe groter de kans op kanker is. Om het aantal replicatiefouten te beperken heeft ons DNA een soort 'spellingschecker' en een reparatie mechanisme, maar helaas haalt die niet alle fouten eruit. De gevolgen van een DNA-fout kun je opdelen in drie categorieën. DNA-varianten die: 1) geen invloed op de functie van de eigenschap hebben, 2) de functie veranderen, of 3) de cel dusdanig schaden dat de cel dood gaat. De eerste en laatste categorie leiden over het algemeen niet tot tumor ontwikkeling. Varianten die de functie veranderen kun je vergelijken met de spellingcontrole van Word; als een woord wel bestaat maar niet past in de zin dan wordt de fout niet herkend. Er hoort bijvoorbeeld te staan: DNA is super interessant! Maar door een paar kleine letter veranderingen komt er te staan: DNA is super irritant! Dan heb ik wel een probleem.

Regelmatige en langdurige blootstelling aan schadelijke stoffen (zoals röntgenstraling, sigarettenrook of asbest) vergroot de kans op DNA-fouten. Deze stoffen beschadigen rechtstreeks het DNA. Deze DNA varianten kun je voorkomen met een gezonde leefstijl.

Krijgen dan alleen rokende bejaarden die naast een kolencentrale wonen kanker? Nee. En hoe komt het dat kinderen kanker krijgen? Dat komt omdat sommige kinderen een DNA variant hebben overgeërfd van hun ouders. Dit betekent dat deze (eerste) DNA variant in alle cellen van hun lichaam zit. De kans dat daar een tweede, derde en uiteindelijke vierde fout bij komt is veel groter dan als je geen overgeërfde DNA variant had. Bij kinderen en jongvolwassen spelen omgevingsfactoren en replicatiefouten een kleinere rol waardoor de rol van erfelijke factoren waarschijnlijk groter is. Kanker is niet erfelijk, maar de aanleg daarvoor wel. We spreken dan ook van een tumor predispositie syndroom. Kenmerken die kunnen wijzen op een erfelijke vorm van kanker zijn: 1) kanker op jonge leeftijd, 2) meerdere familieleden met dezelfde soort kanker, 3) vaker dan een keer kanker krijgen, en 4) combinaties van tumoren die passen bij een bepaald syndroom.

Zoals je inmiddels misschien wel duidelijk is geworden, betreft het een complex samenspel van vele factoren. Genetisch onderzoek levert niet alleen nieuwe inzichten op over tumorontwikkeling maar kan ook direct worden toegepast in de klinische praktijk. Hierbij is de samenwerking tussen de verschillende betrokken medisch specialismen essentieel (figuur 2).



Figuur 2. Verschillende aspecten van patiënt- en familie gerichte zorg voor endocriene tumoren. Geïnspireerd door de vragen van Julia en haar ouders: "Waarom heb ik kanker? [Wie?], kunnen familieleden dat ook krijgen? [Wie?], En zo ja, kunnen we kanker voorkomen? [Hoe?]"

De belangrijkste doelstellingen voor dit proefschrift waren:

1. Het onderzoeken van de rol van DNA tumor testen in de diagnostiek en behandeling van schildklierkanker.
2. Het verbeteren van onze kennis over de erfelijke aanleg voor schildklierkanker door:
   - het bepalen van de bijdrage van varianten in bekende genen bij kinderen met schildklierkanker, en
   - het zoeken naar nieuwe genen geassocieerd met schildklierkanker
3. Het gedetailleerd beschrijven van het klinische beeld van endocriene tumor predispositie syndromen (DICER1-syndroom, MEN2a-syndroom, CDC73-gerelateerde ziekte en SDHA-geassocieerde paragangliomen).

Deel 1 van dit proefschrift richt zich op de rol van moleculaire testen bij schildklierkanker diagnostiek en besluitvorming bij de behandeling.

In **hoofdstuk 2** onderzochten we in samenwerking met Canadese collega's 10 jonge patiënten met DICER1 syndroom en schildklierkanker. Een zeldzaam syndroom waarbij vooral kinderen een verhoogd risico hebben op het ontwikkelingen van verschillende soorten goed- en kwaadaardige tumoren. Gecombineerde analyse van medische gegevens, tumorweefsel en DNA liet zien dat deze tumoren zich anders gedragen dan schildkliertumoren bij kinderen zonder *DICER1*-variant en daarom mogelijk ook anders behandeld zouden moeten worden. Gezien de zeldzaamheid adviseren we om deze patiënten te behandelen in een expertisecentrum.

Terwijl schildklierkanker over het algemeen een goede prognose heeft, is er een kleine groep patiënten die geen profijt heeft van de standaard behandeling (operatie en radioactief

jodium). Behandelopties in deze groep patiënten zijn tot nu toe beperkt. Daarom verrichtten we in **hoofdstuk 3** uitgebreide DNA/RNA-analyse in schildkliertumoren van 132 volwassen patiënten met vergevorderde ziekte. In een specifieke subgroep vonden we frequent gen-fusies. Deze geselecteerde patiënten kunnen eventueel behandeld worden met nieuwe medicijnen, specifiek gericht tegen deze gen-varianten. Of dit ook in de praktijk goed werkt, moet uit vervolgonderzoek blijken.

Deel 2 van dit proefschrift richt zich op de genetische aanleg voor schildklierkanker bij kinderen.

... Terug naar de vragen van Julia en haar ouders. We hebben haar complete DNA-code bekeken, alle 6 miljard letters. Dit is een computerbestand van ongeveer 180Gb. Haar DNA-code hebben we vervolgens vergeleken met een 'standaard' code (*referentie*). Maar wat is eigenlijk 'normaal'? We zijn allemaal immers zo verschillend. Hoewel, jij en ik hebben meer dan 99.9% van ons DNA gemeenschappelijk. Gek hè? Of toch niet? Jij en ik hebben allebei twee ogen, tien vingers met tien nagels en twee longen. En we kunnen allebei lezen, eten, lachen en huilen. Die 0.1% verschillen in de DNA-code is omgerekend dus 4 miljoen DNA varianten. Dat is dan toch best veel! Zoeken naar die ene DNA variant die ervoor zorgde dat Julia schildklierkanker kreeg, lijkt misschien zoeken naar een speld in een hooiberg, ik vind dat juist een uitdaging!

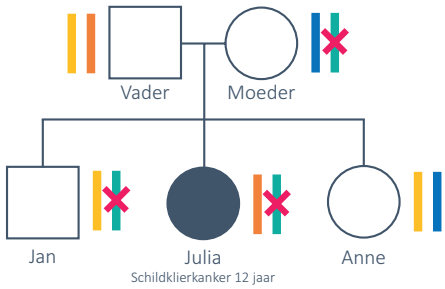
Het nadeel van zo breed DNA onderzoek, is dat je soms ook varianten vindt waarvan de betekenis onduidelijk is. Of varianten die geen relatie hebben tot de ziekte, dus waar je eigenlijk niet naar opzoek was (*nevenbevindingen*). Deze DNA varianten kunnen echter wel consequenties hebben voor iemands gezondheid, daarom is uitgebreide erfelijkheidsvoorlichting voor een DNA test ontzettend belangrijk.

Gelukkig hoeven we deze 4 miljoen varianten niet een voor een met de hand na te lopen maar konden we dit systematisch doen.

We hebben eerst gekeken naar erfelijke eigenschappen (*genen*) waarvan we al weten dat ze belangrijk zijn bij het ontstaan van kanker. We kijken daarbij alleen naar varianten die maar zelden (<0.1%) voorkomen in een grote DNA-database met gezonde personen. Immers, als veel mensen die variant zouden hebben dan zouden er ook veel meer mensen schildklierkanker krijgen. Vervolgens kijken we in het bijzonder naar varianten die de functie van het gen ernstig verstoren.

Een DNA variant kan ervoor zorgen dat een gen niet meer (goed) werkt. Dat is vervelend als het bijvoorbeeld gaat om het DNA reparatie apparaat. Deze genen zijn juist bedoeld om tumoren te voorkomen. Als deze niet goed werken, ontstaan er meer fouten dan 'normaal' en daardoor heb je een verhoogd risico op kanker. Gelukkig hebben we al onze erfelijke eigenschappen dubbel. Zo hebben we tenminste nog 1 goed werkende kopie. Vaak zie je dat in tumoren deze tweede kopie ook beschadigd is ('2-hit-model'). Aan de andere kant kan een DNA fout er ook voor zorgen dat een eigenschap juist overactief werkt. Als dit gebeurd bij een eigenschap is die er voor zorgt dat cellen zich gaan vermenigvuldigen of verspreiden door het lichaam, kan dit kanker veroorzaken. Dan helpt het hebben van een tweede (normale) kopie niet.

.... Bij Julia waren we in staat om de ziekte veroorzakende DNA variant (*mutatie*) te vinden. Zodra we de erfelijke DNA variant hebben gevonden kunnen we ook andere familieleden onderzoeken (zie figuur 3, stamboom). Nu hoefden we niet de gehele DNA-code te bekijken. We kijken direct op die ene specifieke plek waar de DNA variant bij Julia is gevonden. Uit aanvullend onderzoek bleek dat Julia deze DNA variant heeft overgeërfd van haar moeder. Haar moeder is gezond. Gek zou je denken? Nee dat is niet gek. Het is niet zo dat iedereen met deze DNA variant ook schildklierkanker ontwikkeld (*onvolledige penetrantie*). Zoals eerder gezegd, kanker ontstaat door een opstapeling van DNA veranderingen. Naast de erfelijke aanleg moeten er dus



Figuur 3. Stamboom familie Julia. *Rondjes zijn vrouwen; vierkantjes mannen; iedereen heeft twee kopieën van het DNA aangeven met gekleurde staafjes; één kopie overgeërfd van vader en één kopie van moeder; mutatie aangegeven met kruis. Kinderen van een mutatiedrager hebben 50% kans om de mutatie te erven.*

nog nieuwe DNA veranderingen bijkomen om ook echt ziek te worden. In het geval van Julia is dit waarschijnlijk pech. Zij is voor zover bekend niet blootgesteld aan schadelijke stoffen. Haar broer Jan heeft dezelfde variant als Julia. Jan is gezond. We maken jaarlijks een echo van zijn schildklier om eventuele afwijkingen zo vroeg mogelijk op te sporen. Julia haar zusje Anne heeft de variant niet geërfd. Zij kan gerustgesteld worden en hoeft geen extra controles te krijgen.

Net als je de ene vraag beantwoord hebt, volgen er alweer een nieuwe vragen: hoeveel procent van de kinderen met schildklierkanker heeft een erfelijke aanleg? Welke erfelijke eigenschappen spelen een rol? En hoeveel procent van de mensen met deze erfelijke belasting wordt daadwerkelijk ziek? Dat weten we eerlijk gezegd niet, dus dat gaan we verder onderzoeken. De antwoorden op deze vragen kunnen direct worden toegepast in de klinische praktijk. Moeten alle kinderen met schildklierkanker verwezen worden voor DNA onderzoek? En wat moeten we zeggen tegen mensen die de erfelijke aanleg hebben maar nog niet ziek zijn? Hoe vaak moeten we hen controleren en vanaf welke leeftijd? Vragen die niet alleen belangrijk zijn voor de patiënt maar ook voor diens familie.

In **hoofdstuk 4** beschrijven we de eerste resultaten van een studie bij 100 kinderen vergelijkbaar met Julia. Dit was mogelijk vanwege een onderzoekssubsidie van Stichting Kinderen Kankervrij (KiKa). Bij 6 kinderen konden we tot nu toe de erfelijke aanleg aantonen. Bij deze patiënten viel op dat de tumor onder de microscoop bijzondere kenmerken had. Het herkennen van deze kenmerken door dokters die het weefsel onderzoeken (pathologen) is niet eenvoudig omdat het zo extreem zeldzaam is. Ervaren pathologen spelen mogelijk een belangrijke rol bij het herkennen van deze patiënten en verwijzing voor DNA onderzoek. Bij de andere 94 patiënten gaan we de komende periode nog specifieker naar de DNA-code kijken. Een enorme klus, 94x6 miljard letters, 94x180Gb aan data, maar ontzettend dankbaar werk!

Deel 3 van dit proefschrift richt zich op de erfelijkheidsvoorlichting bij endocriene tumor predispositie-syndromen (CDC73-gerelateerde ziekte, SDHA-geassocieerde paragangliomen en MEN2a syndroom). In dit deel geven we antwoord op een gedeelte van bovenstaande vragen ten aanzien van erfelijke bijschildkliertumoren, paragangliomen en een specifiek type schildklierkanker.

Hoofdstuk 5 beschrijft patiënten met een DNA variant in het *CDC73* gen. Mutaties in dit gen verhogen het risico op het ontwikkelen van bijschildkliertumoren en, in mindere mate, kaak, nier-,

en baarmoeder afwijkingen. Op basis van de verzamelde gegevens konden we een voorzichtige uitspraak doen over 1) bij welke patiënten DNA onderzoek verricht moet worden, en 2) hoe groot het risico op ziekte is bij patiënten met een *CDC73* variant en 3) hoe we deze patiënten het beste kunnen controleren.

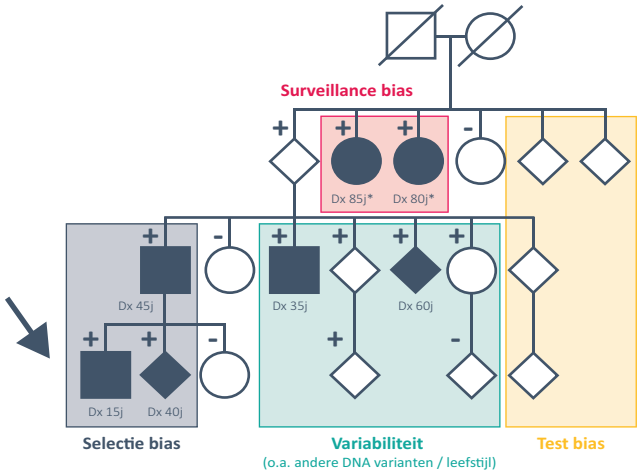
We onderzochten 89 patiënten met bijschildkliertumoren verwezen voor DNA onderzoek. Bij 11 patiënten werd een *CDC73* mutatie aangetoond (12%). Op basis van deze gegevens adviseren we DNA onderzoek bij patiënten met bijschildkliertumoren op jonge leeftijd (<35 jaar), bij familiare belasting, bij kwaadaardig tumoren, en bij de combinatie van bijschildklier- en kaaktumoren. Door onze gegevens over kwaadaardige bijschildkliertumoren te vergelijken met het nationale pathologie archief (PALGA) konden we aantonen dat nog niet alle patiënten waarbij DNA onderzoek geadviseerd wordt op dit moment verwezen worden voor genetische analyse. Terwijl vroege opsporing en gerichte behandeling van kwaadaardige bijschildkliertumoren erg belangrijk is.

Analyse van 77 familieleden leverde nog eens 43 mutatiedragers op. Op dat moment hadden 24 van de 43 mutatiedragers (56%) ook CDC73-gerelateerde ziekte. De klachten van goedaardige bijschildkliertumoren zijn soms moeilijk te herkennen waardoor mensen lang met klachten rond blijven lopen. Herkennen van patiënten met verhoogd risico op bijschildkliertumoren kan bijdragen aan vroege opsporing en behandeling. Het risico op ziekte bij een *CDC73* mutatie wordt geschat op ongeveer 11% op de leeftijd van 25 jaar, 65% op leeftijd 50 jaar en 83% op leeftijd 70 jaar. Recent is ons richtlijnvoorstel voor diagnostiek en controle bij *CDC73* mutaties goedgekeurd door de Vereniging voor Klinische Genetica Nederland.

Het onderzoek beschreven in **hoofdstuk 6** richtte zich op erfelijke vormen van paragangliomen veroorzaakt door mutaties in het *SDHA*-gen. Paragangliomen zijn zeldzame, over het algemeen langzaam groeiende, goedaardige vaatrijke tumoren die dicht tegen zenuwen en bloedvaten aanliggen. Het beloop van de ziekte is in de meeste gevallen gunstig, maar afhankelijk van hun plek in het lichaam of eventuele stresshormonen die ze aanmaken, kunnen ze wel klachten veroorzaken. *SDHA* mutaties werden aangetoond bij 30 uit 393 patiënten(7%) met een paraganglioom waarbij tot nu toe geen genetische oorzaak was aangetoond. Analyse van 94 familieleden leverde nog eens 56 mutatiedragers op. Opvallend was dat het risico op ziekte bij familieleden met de *SDHA* mutatie veel lager is dan voor de andere bekende paraganglioom-genen. Slechts 3 van de 56 mutatiedragers (5%) hadden *SDHA*-gerelateerde ziekte. Het risico op ziekte bij (gezonde familieleden die drager zijn van) een *SDHA* mutatie wordt geschat op 0% op de leeftijd van 25 jaar, 2% op leeftijd 50 jaar en 10% op leeftijd 70 jaar. Dit leidt tot discussie over de frequentie en leeftijd waarop gestart zou moeten worden met periodieke controles. We adviseren om de richtlijnen voor controles in de toekomst gen-specifiek te maken.

Hoofdstuk 7 beschrijft een familie met een *RET* mutatie (MEN2a syndroom). Tot nu toe werd gedacht dat iedereen met deze *RET* mutatie medullair type schildklierkanker ontwikkelt. Een 93-jarige mutatiedrager had geen schildklierproblemen. Terwijl zijn zoon en kleinzoon op respectievelijk 51- en 19-jarige leeftijd wel geopereerd moesten worden. Dit onderzoek laat de variatie zien binnen families en het belang van DNA onderzoek bij gezonde familieleden.

Tot slot, bespreken we in **hoofdstuk 8** de belangrijkste bevinden van de studies in dit proefschrift in de context van recente literatuur. De studies in **hoofdstuk 5 en 6** zijn retrospectieve studies (gegevens terugkijken in de tijd). Data in dit soort type studies kunnen vertekend zijn (*bias*) en moeten kritisch beoordeeld worden. Accurate berekening van het risico op ziekte is belangrijk voor het vaststellen van richtlijnen voor controle adviezen. In **hoofdstuk 8** gaan we dieper in op mogelijke vormen van bias (geïllustreerd in figuur 4). We bespreken bias veroorzaakt door 1) selectie van patiënten, 2) het testen van familieleden en 3) periodieke controles.



Figuur 4. Stamboom met mogelijke vormen van bias en variabiliteit binnen families. *Rondjes zijn vrouwen; vierkantjes mannen; ruiten onbekend geslacht; mutatiedrager (+); geen mutatiedrager (-), index patiënt aangegeven met de pijl, Dx; leeftijd diagnose ziekte (ingekleurd); * ziekte ontdekt bij periodieke controle na DNA onderzoek*

1. Families met veel aangedane familieleden en/of diagnose op jonge leeftijd worden eerder verwezen voor genetische analyse op basis van klinische verwijscriteria (*selectie bias*). Inclusie van enkel deze families geeft mogelijk een overschatting van het risico. Bij veel vormen van erfelijke kanker zien we dat, nu de verwijscriteria minder streng worden, ook de risicoberekeningen teruglopen.
2. Aangedane familieleden worden mogelijk eerder verwezen voor DNA onderzoek dan gezonde familieleden (*test bias*), ook dit leidt tot een overschatting van het risico. Daarnaast worden 'index' patiënten vaak meegenomen in risico berekeningen terwijl deze per definitie aangedaan zijn en derhalve geselecteerd zijn op de ziekte.
3. Als onderdeel van het erfelijkheid onderzoek worden mutatiedragers periodieke controles aangeboden. Dit leidt mogelijk tot identificatie van kleine, niet klinische relevante afwijkingen die anders niet aan het licht waren gekomen (*surveillance bias*).

Tevens blikt **hoofdstuk 8** vooruit op de toekomst van DNA onderzoek in een bredere context.

De ontwikkelingen in de genetica gaan onvoorstelbaar snel. Zo snel dat moet worden opgemerkt dat de 'toekomst' hieronder beschreven na het drukken van dit proefschrift mogelijk alweer achterhaald is.

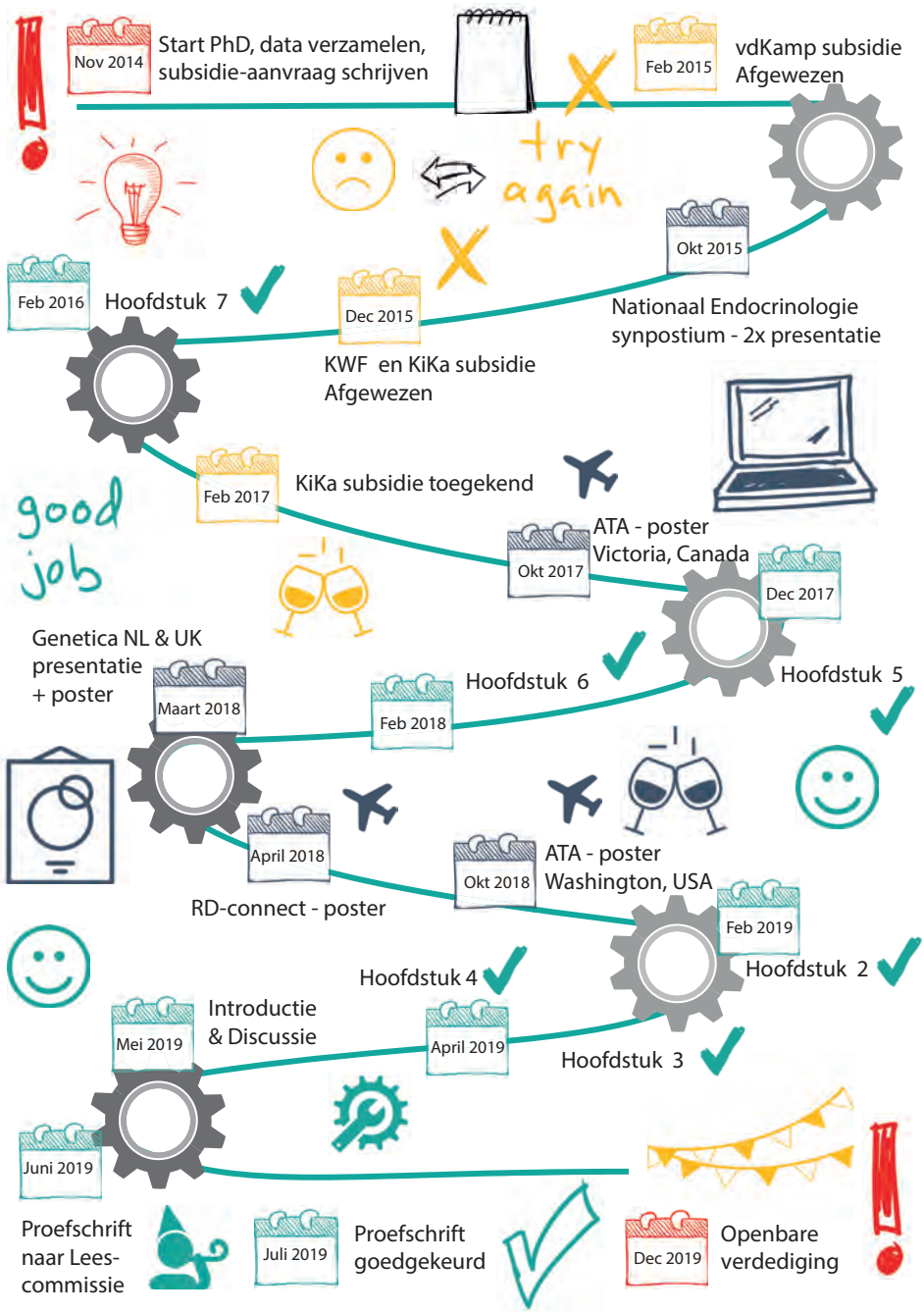
In 2003 werd voor het eerst de gehele DNA-code van de mens gepresenteerd. Dit baanbrekende project duurde bijna 15 jaar en kostte 3,4 miljard dollar. Nu, iets meer dan 15 jaar later, kunnen we de gehele code bekijken in minder dan een week, voor minder dan 1000 dollar. Ik kan me niet voorstellen hoe de situatie over 15 jaar zal zijn...

... Krijgen baby's in 2035 vlak na hun geboorte een genenpaspoort waar al je DNA-varianten in staan, of is dat geen goed idee? Wat zijn de consequenties van de uitslag voor het individu, voor diens familie en eventueel nageslacht? Wil je weten of je een verhoogd risico op ziektes hebt? Misschien wel als het gaat om een ziekte waarvoor preventie zinvol is of waar een goede behandeling voor is. Maar zou je het ook willen weten als je mogelijk op jonge leeftijd dement

wordt en we daar niets tegen kunnen doen? Heeft het zin om dat te weten? Maak je dan andere keuzes? Bijvoorbeeld over werk, carrière of het krijgen van kinderen? Of word je alleen maar heel erg ongelukkig van deze kennis? Dat zijn zeer persoonlijke afwegingen. De eenvoud van het opsturen van wat speeksel voor een commerciële DNA test voor 100 euro, doet geen recht aan deze lastige vragen. Hoewel we steeds meer weten van de DNA-code, is er is nog steeds meer dat we niet weten dan dat we wel weten. Hoe ga je om met die onzekerheid? Je kunt waarschijnlijk nog steeds meer over mij te weten komen als je 10 minuten met mij praat, dan wanneer je mijn complete DNA-code bekijkt. En zelf als we het wel weten, dan zegt ons DNA niet alles, omgevingsfactoren spelen ook een belangrijke rol bij het ontstaan van ziektes.

... "Dokter, wij willen graag een meisje met blond haar en blauwe ogen, bij voorkeur gaat ze naar het VWO, kan ze goed hockeyen en viool spelen" De aanstaande vader voegt toe: "Als het even kan heeft ze een talen- en wiskundeknobbel, want ja dan kan ze later nog alle kanten op". Is dit complete fictie of toekomstmuziek? Afgelopen jaar werd er regelmatig geschreven over een techniek genaamd CRISPR-Cas. Een techniek waarmee het DNA eenvoudig, snel en goedkoop kan worden aangepast. Simpel gezegd is het een knip-en-plak tool, die heel specifiek een 'fout' stukje DNA verwijdert en er een 'goed' stukje terugplaatst. In de medische wereld werd enerzijds enthousiast gereageerd op de mogelijkheid om met deze techniek ziekte te behandelen of erfelijke aandoeningen te voorkomen. Anderzijds zijn er ook twijfels over de veiligheid en lange termijn gevolgen, nog los van de vraag of het ethisch verantwoord is om aan het menselijke DNA te sleutelen. De bovenbeschreven zogeheten 'designer-baby' is een illusie. De beschreven eigenschappen zijn te complex om met deze techniek te beïnvloeden maar er zijn zeker toepassingen waarvoor deze techniek 'misbruikt' kan worden. Er bestond dan ook consensus onder wetenschappers dat deze techniek (nog) niet gebruikt mag worden voor het aanpassen van het erfelijk materiaal (*kiembaan modificatie*). Daarnaast zou daarvoor de wetgeving in veel landen moeten worden aangepast. Des te meer was de wereld geschokt toen in november 2018 de Chinese wetenschapper Jiankui He via YouTube bekend maakte dat hij kiembaanmodificatie had toegepast op een (gezonde) tweeling. Misschien nog belangrijker dan de vraag "waar ligt de grens?", is: "wie gaat die grens bepalen?". Zijn dat de wetenschappers en artsen? Medisch ethici? Of zijn dat zorgverzekeraars? Welke rol speelt de overheid en ons rechtssysteem hierin? Richten we een commissie op met wijze dames en heren die daar beleid over gaan schrijven? Of hebben patiënten en (aanstaande) ouders hierin het laatste woord? Nu is het moment waarop jij en ik moeten nadenken over of we alle dingen die mogelijk zijn, ook wel moeten willen. De wetenschap geeft hier niet de antwoorden, maar stelt jou de vragen.

Concluderend, genetisch onderzoek levert niet alleen nieuwe inzichten op over tumorontwikkeling maar kan ook direct worden toegepast in de klinische praktijk. Om zodoende de diagnostiek en behandeling van patiënten met zeldzame endocriene tumoren te verbeteren en personen met een verhoogd risico te identificeren. Bovenbeschreven onderzoek heeft bijgedragen aan ontwikkeling van richtlijnen voor DNA-onderzoek en periodieke controles. DNA onderzoek is onderdeel van de geneeskunde, niet exceptioneel, speciaal of iets om bang voor te zijn. Het is net als bij andere onderzoeken belangrijk om de voor- en nadelen goed te bespreken. Nationale samenwerking tussen o.a. de afdelingen endocrinologie, pathologie en klinische genetica was essentieel voor het werk naar DNA varianten in zeldzame endocriene tumoren beschreven in dit proefschrift. Intensievere samenwerking met internationale onderzoeksgroepen en patiëntenorganisaties zou het onderzoek in de toekomst verder kunnen helpen. Tenslotte illustreert figuur 5 de mijlpalen bij de totstandkoming van dit proefschrift.



Figuur 5. Overzicht mijlpalen promotietraject (zie ook PhD portfolio in appendix)

APPENDIX

LIST OF PUBLICATIONS

van der Tuin K, Ventayol Garcia M, Corver WE, Khalifa MN, Ruano Neto D, Corssmit EPM, Hes FJ, Links TP, Smit JWA, Plantinga TS, Kapiteijn E, van Wezel T, Morreau H. Targetable gene fusions identified in radioactive iodine refractory advanced thyroid carcinoma. Eur J Endocrinol. 2019 Apr 1;180(4):235-241.

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GENETIC GLOSSARY

Autosomal dominant inheritance: refers to disorders caused by mutated genes located on the non-sex chromosomes (autosomes), thereby affecting both males and females. The disease or mutant alleles are dominant to the wild-type alleles, so the disorder is manifest in the heterozygote (i.e., an individual who possesses both the wild-type and the mutant allele) and shows vertical transmission. This disease can also occur as a new condition in a child when neither parent has the abnormal gene (*de novo*). A person with an autosomal dominant disorder has a 50% chance of having an affected child. Children who do not inherit the abnormal gene will not develop or pass on the disease.

Mutation carrier: is used to indicate an individual who has one correct gene copy and one mutated gene copy. The term is used to indicate an individual with a heterozygote germline mutation related to a monogenetic disorder. In this situation mutation carriers are at increased risk to develop a certain disease. The term is also used to indicate carriers of a recessive mutation, they are usually not affected but they are at risk for passing on the mutated gene to their offspring.

De novo mutation: is a genetic alteration that is present for the first time in one family member as a result of a variant in a germ cell (egg or sperm) of one of the parents, or a variant that arises during early embryogenesis.

Driver mutation: mutation that directly or indirectly confers a selective growth advantage to the cell in which it occurs.

Frameshift variant: is a type of mutation caused by the insertion or deletion of a number of nucleotides that is not divisible by three in a nucleic acid sequence. Because of the triplet nature by which nucleotides code for amino acids, a mutation of this sort causes a shift in the reading frame of the nucleotide sequence, resulting in the sequence of codons downstream of the mutation site being completely different from the original. Frameshift mutation often lead to a premature stop codon and therefore loss of function.

Gene: is the basic physical and functional unit of hereditary information that occupies a fixed position (locus) on a chromosome. Genes used to be defined as stretches of DNA that contain instructions that are copied into RNA and then turned into proteins.

Genotype (from the Greek *genos*, meaning race, offspring): The complement of alleles present in a particular individual's genome that give rise to the individual's phenotype.

Genotype-phenotype correlations: a statistical relationship that predicts a physical trait in a person or abnormality in a patient (phenotype) with a given mutation or a group of similar mutations (genotype).

Germline mutation: a heritable change in the DNA that occurred in a germ cell (a cell destined to become an egg or in the sperm). Germline mutations can be passed on to future generations.

Heritability / inherited: the transmission of genetic information from a parent to a child.

Heterogeneous: refers to the occurrence of clinically different types of genetic conditions due to mutations in the same gene.

Heterozygous: refers to having inherited different alleles at a particular gene locus from each parent.

Human genome project (HGP): international scientific effort that began in the 1980s to 'read' the order of bases (sequence) as they appear in the DNA of human chromosomes. The objective is to create a directory of the genes that can be used to answer questions such as what specific genes do and how they work.

Imaging techniques of (neuro) endocrine tumors

- Ultrasound is primarily used in thyroid and parathyroid imaging. This technique has the advantages of near-universal availability, intraoperative utility, minimal expense and lack of radiation.
- Computed tomography (CT) is used for disease staging and surgical planning as they provide more anatomic detail of the tumors themselves and surrounding structures.
- Magnetic resonance imaging (MRI) is not a first-choice imaging tool for most endocrine tumor, however might be used to image curtain metastasis.
- ^{18}fluoro-deoxy-glucose PET (FDG PET) is used to detect malignancy for a variety of tumor types, based on metabolic activity.
- ^{123}I-metaiodobenzylguanidine (MIBG) is an analog of norepinephrine that is used to image catecholamine-secreting paragangliomas.
- Somatostatin receptor-based imaging techniques (e.g. OctreoScan (^{111}In-DPTA-D-Phe-1-octreotide) and ^{68}Ga-DOTATATE) are used to detect neuro-endocrine tumors, for staging, follow-up for disease recurrence and to select patients for peptide receptor radionuclide therapy (PRRT).
- ^{99m}Tc-sestamibi scintigraphy is a radiotracer imaging techniques for preoperative location of parathyroid tumors.

Micro RNAs (miRNAs): are a small non-coding RNA molecules that functions in RNA silencing and post-transcriptional regulation of gene expression.

Missense mutation: is a single-nucleotide substitution (e.g., C to T) that results in an amino acid substitution (e.g., histidine to arginine). Also referred to as non-synonymous variant.

Mutually exclusive mutational patterns: refers to the situation that mutations in two different genes do not occur simultaneously or occurs very rarely together in the same patient. Major driving oncogenes are commonly mutually exclusive.

Nonsense mutation: is a single-nucleotide substitution (e.g., C to T) that results in a stop codon. Also referred to as non-synonymous variant.

Oncogene: is a gene that, when activated by mutation, increases the selective growth advantage of the cell in which it resides.

Penetrance: refers the proportion of individuals carrying a particular gene mutation (genotype) that also express an associated disorder (phenotype). Penetrance less than 100%, is referred to as reduced or incomplete penetrance.

Phenotype (from the Greek *phaino-*, from *phainein*, meaning to show): the physical and/or biochemical characteristics of a person, determined by their genotype and/or environment.

Polygenic: condition or characteristic that is caused by many different genes acting together.

Polymorphisms / benign variant: DNA variant that is observed in natural populations and do not cause any harm to the individual. A gene locus is in general defined as polymorphic if a allele has a frequency of 0.01 (1%) or more.

Prediction software: is used to analyze the effect of a gene mutation. Often used is the Alamut software (Interactive Biosoftware, Rouen, France), which incorporates e.g. Align GVGD, SIFT, and PolyPhen2.

- Align GVGD is a web-based program that combines the biophysical characteristics of amino acids and protein multiple sequence alignments.
- SIFT (Sorting Intolerant From Tolerant) predicts whether an amino acid substitution affects protein function based on sequence homology and the physical properties of amino acids.
- PolyPhen-2 (Polymorphism Phenotyping v2) predicts possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations.

Public genomic databases: aggregate and harmonize exome sequencing data from a variety of large-scale sequencing projects as part of various disease-specific and population genetic studies (i.e. Exome aggregation consortium [ExAC], The Cancer Genome Atlas [TCGA], and Genome of the Netherlands [GoNL]).

Predisposition: refers to having genetic factor(s) that may make an individual more likely to develop a particular condition than the general population.

Preimplantation genetic diagnosis (PGD): is an adjunct to the IVF process where the embryo undergoes genetic testing before it is transferred (implanted) into to uterus.

Pre-symptomatic testing: determines if a person, who does not have any symptoms of the condition at the time, has inherited the mutation (present in their family).

Proband: is the person that serves as the starting point for the genetic study of a family, also referred to as index patient.

Pseudogenes: are characterized by a combination of homology to a known gene but loss at least some functionality. High-sequence similarity between pseudogenes and their functional partners poses a challenge for interpretation of sequencing data. Next-generation sequencing reads are usually few hundred bases in length and cannot be accurately aligned to either in the pseudogene or the real gene. Sequencing errors might cause mismapping of the variable pseudogene sequences (not under selective pressure, hence, they accumulate more variations) and interference with the results obtained for the real gene. Due to high degree of sequence similarity, it is difficult to design Sanger sequencing primers that would not cross-react with pseudogene sequences.

Sanger sequencing: is the gold standard for determining the nucleotide sequence of DNA. This method is based on the selective incorporation of chain-terminating dideoxynucleotides by DNA

polymerase during in vitro DNA replication. It is the most widely used method for the detection of single nucleotide variants (SNVs), often in single genes.

Spice site mutation: occur in the small regions of genes that are juxtaposed to the exons and direct exon splicing. Mutations in these regions may lead to retention of large segments of intronic DNA by the mRNA, or to entire exons being spliced out of the mRNA. These changes could result in production of a nonfunctional protein.

Somatic mutation: occur in any non–germ cells such as those that initiate tumorigenesis. Somatic mutations cannot be passed on to future generations.

Syndrome: group of characteristics and/or symptoms that occur together in a recognizable pattern.

Structural genomic variant: includes any genetic variant that alters chromosomal structure, including inversions, translocations, duplications and deletions. Duplications and deletions, collectively known as copy number variation are the most common form of structural variation in the human genome.

Synonymous vs non-synonymous: a synonymous change in the DNA sequence does not result in the change in the amino acid sequence, e.g. GTT>GTC both code for Valine. A nonsynonymous change does results in the coding of a different amino acid e.g. GTT>GAT results in Val>Asp. These nonsynonymous changes include missense, nonsense, frameshift, splice site, and indel mutations.

Tumor suppressor gene: makes a protein that helps control cell growth. Inactivating mutations in tumor suppressor genes increases the selective growth advantage of the cell in which it resides and therefore may lead to cancer.

Variant pathogenicity classification (according Plon et al. 2013): intended to improve the clinical utilization of genetic testing results, to maximize the opportunity to learn more about variants for the benefit of other families and to minimize the risk of incorrect interpretation of variants in the clinical setting.

Class	Description	Probability of being pathogenic	DNA-test / surveillance at-risk asymptomatic relatives	Research Testing of Family Members
5	Pathogenic	>0.99	DNA test and full surveillance	Not indicated
4	Likely Pathogenic	0.95–0.99	DNA test* and full surveillance	May be helpful to further classify variant
3	Uncertain significant (VUS)	0.05–0.949	No DNA test* and surveillance based on family history (and other risk factors)	May be helpful to further classify variant
2	Likely Benign	0.001–0.049	No DNA test* and treat as "no mutation detected" for this disorder	May be helpful to further classify variant
1	Benign	<0.001	No DNA test* and treat as "no mutation detected" for this disorder	Not indicated

*Consider continuing to test probands for any additional testing modalities available for the disorder in question
Table adjusted from Plon et al. Hum Mutat. 2008

Whole exome sequencing: technique for sequencing all of the protein-coding region of genes in a genome (known as the exome). Humans have about 20.000 genes with in total 180.000 exons, constituting about 1% of the human genome.

Whole genome sequencing: process of determining the complete DNA sequence of an organism's genome at a single time, including the protein-coding and non-coding regions. For a human, a whole genome is approximately 3 billion base pairs, haploid—so 6 billion base pairs to capture the whole diploid complement per cell. Non-coding DNA is not part of an active gene that contains a code for making a protein, also referred to as 'junk DNA'. Recent evidence shows that at least some non-coding DNA is involved in biological processes such as regulation of gene expression and chemical signaling among cells.

LIST OF ABBREVIATIONS

ATC	anaplastic thyroid carcinomas
CBME	ciliary body medullo-epithelioma
CMV-PTC	cribriform-morular variant
CN	cystic nephroma
DTC	differentiated thyroid carcinoma
FAP	familiar adenomatous polyposis
FDA	food and drug administration
FFPE	formalin-fixed, paraffin-embedded
FIHP	familiar isolation hyperparathyroidism
FMNTC	familial non-medullary thyroid carcinoma
FMTC	familial medullary thyroid carcinoma
FTC	follicular thyroid carcinoma
FVPTC	follicular variant of PTC
GIST	gastrointestinal stromal tumor
HCC	hürthle cell carcinomas
HE	hematoxylin-and-eosin
HNPGL	head and neck paraganglioma
HPT	hyperparathyroidism
HPT-JT	hyperparathyroidism–jaw tumor
IHC	immunohistochemistry
LOH	loss of heterozygosity
LOVD	leiden open variation database
MAPK	mitogen-activated protein kinase
MEN	multiple endocrine neoplasia
MNG	multi nodular goiter
mTOR	mammalian target of rapamycin
NET	neuroendocrine tumor
NGS	next-generation sequencing
NIFTP	noninvasive follicular thyroid neoplasm with papillary-like nuclear features
NMTC	non-medullary thyroid carcinoma
PA	parathyroid adenoma
PC	parathyroid carcinoma
PDTC	poorly differentiated thyroid carcinoma
PGL	paragangliomas
PHEO	pheochromocytoma
pHPT	primary hyperparathyroidism
PPB	pleuropulmonary blastoma

PTC	papillary thyroid carcinoma
RAI	radioactive iodine
RAI-R	radioactive iodine refractory
RCC	renal cell carcinoma
SDH	succinate dehydrogenase
SLCT	sertoli-leydig cell tumor ovarian
SPGL	sympathetic paraganglioma
TC	thyroid cancer
TCGA	the cancer genome atlas
VUS	variant of uncertain significance
WES	whole exome sequencing
WGS	whole genome sequencing

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Karin van der Tuin was born on September 19th 1987 in Groningen, the Netherlands. In 2005 she completed athenaeum secondary education at Dr. Aletta Jacobs College in Hoogezand-Sappemeer. In the same year she started her studies Biomedical Sciences at the Leiden University Medical Center (LUMC). In 2007 she started Medical School in parallel to her study Biomedical Sciences at the LUMC. She wrote her Biomedical Sciences undergraduate thesis on patients with diabetes mellitus or hypertension at risk for development of chronic kidney diseases in primary health care setting under supervisor of Prof. F.W Dekker. As a medical student, she worked as student-assistant in (neuro) physiology and epidemiology education at the LUMC. In 2012, she obtained her Bachelor degree in Biomedical Sciences and Medicine. Between 2011-13 she was a member of the Medicine Curriculum Review Task Force at the LUMC. She obtained her Medical Master Degree in 2014 after a senior internship at het Department of Clinical Genetics of the LUMC. Directly followed by a 6 months residency in onco-genetics at the same department. In November 2014 she started working on her PhD project on rare endocrine tumors under supervision of Prof. Hans Morreau and Dr. Frederik Hes, without allocated financing. In February 2017, after three rejected grant proposals, she and her supervisors received in collaboration with Prof. Thera Links from the University Medical Centre Groningen a grant from the Dutch Pediatric Cancer Society (KiKa), for the project titled: "The Genetic Background of Non-Medullary Paediatric Thyroid Carcinoma" to further continue her PhD research. During her PhD period, she orally presented her research at several national and internal conferences and supervised several students during their internships. She participated in multidisciplinary endocrine cancer patients meetings and counseled patients for research projects. Furthermore, she initiated and participated in several societal impact scientific projects besides the PhD and she was an invited speaker at many public events. In February 2019, she started her postgraduate training as a clinical geneticist (residency) under supervision of Dr. Emilia Bijlsma at the LUMC. In the coming years she would like to combine her residency with endocrine cancer genetics research.

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Education and Courses

General academic skills

Introduction PhD course	2014
Basic course in legislation and organization for clinical researchers (BROK®)	2016
Basic methods and reasoning in Biostatistics (1.5 ECTS, mark 9)	2017

Research skills

Courses in the program of the Boerhaave Continuing Medical Education, Leiden University Medical Center or Graduate school Medical Genetics Centre South-West Netherlands

Introduction genetic epidemiology	2014
MGC Next Generation sequencing (1.4 ECTS)	2016
MCG Genome Maintenance and Cancer (0.8 ECTS)	2016
Practical Linux (0.4 ECTS)	2017
Introduction in Shark	2017

Other courses in the program of the Leiden University

Writing for a broader audience	2017
Social media	2017
Writing grand proposal	2018

(Inter) national conferences

Annual International Society of Pediatric Oncology (SIOP) Meeting, Cape town, South-Africa (attendance)	2015
Annual Young Dutch Endocrine Meeting, Leiden, the Netherlands (2x oral presentation)	2015
Annual American Thyroid Association Meeting, Victoria, Canada (poster presentation)	2017
Joint meeting UK / Dutch Clinical Genetics Societies & Cancer Genetics Groups, Utrecht, the Netherlands (oral and poster presentation).	2018
Annual RD-connect meeting, Athens, Greece (poster presentation)	2018
Annual American Thyroid Association Meeting, Washington, USA (poster presentation)	2018
Annual International Society of Pediatric Oncology (SIOP) Meeting, Lyon, France (poster)	2019

Symposia and Meetings

Weekly seminars department of Pathology and Clinical Genetics, Leiden University Medical Center (several oral presentations)	2014-19
Weekly molecular tumor genetics meeting, Leiden University Medical Center (several oral presentations)	2014-19
Yearly science and education day department of Clinical Genetics, Leiden University Medical Center (several oral presentation)	2014-19
Science and Society meeting ZonMw, Utrecht, the Netherlands (invited speaker).	2017
Introduction day Biomedical Science students, 2018, Leiden, the Netherlands (invited speaker).	2018
Research lunch meeting Biopharmaceutical Science students, 2018, Leiden, the Netherlands (invited speaker).	2018
Adrenal Masterclass, 2019, Amsterdam, the Netherlands (invited speaker).	2019
DNA lab day for biology and chemistry teachers, 2019, Delft, the Netherlands (invited speaker).	2019

Teaching

Hereditary cancer course, Medicine, second year students	2014-19
Critical appraisal of a topic course, Medicine, third year students	2014-19
Student internship projects guidance	2014-19

Public science projects

Lowlands Science

- Thrill-seeking gen (DRD4) [in Dutch] *(zie fragment New Scientist op YouTube)* — 2015
  - [in Dutch] Galileo *(zie fragment op YouTube)*
  - [in Dutch] Klokhuis *(kijk terug via NPO)* — 2017
- DNA dating [in Dutch] *(zie fragment op YouTube)*

Science Battle — 2017-19

Face of Science, Royal Netherlands Academy of Arts and Sciences — 2018-19

- Schildkliekanker bij kinderen [in Dutch] *(zie fragment op YouTube)*

Public lectures

[in Dutch] 'Leve adventure DNA', Cafe Scientifique, Amsterdam	2016
[in Dutch] 'Zit de voorkeur voor hutspot, haring en bier in je genen?', Leiden	2016
[in Dutch] 'Heeft u het in zich om ooit Olympisch goud te winnen?' Wetenschapsdag LUMC, Leiden	2016

'Exploring the genetic background of pediatric thyroid carcinoma using whole genome sequencing' FameLab, Leiden, the Netherlands	2017
[in Dutch] 'Leve Adventure DNA!' Wereld DNA dag, Corpus, Leiden	2017
[in Dutch] 'DNA-daten', Wetenschapsdag LUMC, Leiden	2017
[in Dutch] 'Is DNA daten, het daten van de toekomt?', Nacht van Kunst en Kennis, Leiden	2017
[in Dutch] DNA en Sport; Kun jij olympisch goud winnen?, Corpus, Leiden	2018
'Your DNA in the cloud', TEDx Leiden University, Den-Haag, the Netherlands *(see fragment on YouTube)*	2018
[in Dutch] 'Talkshow van de Toekomst – Voortplanting', Tivoli Vredenburg, Utrecht (zie fragment op YouTube)	2018
[in Dutch] Is DNA daten, het daten van de toekomt?, Expeditie Next Festival, Rotterdam	2019

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