RESEARCH

Somatic genetic alterations in a large cohort of pediatric thyroid nodules

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Abstract

There is a rise in the incidence of thyroid nodules in pediatric patients. Most of them are benign tissues, but part of them can cause papillary thyroid cancer (PTC). The aim of this study was to detect the mutations in commonly investigated genes as well as in novel PTC-causing genes in thyroid nodules and to correlate the found mutations with clinical and pathological data. The cohort of 113 pediatric samples consisted of 30 benign lesions and 83 PTCs. DNA from samples was used for next-generation sequencing to identify mutations in the following genes: HRAS, KRAS, NRAS, BRAF, IDH1, CHEK2, PPM1D, EIF1AX, EZH1 and for capillary sequencing in case of the TERT promoter. RNA was used for real-time PCR to detect RET/PTC1 and RET/PTC3 rearrangements. Total detection rate of mutations was 5/30 in benign tissues and 35/83 in PTCs. Mutations in RAS genes (HRAS G13R, KRAS G12D, KRAS Q61R, NRAS Q61R) were detected in benign lesions and HRAS Q61R and NRAS Q61K mutations in PTCs. The RET/PTC rearrangement was identified in 18/83 of PTCs and was significantly associated with higher frequency of local and distant metastases. The BRAF V600E mutation was identified in 15/83 of PTCs and significantly correlated with higher age of patients and classical variant of PTC. Germline variants in the genes IDH1, CHEK2 and PPM1D were found. In conclusion, RET/PTC rearrangements and BRAF mutations were associated with different clinical and histopathological features of pediatric PTC. RAS mutations were detected with high frequency in patients with benign nodules; thus, our results suggest that these patients should be followed up intensively.

Key Words

- papillary thyroid cancer
- pediatric
- mutations
- benign
- next-generation sequencing

Introduction

Thyroid nodules affect around 1% of the pediatric population. The incidence has been steadily increasing over the last decades, by approximately 1.1% per year worldwide (1, 2). Possible reasons for this increase are improvements in medical care including ultrasonography and fine-needle aspiration biopsy, environmental carcinogens, endocrine disruptors, inadequate iodine intake and exposure to radiation (3). Thyroid nodules have benign or malignant character. The risk of malignancy is higher in pediatric nodules compared with adults, 26 vs 7–15% (2, 4).

Pediatric thyroid cancer is a rare disease occurring mostly in females than in males (1). The most common type is papillary thyroid cancer (PTC), which represents 90% or more of pediatric thyroid cancer (4). Medullary (MTC) and follicular thyroid cancer (FTC) are relatively rare. MTC is mostly familial than sporadic in children and adolescents. Poorly differentiated cancer and anaplastic
thyroid cancer (ATC) have only been identified in several pediatric patients (5, 6).

PTCs in children and adolescents are more aggressive than adult PTCs according to many studies (7, 8, 9). On the other hand, they have better outcomes, a lower rate of dedifferentiation and a disease-specific mortality lower than 2% (10). Due to many differences between pediatric and adult PTCs, ATA guidelines specifically for children and adolescents were developed (4).

Differences between pediatric and adult PTCs are not only in clinical-pathological features, but also in genetic alterations. Main PTC-activating somatic mutations in the \textit{RAS}, \textit{BRAF} and \textit{TERT} genes and \textit{RET/PTC} rearrangements cause uncontrolled activation of MAPK and PI3K signaling pathways. It was reported that pediatric PTCs harbor more frequently rearrangements and less frequently point mutations than adult PTCs (11, 12). However, published studies have been performed only in a limited number of pediatric patients or on a low number of investigated genes.

In the Thyroid Cancer Genome Atlas (TCGA) project, genetic alterations of 496 PTCs were detected and mutations in the \textit{CHEK2}, \textit{PPM1D} and \textit{EIF1AX} genes were identified as the novel PTC-causing genes. If these genes play a role in pediatric carcinogenesis is still unknown, because only nine pediatric patients were included in the TCGA project (13).

To the other genes that are associated with thyroid nodules, \textit{IDH1} and \textit{EZH1} genes belong. Mutations in the \textit{IDH1} gene are present in many different types of cancer, including PTC. Several variants have been revealed in the conserved part of the \textit{IDH1} gene with an association with follicular variant of PTC (14). In the \textit{EZH1} gene a hotspot Q571R mutation was detected that causes increased proliferation of thyroid cells (15). This mutation was found predominantly in benign thyroid nodules with follicular pattern and without \textit{RAS} mutation (16).

The purpose of this study was to identify main genetic alterations in one of the largest pediatric cohorts of thyroid nodules. This study investigated not only commonly screened genes \textit{HRAS}, \textit{KRAS}, \textit{NRAS}, \textit{BRAF}, \textit{TERT} but also newly identified genes \textit{IDH1}, \textit{CHEK2}, \textit{PPM1D}, \textit{EIF1AX} and \textit{EZH1}. From fusion genes the most common \textit{RET/PTC1} and \textit{RET/PTC3} rearrangements were tested.

Material and methods

Patients and data collection

The cohort consisted of 113 samples from 83 PTCs and 30 benign lesions. Samples were collected from pediatric patients who underwent surgery from 2003 to 2017 at the Department of Ear, Nose and Throat, 2nd Faculty of Medicine, Charles University and Motol University Hospital in Prague. The thyroid tumor samples were histologically evaluated. All the specimens were snap-frozen and stored at ~80°C until used for DNA and RNA isolation. Clinical and histopathological data were obtained from clinical and pathological records. Patients or their legal representatives signed an informed consent for genetic studies approved by the Ethics Committee of the Institute of Endocrinology.

DNA and RNA extraction

DNA and RNA isolations were performed using the AllPrep DNA/RNA/Protein Mini kit (Qiagen) according to the manufacturer’s instructions. The concentration and purity of DNA and RNA was measured using a spectrophotometer (NanoPhotometer P330; Implen GmbH, München, Germany) and a fluorometer (Qubit 2.0; Invitrogen). The RNA integrity was evaluated using a Bioanalyzer 2100 and Agilent RNA 6000 Nano Kit (Agilent Technologies).

Next-generation sequencing

The analyzed genes were \textit{HRAS} (exons 2, 3), \textit{KRAS} (exons 2, 3), \textit{NRAS} (exons 2, 3), \textit{BRAF} (exon 15), \textit{TERT} (promoter), \textit{IDH1} gene (exons 4, 6), \textit{CHEK2} (exons 3, 4, 7, 11, 13), \textit{PPM1D} (exons 1, 4, 5, 6), \textit{EIF1AX} (exons 1, 2, 5, 6), \textit{EZH1} (exons 16, 17). Exons of \textit{RAS}, \textit{BRAF}, \textit{CHEK2}, \textit{PPM1D}, \textit{EIF1AX} genes and the \textit{TERT} promoter were selected for analysis because they were the most mutated regions in the TCGA study (13). Exons of \textit{IDH1} and \textit{EZH1} genes were chosen according to previous publications (14, 16). Exons were amplified using PCR with sequence-specific primers. The primer sequences and PCR conditions are available on request. The PCR products were purified using Agencourt AMPure (Beckman Coulter) and used for preparation of next-generation sequencing (NGS) libraries using Nextera XT Sequencing Kit (Illumina) according to the manufacturer’s sample preparation protocol and modifications as described in our previous article (17). The NGS libraries were paired-end sequenced for 500 cycles by MiSeq Reagent kit v2 (Illumina) using MiSeq sequencer platform (Illumina).

All variants of the novel PTC-causing genes from TCGA study were also analyzed in DNA samples extracted from peripheral blood of the same patient to distinguish somatic and germline detected genetic changes.
Somatic mutations in pediatric thyroid nodules

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The study consisted of 113 specimens of pediatric patients with predominance of females (73.5%). The age ranged 6–20 years at the time of diagnosis. As detailed in Fig. 1 and Table 1, 30 samples of benign lesions were collected (nine solitary thyroid nodules, eight follicular adenomas, five multidular goiters, five chronic lymphocytic thyroiditis, two thyroid cysts, one oncocyotic adenoma). The cohort of benign patients had a mean age at diagnosis 14.7 ± 2.6 years and most of the group consisted of females (24/30). The half of the patients underwent hemithyroidectomy and the other half total thyroidectomy, and in two of them the lymph node dissection was performed. Multifocal nodules in six cases were noted.

Medical records of 83 patients with PTCs were studied for family history, another serious disease and radiation exposure before PTC diagnosis. Thyroid disease in family history had 25 patients. Except two, no other patient had thyroid cancer in family. Two patients had known previous history of radiation exposure. The first one was diagnosed with acute lymphoblastic leukemia and the therapeutic whole-body radio-exposition was prior to thyroid cancer diagnosis. The second patient was diagnosed with Hodgkin’s lymphoma and was treated with chemotherapy and radiotherapy. One patient was diagnosed with Cowden syndrome, which is associated with a higher risk of thyroid cancer due to mutation in the PTEN gene.

Detailed patient’s characteristics of the PTC cohort are summarized in Fig. 1 and Table 2. PTC patients were predominantly females (59/83). Total thyroidectomy was performed in 72 PTC cases and hemithyroidectomy in 11 PTC cases. Hemithyroidectomy was completed to total thyroidectomy in ten cases due to diagnosis of PTC. Mean size of tumor was 22.2 ± 13.6 mm. Microcarcinomas defined as tumors with diameter 10 mm or smaller were detected in 16/83 cases. The most common variant of PTC was follicular variant followed by the classical variant. Many rare variants such as columnar, clear cell, tall cell and diffuse sclerosing variant were identified. More than half PTCs were multifocal (46/83) and nearly half had extrathyroidal extension (37/83) and were classified as T3/T4 (38/83). Lymph node metastases were detected in 52/83 and distant metastases in 10/83 cases. All distant metastases were affecting lungs. Most of the PTC patients (72/82) underwent radioactive iodine (RAI) treatment. One 7-year-old patient died from the disease after surgery and before RAI treatment. Maximum number of RAI ablations was 6, which was used in two cases. The RAI treatment was applied in the first case during 9 years and in the second case during 11 years. In the first case, it was PTC with mix of classical, follicular and solid variant and in the second case, it was solid variant of PTC. Reoperations of lymph node metastases were performed in five patients; two of them underwent reoperations three times.

Median follow-up was 72 months (range 1–170 months). Thyreoglobulin (Tg) blood serum level
and thyroglobulin antibodies (a-Tg) were monitored for whole postoperative care. Remission was defined as a condition of the patient with any suspect object on ultrasound or whole-body scintigraphy, the Tg levels were lower than 1 µg/L and the a-Tg levels were not detectable. Recurrence and persistence were defined as a condition of a patient with malignant object or objects on ultrasound or whole-body scintigraphy at least 1 year after surgery or patient who entered remission and then malignant object was appeared. Biochemical persistence was defined as a condition of the patient with the level of Tg higher than 1 µg/L or detectable level of a-Tg with no evident tumor recurrence or persistence. Eight patients could not be classified due to short-term follow-up (range 0–12 months after surgery).

**Table 1** Characteristics of pediatric benign cohort.

| Pediatric benign cohort (n = 30) |
|----------------------------------|
| **Patients**                     |
| Females                          | 24 |
| Males                            | 6  |
| Age at diagnosis (years, mean ± s.d.) | 14.7 ± 2.6 |
| **Type of disease**              |
| Solitary thyroid nodule           | 9  |
| Follicular adenoma                | 8  |
| Multinodular goiter               | 5  |
| Chronic lymphocytic thyroiditis   | 5  |
| Thyroid cyst                      | 2  |
| Oncocytic adenoma                 | 1  |
| **Surgery**                      |
| Hemithyroidectomy – left lobe    | 8  |
| Hemithyroidectomy – right lobe   | 7  |
| Total thyroidectomy without LND  | 13 |
| Total thyroidectomy with LND     | 2  |
| **Tissue characteristics**        |
| Mean ± s.d. (mm)                 | 22.2 ± 12.1 |
| Multifocality                    | 6  |

LND, lymph node dissection.

**Figure 1**
Tile plot of genetic alterations detected in patients with benign lesions (A) and with PTC (B). Clinical and pathological data as gender, age at diagnosis, histological variant, tumor stage, lymph node metastases and distant metastases are shown. C, classical variant; CF, classical and follicular variant; DM distant metastases; F, female; FV, follicular variant; LNM, lymph node metastases; M, male; O, other variant; PTC, papillary thyroid cancer; T, tumor size and extension.

**Mutational analysis**

The detection rate of mutations in benign and malignant cohorts is noted in Table 3. The total detection rate of mutations in the cohort of benign tissues was 5/30 and in the cohort of PTCs was 35/83, of which RET/PTC rearrangement (18/83), *BRAF* V600E mutation (15/83) and *RAS* mutations (2/83) were identified (Fig. 1). All mutations in benign cohort were in *RAS* genes (Fig. 1). Mutations of *RAS* genes in benign lesions included *HRAS* G13R mutation found in one follicular adenoma with uncertain behavior and in one solitary thyroid nodule with oncocytic changes, *KRAS* G12D mutation in the multinodular goiter, *KRAS* Q61R mutation in the follicular adenoma and *NRAS* Q61R mutation in the multinodular goiter with oncocytic changes. Only two mutations in *RAS* genes were identified in PTCs: *HRAS* Q61R and *NRAS* Q61K. *NRAS* Q61K mutation was found in tall cell variant of PTC and *HRAS* Q61R mutation in the mix of
Table 2 Characteristics of pediatric PTC cohort.

| Characteristics                  | Pediatric PTC (n = 83) |
|----------------------------------|------------------------|
| Patients                         |                        |
| Females                          | 59                     |
| Males                            | 24                     |
| Age at diagnosis (years, mean ± s.d.) | 14.2 ± 3.4             |
| History of radioexposure         | 2                      |
| Tumor size                       |                        |
| Mean ± s.d. (mm)                 | 22.2 ± 13.6            |
| Microcarcinoma (≤10 mm)          | 16                     |
| Histological variant*            |                        |
| Classical                        | 23                     |
| Classical and follicular          | 16                     |
| Follicular                       | 27                     |
| Solid                            | 3                      |
| Classical, follicular and solid  | 3                      |
| Diffuse sclerosing               | 2                      |
| Columnar                         | 2                      |
| Tall cell                        | 1                      |
| Clear cell                       | 1                      |
| Pathological characteristics     |                        |
| Multifocality                    | 46                     |
| Extrathyroidal extension         | 37                     |
| Intravascular invasion           | 22                     |
| T1/T2 classification             | 45                     |
| T3/T4 classification             | 38                     |
| Lymph node metastases (N)        | 52                     |
| Distant metastases (M)           | 10                     |
| Chronic lymphocytic thyroiditis  | 42                     |
| Radioiodine therapy^6            |                        |
| Without radioiodine therapy      | 9                      |
| One dose                         | 41                     |
| Two or more doses                | 31                     |
| Follow-up^5                      |                        |
| Reoperation of recurrent metastases | 5                    |
| Remission                        | 49                     |
| Recurrence or persistence        | 9                      |
| Biochemical persistence          | 16                     |
| Disease-specific mortality       | 1                      |

*In five cases histological variant was not available; ^In two cases radioiodine therapy records were not available; ^Eight cases were not classified due to short-term follow-up.

PTC, papillary thyroid cancer.

classical and follicular variant of PTC. Both patients with RAS mutation had lymph node metastases and multifocal carcinoma. The patient with HRAS mutation was diagnosed with Hodgkin’s lymphoma three years prior to diagnosis of PTC. The tumor had very invasive character; intravascular invasion and lung metastases were detected.

Clinical-pathological features of RET/PTC and BRAF-positive patients are summarized and compared in Table 4. The most common mutation was the RET/PTC rearrangement detected in 18/83 PTCs, including RET/PTC1 in 11 and RET/PTC3 in 6 samples. In single case, novel RET/PTC rearrangement named RET/PTC1ex9, which included part of exon 9 of the RET gene, was also identified (18). The ratio between females and males in RET/PTC-positive patients was lower compared to BRAF positive patients, but not statistically significant (P = 0.070). Samples with RET/PTC rearrangement were significantly associated with mix of classical and follicular variant of PTC (P = 0.009) and higher frequency of lymph node metastases (P = 0.020) and distant metastases (P = 0.005). All six RET/PTC3-positive patients had lymph node metastases, four of these six patients had lung metastases and one patient died from disease. The BRAF V600E mutation was detected in 15/83 PTC samples. In BRAF positive patients the mean age at the time of diagnosis was significantly higher than in RET/PTC positive patients (P = 0.012). The BRAF mutation also correlates with the classical variant of PTC (P < 0.001). The number of patients, who underwent reoperation of recurrent lymph node metastases, was higher, but not significantly (P = 0.073). Five patients did not respond to RAI treatment because they did not accumulate RAI. Mutations in the BRAF gene and RET/PTC rearrangements were exclusively found in malignant samples.

The TERT promoter was analyzed only in malignant samples and no mutation was found.

Detected variants in the IDH1, CHEK2 and PPM1D genes are summarized in Fig. 1 and Table 5. Variants G105G and V178I in the IDH1 gene always co-occurred and were detected with comparable frequency 9/83 in malignant and 3/30 in benign samples. The third genetic variant in the IDH1 gene was Y183C. In the CHEK2 gene R117G substitution and T367Mfs*15 deletion was identified only in PTC samples. I157T variant was the most common variant in the CHEK2 gene and was found predominantly in malignant samples. L467F variant was
Table 4  The comparison of clinical and pathological features between RET/PTC- and BRAF-positive patients.

|                | RET/PTC (n = 18) | BRAF (n = 15) | P value |
|----------------|------------------|---------------|---------|
| Patients       |                  |               |         |
| Females/males  | 10/8 (1.25:1)    | 13/2 (6.5:1)  | 0.070   |
| Age at diagnosis (mean ± s.d.) | 13.4 ± 3.6 | 16.3 ± 2.4 | 0.012   |
| Tumor size     |                  |               |         |
| Mean ± s.d. (mm)| 29.6 ± 18       | 20 ± 10.6     | 0.079   |
| Microcystoma   | 3                | 4             | 0.674   |
| Microcystoma (≤10 mm) | 1          | 1             |         |
| Histological variant |            |               |         |
| Classical      | 4                | 11            | <0.001  |
| Classical and follicular | 8          | 0             | 0.099   |
| Follicular     | 1                | 1             | 1.000   |
| Other          | 4                | 0             | 0.121   |
| Pathological characteristics |          |               |         |
| Multifocality  | 11               | 8             | 0.733   |
| Extrathyroidal extension | 12          | 6             | 0.170   |
| Intravascular invasion | 7           | 3             | 0.283   |
| T1/T2 classification | 5           | 9             | 0.085   |
| T3/T4 classification | 13          | 6             |         |
| Lymph node metastases (N) | 16          | 7             | 0.020   |
| Distant metastases (M) | 6           | 0             | 0.005   |
| Chronic lymphocytic thyroiditis | 9           | 4             | 0.284   |
| Radioiodine therapy |                  |               |         |
| Without radioiodine therapy | 2           | 2             | 1.000   |
| One dose       | 8                | 5             | 0.725   |
| Two or more doses | 8            | 7             | 1.000   |
| Follow-up\(^a\) |                  |               |         |
| Reoperation of recurrent metastases | 3           | 0             | 0.073   |
| Remission      | 10               | 6             | 0.285   |
| Recurrence or persistence | 1           | 3             | 0.295   |
| Biochemical persistence | 6           | 5             | 1.000   |
| Disease-specific mortality | 1           | 0             | 1.000   |

\(^a\)In three BRAF-positive cases and in one RET/PTC positive case histological variant was not available; \(^b\)In one BRAF-positive case radioiodine therapy record was not available; \(^c\)One BRAF-positive case was not classified due to short-term follow-up. Bold indicates statistical significance.

Table 5

| Genes          | RET/PTC (n = 18) | BRAF (n = 15) | P value |
|----------------|------------------|---------------|---------|
| KRAS           | 6                | 3             |         |
| NRAS           | 12               | 4             |         |
| HRAS           | 71               | 25            |         |
| BRAF           | 11               | 1             |         |
| IDH1           | 1                | 0             |         |
| CHEK2          | 2                | 1             |         |
| PPM1D          | 1                | 0             |         |

In our cohort, the most frequently detected MUT were in KRAS (71%), NRAS (45%), and HRAS (31%) genes. In the BRAF group, the most frequent MUT was detected in the BRAF gene (11%). The differences between malignant and benign cohorts were not statistically significant (Table 5).

Discussion

Thanks to the development of NGS and using this technique in molecular genetics laboratories, it is possible to study the precise and comprehensive genetic landscape of pediatric thyroid nodules. It helps not only to increase detection rate, but also to identify new molecular markers or cancer gene predispositions. Several years ago, only small cohorts of pediatric patients and few selected numbers of genetic alterations were investigated. Recently, studies with larger cohorts of pediatric patients have appeared with enlarging the spectrum of investigated genetic mutations using NGS panels to find mutations or gene fusions (12, 19, 20). Our study with the size of 113 samples of thyroid nodules is according to our best knowledge the largest pediatric cohort that was analyzed by NGS and provides very important genetic, clinical and pathological data. Unlike our study, in previous studies where NGS panels were also used, presented a limited number of pediatric PTC specimens that ranged from 13 to 25 (12, 19, 20). On the other hand, NGS panels included more genes (14–250) in these studies (12, 19, 20). Moreover, mutations in genes TET, TSHR (12) and CDKN2A (19) and rearrangements of ALK, NTRK1, NTRK3, PPARγ genes were revealed (12, 20), besides point mutations in the genes identified also in our study.

Mutations in RAS genes occur in malignant and benign pediatric thyroid nodules overall with very low frequency (20, 21, 22, 23). In our cohort, NRAS and HRAS mutations were detected with no other co-mutation in PTCs with aggressive character, and surprisingly, five RAS-positive samples were identified in benign lesions. Clinical impact and prognostic importance of RAS mutations is not clearly established, because they appear in follicular adenoma and also in ATC. The risk of malignancy varies among RAS genes (HRAS 92%, NRAS 74%, KRAS 61%) in adult population (24). When we consider that pediatric patients have overall higher risk of malignancy than adults, the numbers could be even higher. In our benign cohort, oncocyctic changes or uncertain behaviour were identified in HRAS and NRAS-positive samples, but not in KRAS-positive samples. RAS mutations could play a role in early cancerogenesis (25). It implies that the pediatric patients with benign lesions harboring RAS mutations should be more intensively followed.
The RET/PTC rearrangement was the most frequent mutation in our cohort of pediatric PTCs, detected in 18/83 malignant samples exclusively. The prevalence of RET/PTC in pediatric PTCs is worldwide in the range of 15–67% (7, 8, 26, 27). However, these fusion genes were also found in benign pediatric samples (28). RET/PTC is commonly associated with radiation-induced PTC, in which the prevalence is higher (7). In our study, all RET/PTC-positive patients were without radiation history. In literature, RET/PTC rearrangements do not harbor in general other driver co-mutation in the BRAF or RAS genes, which was also confirmed in our study. This co-occurrence was found only in few cases and it was associated with more aggressive disease (8, 29). RET/PTC-positive samples were associated (not statistically significant) with a higher rate of tumor growth (mostly T3/T4 classification), more frequent extrathyroidal extension, intravascular invasion and multifocality compared to BRAF-positive samples. They were significantly associated with more frequent local and distant metastases, especially in cases of RET/PTC3 rearrangement. No patient underwent reoperation due to recurrent metastases.

The BRAF V600E mutation was completely associated with PTC. In our cohort of pediatric PTCs, the prevalence of the BRAF V600E was 15/83, which falls within the range of 0–63% of the prevalence BRAF mutation in pediatric cases published earlier (7, 9, 30, 31). Possible reasons for wide dispersal of prevalence are different used methods, geographical and environmental factors, size of cohort and age border of pediatric patients. Our study also confirmed that the BRAF mutation correlates with the classical variant of PTC and the higher age of patients (32). The association of the BRAF V600E mutation with more frequent T1/T2 classification than T3/T4 classification and with higher rate of recurrence was seen, but was not statistically significant (Table 4). BRAF V600E-positive patients had more lymph node metastases reoperations due to radiorefractority than RET/PTC-positive patients, but the difference was not statistically significant.

In the IDH1 gene, the germline variants G105G and V178I in similar frequencies in patients with benign and malignant nodules were found. Clinical significance of these variants seems according to ClinVar database and SIFT/PolyPhen in silico analysis benign on thyroid tumorigenesis. The V178C variant was detected with higher frequency in benign nodules. However, this variant was assumed as deleterious/probably damaging by SIFT and PolyPhen analysis. Clinical effect of Y183C variant is still unknown. None of the previously described variants

### Table 5

| Gene     | Exon | Amino-acid | SNP ID          | Clinical significance | SIFT    | PolyPhen |
|----------|------|------------|-----------------|-----------------------|---------|----------|
| IDH1     | 4    | G105G      | rs1554637       | Benign                | Tolerated | NA       |
|          | 6    | V178C      | rs2018846       | Tolerated             | Deleterious | NA       |
| CHEK2    | 3    | R117G      | rs3489982       | Benign                | Tolerated | NA       |
|          | 11   | T367Met>His | rs28599082      | Benign                | Tolerated | NA       |
|          | 13   | L467F      | rs1459400522    | Benign                | Tolerated | NA       |
| PPM1D    | 1    | A152A      | rs34599179      | Tolerated             | Deleterious | NA       |
|          | 6    | I196V      | rs35491690      | Tolerated             | Deleterious | NA       |

*ETF: Conflict interpretations of pathogenicity. Uncertain significance/damaging/likely benign/*PolyPhen*/SIFT; Single Nucleotide Polymorphism
around codon R132 was found, where the active site of the enzyme occurs (14).

In the TCGA study PTC-causing somatic mutations in CHEK2, PPM1D and EIF1AX genes were identified. Our study revealed several variants in CHEK2 and PPM1D genes that were detected in benign as well as in malignant nodules. These variants did not correspond to those found in the TCGA study (13). All our detected variants in the CHEK2 and PPM1D genes were germline. 1157T variant in the CHEK2 gene was detected in one benign tissue and in five PTCs, which falls in the worldwide range 4.5–15.6% of PTC (33, 34, 35). It was reported that this variant increases the risk of PTC almost twice (OR = 1.81) (35). In PTCs, also other variants R117G and T367Mfs*15 in the CHEK2 gene with probably deleterious effect on protein function were detected (36). It was published that truncating variants are associated with higher risk of thyroid cancer (OR = 5.7) than 1157T missense variants (OR = 2.8) (34). In the PPM1D gene two variants were detected in our cohorts with probably benign significance on thyroid tumorigenesis. In our study, any variant in the EIF1AX gene was revealed. Frequency of mutations in this gene is generally very low and we assume according to our results, that there is probably no correlation between pediatric thyroid nodules and EIF1AX mutations. In one recent study, these novel genes were investigated in pediatric and adult cohorts with PTCs. In pediatric patients, they detected five variants in the CHEK2 gene, which were presented in all samples, two variants in the PPM1D gene and none in the EIF1AX gene. Found variants in the PPM1D gene did not match ours (37).

Any mutation in the TERT promoter in pediatric PTCs was detected. Mutations in this gene are strongly associated with the higher age of adult patients (38). In many studies of pediatric PTC, mutation in the TERT promoter has not yet been detected all over the world (9, 19), except one study. They found C228T mutation in 10-year-old patient without extrathyroidal invasion and metastases (39).

In the EZH1 gene, no mutation was found in benign and malignant specimens in our cohorts. The reason was probably a small cohort of benign samples, because mutations in the EZH1 gene were detected almost 20 times more frequently (13.5 vs 0.7%) in benign than in malignant thyroid nodules (16). Mutations in the EZH1 gene were identified only in two PTC cases in the TCGA study (13).

In summary, we revealed genetic cause in 5/30 of benign nodules and in 35/83 of pediatric PTCs; mutations in the BRAF gene, RAS genes and RET/PTC rearrangements were included. There were significant differences in clinical courses in RET/PTC and BRAF-positive PTC patients. Patients with RET/PTC had more aggressive disease (more frequent local and distant metastases) than patients with BRAF mutation. On the basis of the known genetic change in tumor, it could be possible to stratify and individualize the treatment. The role of RAS mutations, especially in benign lesions, is not yet fully understood, so it should be kept in mind that they may predispose to cancer and the intensive follow-up of these patients is recommended. Some of the identified variants in the CHEK2 gene are pathogenic or likely pathogenic and the influence of found variants in the IDH1 and PPM1D gene on thyroid nodules is still uncertain and probably benign.

The genetic landscape of more than half of our PTC cases is still unknown; therefore, further investigation is required, for example in PTEN or PIK3CA genes, in which somatic mutations in pediatric PTCs were detected (22). Furthermore, other fusion genes need to be identified by RNA sequencing, and copy number variants should also be detected, which could be another trigger of thyroid cancer. The genetic molecular testing seems to be the benefit for pediatric patients for their diagnosis and prognosis. Hopefully, it will improve the quality of life of pediatric patients with thyroid nodules.

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

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