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

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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

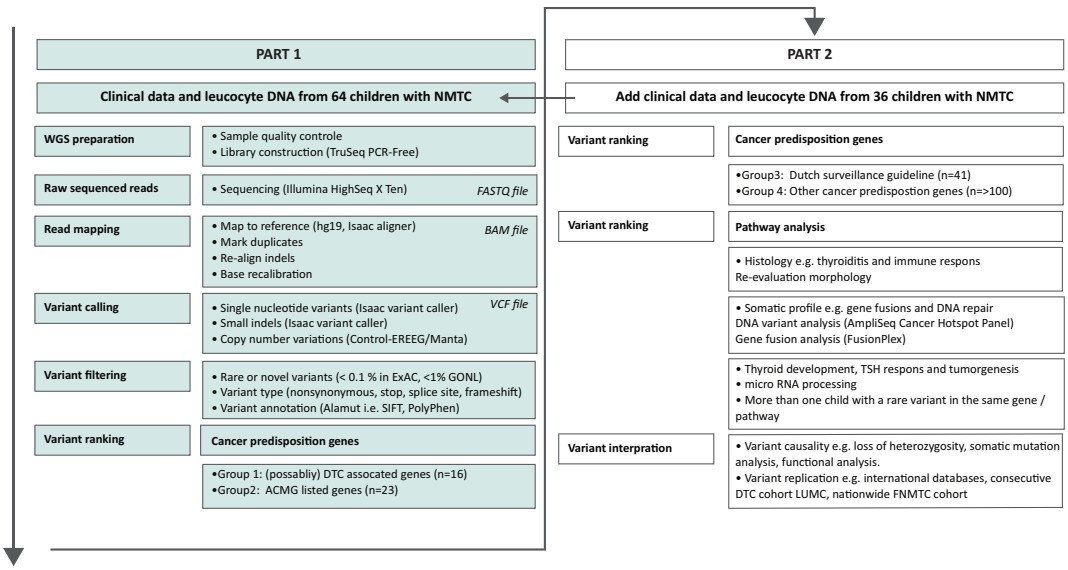

Figure 1. Full project work flow. The method and results of part 1 is described in this chapter, furthermore the plan of investigation of part 2 is described on the end of this chapter.

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

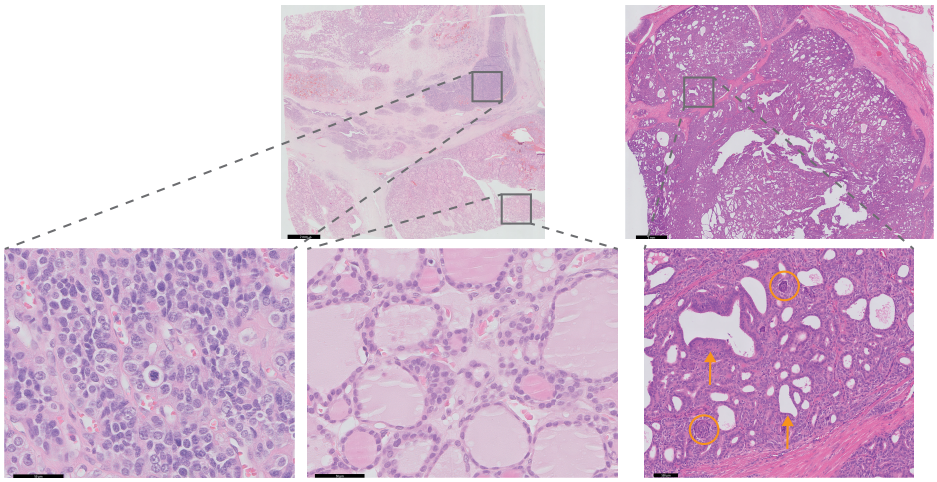

Figure 2. Clinical, histological and molecular features of hereditary syndromes associated with non-medullary thyroid cancer.

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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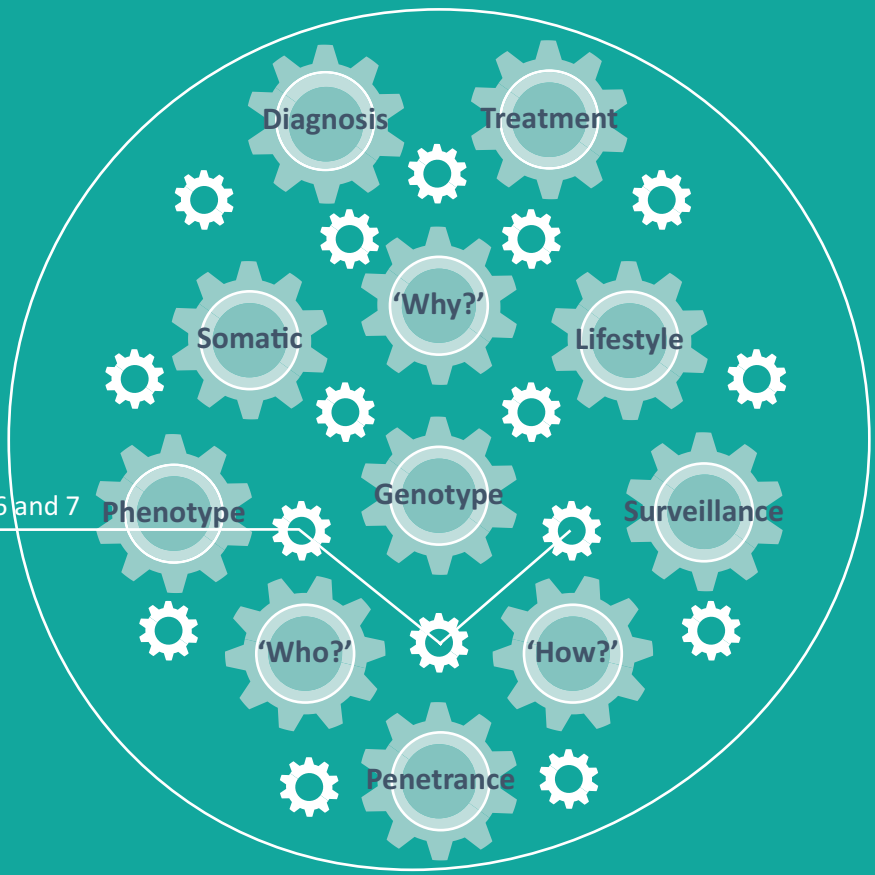

Part III
Chapter 5, 6 and 7

PART III

GENETIC COUNSELING IN ENDOCRINE TUMOR PREDISPOSITION SYNDROMES