Search for New Genetic Biomarkers in Poorly Differentiated and Anaplastic Thyroid Carcinomas Using Next Generation Sequencing

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Abstract. *Background: Poorly differentiated thyroid carcinoma (PDTC) and anaplastic thyroid carcinoma (ATC) are very rare tumors with extremely aggressive behavior. Their comprehensive genetic background is still unclear. Some of the main genetic changes of differentiated thyroid carcinomas, such as mutations in BRAF and RAS genes, as well as changes in CTNNB1, PIK3CA, TP53, AXIN1, PTEN or APC genes leading to the dedifferentiation of the tumors, are described. Materials and Methods: DNAs from fresh frozen thyroid tissues of 3 PDTCs and 5 ATCs were extracted. The next-generation sequencing (NGS) approach was used to target 94 genes involved in cancer. The samples were prepared using a TruSight Cancer panel and sequenced with a MiSeq sequencer. Analysis of variants was performed by the MiSeq Reporter and NextGENe software and stringent criteria for prioritization of the variants were used in the Illumina VariantStudio software. Results: Using NGS, we identified 26 genetic changes in 18 genes, novel variants included. Conclusion: NGS is a useful tool for searching for new variants and genes involved in PDTC and ATC. It seems that each of these rare tumor types has its own specific genetic background. These data could be helpful for recognizing new genetic markers and targets for future personalized therapy.*

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Key Words: Anaplastic thyroid carcinoma, poorly differentiated thyroid carcinoma, genetics, next-generation sequencing.

Thyroid carcinomas represent the most common endocrine malignancy and could be classified according to the origin of their tumor cells. Well-differentiated thyroid carcinomas (DTC)–papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC), as well as more aggressive carcinomas–poorly differentiated thyroid carcinoma (PDTC) and anaplastic thyroid carcinoma (ATC), arise from follicular thyroid cells. Medullary thyroid carcinoma (MTC) is derived from parafollicular C-cells.

PDTC and ATC are extremely rare and very aggressive tumors with a prevalence of about 1-2% of all thyroid cancer. Both can develop from normal follicular thyroid cells or by dedifferentiation from DTC, which is supported by the presence of well-differentiated areas and specific genetic changes for DTC (1, 2).

PDTC is characterized by partial dedifferentiation and usually more aggressive behavior than DTC and less aggressive behavior than ATC. Morphologically, it is an intermediate entity between well-differentiated and undifferentiated thyroid carcinomas. PDTC was originally

introduced by Sakamoto *et al*. in 1983 (3) but characterized as a separate entity in 2004 by the World Health Organization (WHO) as "follicular-cell neoplasms that show limited evidence of structural follicular cell differentiation and occupy both morphologically and behaviorally an intermediate position between differentiated (follicular and papillary carcinomas) and undifferentiated (anaplastic carcinomas)". The diagnostic algorithm was suggested in 2006 at an international conference in Turin by "a solid/trabecular/insular pattern of growth, absence of conventional nuclear features of papillary carcinoma and presence of at least one of the following features: convoluted nuclei, mitotic activity (≥3×10 HPF), necrosis" (4).

Anaplastic carcinoma is a rapidly growing tumor with massive extrathyroidal extension and distant metastases, and represents the most aggressive thyroid tumor, with a median overall survival of about six months. It is one of the most lethal human cancers due to incomplete surgical resection or inoperability. ATC is often identified in older patients with longstanding goiters or incompletely treated DTC. In younger patients (<50 years), ATC occurs rarely and patients have a slightly more favorable prognosis, with longer survival (5). ATC rarely responds to the conventional treatment and, due to dedifferentiation, is resistant to radioiodine therapy. Consequently, a multimodality treatment, as well as novel therapeutic approaches, are needed. Molecular targeted therapy is the most promising; however, the genetic background of the carcinoma is important for this purpose.

Several genetic changes which play a role in the pathogenesis of PDTC and ATC have been described. Multiple mutations in oncogenes and tumor-suppressor genes, as well as copy number variants, aberrant microRNA expression and epigenetic changes, are usually detected in PDTC and ATC (6).

Mutations in the *BRAF* and *RAS* genes are the main genetic changes of DTC and are considered to be early genetic events contributing to its dedifferentiation into PDTC and ATC (7, 8). The *BRAF* gene encodes a serin-threonine protein kinase and is aberrantly activated by the point mutation in the exon 15 at codon 600 resulting in the substitution Val600Glu (9). This is the most common genetic change in PTC and an important prognostic marker associated with poorer prognosis and higher mortality and recurrence rates (10, 11).

On the other hand, the *RET*/*PTC* rearrangements frequently detected in PTChave only been detected in a few ATC cases but some of them also contained PTC tissues (12) and, thus, it is assumed no progress occus from *RET*/*PTC*-rearranged PTC towards PDTC and ATC (13, 14).

Mutations in a family of *RAS* oncogenes (*H*-, *K*- and *NRAS* genes) are detected mainly in tumors with follicular morphology, such as follicular adenoma, follicular carcinoma and the follicular variant of PTC and, surprisingly, they were also found in sporadic *RET*-negative MTC (15). *RAS* mutations occur in the GTP-binding domain (at codons 12 and 13), leading to higher affinity to GTP or the GTPase domain (at codon 61) and resulting in the failure of GTPase function. Both types of mutations cause the stabilization of the active form of protein and the continuous stimulation of signaling pathways, mainly MAPK and PI3K/AKT/mTOR.

Recently, mutations 1 295 228 C>T and 1 295 250 C>T (named C228T and C250T) in the promoter of the gene for telomerase reverse transcriptase (*TERT*) were reported in thyroid cancer, particularly in aggressive types, such as ATC, PDTC and PTC with more aggressive behavior (16). Coexistence of *BRAF* Val600Glu and *TERT* C228T mutations has been reported to lead to higher aggressiveness of PTC than each of these mutations alone (17).

Several specific genetic changes leading to the development of PDTC and ATC, probably due to dedifferentiation of the follicular cells, such as mutations in β-catenin (*CTNNB1*), *PIK3CA*, *TP53*, *AXIN1*, *PTEN* or *APC*, were also described (6). However, PDTC and ATC are a heterogenic group with an unclear genetic background. For this purpose, next-generation sequencing (NGS) is a promising methodology for enabling analysis of multiple genes in multiple sample libraries. From these extensive data, it will be possible to identify new genes and variants involved in the development of PDTC and ATC.

Materials and Methods

Sample cohort. Fresh frozen tissues of three PDTCs and five ATCs were analyzed. The thyroid tumor samples were surgically removed at the Department of Otorhinolaryngology and Head and Neck Surgery, First Faculty of Medicine, Charles University in Prague and Motol University Hospital in Prague. The samples were collected and histologically evaluated based on the WHO Classification of Tumors and on the consensus conference in Turin by an experienced pathologist at the Department of Pathology and Molecular Medicine, Second Faculty of Medicine, Charles University in Prague and Motol University Hospital in Prague. All the specimens were snap-frozen and stored at –80˚C until used. Patients' anamnesis and clinical-pathological characteristics were collected. Patients signed an informed consent for genetic studies and the study was approved by the local Ethics Committee.

DNA extraction. DNA extraction from fresh frozen samples was performed using the AllPrep DNA/RNA/Protein Mini kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's instructions. Concentration of DNA and purity was measured using a spectrophotometer (NanoPhotometer P330; Implen GmbH, Munich, Germany).

Sanger sequencing. The *BRAF* mutation Val600Glu was analyzed separately by Sanger sequencing because of its absence in the TruSight Cancer Sequencing Panel (Illumina). Extracted DNA was amplified to generate a 224 bp sequence of *BRAF* exon 15 using

Table I. *Mutations detected using the TruSight Cancer panel in poorly differentiated thyroid carciNoma (PDTC) and anaplastic thyroid carciNoma (ATC) patients.*

Patient[#]	Histology	Gene	Chromosome	Nucleotide change	Protein change	Conserved	Variant	Sift/PolyPhen	dbSNP ID	COSMIC	ClinVar
A (62, F)	PDTC	*CDH1*	16	c.2413G>A	p.Asp805Asn	Yes	MS	T/PD	rs200894246	No	Yes
		FANCD2	3	c.2803A>C	p.Ile935Leu	Yes	MS	T/PsD	rs61751578	No	No
		CHEK2	22	c.1550G>A	p.Arg517His	Yes	MS	D/PD	rs121908706	No	Yes
B (76, F)	PDTC	*ADH1B*	4	c.142C>A	p.His48Asn	No	MS	D/B	No	No	No
		GPC3	X	c.160G>T	p.Glu54Ter	Yes	SG	NA	No	No	No
		TP53	17	c.673-2A>G	NA	No	SAV	NA	No	Yes	No
C (65, F)	PDTC	*TP53*	17	c.861_871delGAATCTCCGCA	p.Asn288GlufsTer14	Yes	DEL	NA	NA	No	NA
		PTEN	10	c.950_953delTACT	p.Thr319Ter	Yes	INS	NA	NA	Yes	NA
D (62, F)	ATC	*ATM*	11	c.8977C>T	p.Arg2993Ter	Yes	SG	NA	No	Yes	No
		HNF1A	12	c.481G>A	p.Ala161Thr	Yes	MS	D/PsD	rs201095611	No	No
		MET	7	c.1579A>G	p.Ser527Gly	Yes	MS	T/PsD	No	No	No
		NF1	17	c.7117C>T	p.Gln2373Ter	Yes	SG	NA	No	No	No
		TP53	17	c.845G>A	p.Arg282Gln	Yes	MS	D/PD	No	Yes	No
		PTEN	10	c.45_46insT	p.Tyr16LeufsTer28	Yes	INS	NA	NA	Yes	NA
		MSH2	2	c.747delG	p.Gly250GlufsTer4	Yes	DEL	NA	NA	No	NA
		RB1	13	c.865delA	p.Asn290MetfsTer11	Yes	DEL	NA	NA	No	NA
E (65, F)	ATC	*NBN*	8	c.511A>G	p.Ile171Val	Yes	MS	T/PD	rs61754966	No	Yes
		NF1	17	c.7895A>G	p.Asp2632Gly	Yes	MS	D/PD	No	No	No
		TP53	17	c.910delA	p.Thr304LeufsTer41	No	DEL	NA	NA	Yes	NA
F (88, F)	ATC	*ATM*	11	c.4424A>G	p.Tyr1475Cys	Yes	MS	T/PsD	rs34640941	Yes	No
		MUTYH	1	c.1276C>T	p.Arg426Cys	Yes	MS	D/B	rs150792276	Yes	Yes
		TP53	17	c.749C>T	p.Pro250Leu	Yes	MS	D/PD	No	Yes	No
		TSC2	16	c.1292C>T	p.Ala431Val	Yes	MS	D/PD	rs202187148	No	No
G (67, M)	ATC	*HRAS*	11	c.181C>A	p.Gln61Lys	Yes	MS	D/B	rs28933406	Yes	Yes
		TP53	17	c.455dupC	p.Pro153AlafsTer28	Yes	INS	NA	NA	Yes	NA
H (78, F)[*]	ATC	*EGFR*	7	c.2024G>A	p.Arg675Gln	Yes	MS	D/PD	rs150423237	No	No

SIFT: T, Tolerated; D, deleterious; PolyPhen: B, benign; PsD, possible damaging; PD, probably damaging; MS; missense variant; SG, stop gained variant; SAV, splice acceptor variant; DEL, deletion; INS, insertion; NA, Not available; [*]patient with *BRAF* Val600Glu mutation; [#]age in years, sex.

Table II. *Genes altered in PDTC and ATC patients.*

Gene	Chromosome	Description of gene
ADH1B	4	Alcohol dehydrogenase 1B (class I), beta polypeptide
ATM	11	Ataxia telangiectasia mutated
CDH1	16	Cadherin 1, type 1, E-cadherin (epithelial)
EGFR	7	Epidermal growth factor receptor
FANCD2	3	Fanconi anemia, complementation group D2
GPC3	X	Glypican 3
HNF1A	12	HNF1 homeobox A
HRAS	11	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
CHEK2	22	Checkpoint kinase 2
MET	7	Met proto-oncogene (hepatocyte growth factor receptor)
MSH2	2	MutS homolog 2
MUTYH	1	MutY homolog
NBN	8	Nibrin
NF1	17	Neurofibromin 1
PTEN	10	Phosphatase and tensin homolog
RB1	13	Retinoblastoma 1
TP53	17	Tumor protein p53
TSC2	16	Tuberous sclerosis 2

polymerase chain reaction (PCR) with primer pairs 5'-tcataatgcttg ctctgatagga-3' and 5' ggccaaaaatttaatcagtgga-3' (18). Thirty ng DNA were amplified with 0.1 μM of each primer, 160 μM deoxynucleotide triphosphates, 2 mM $MgCl_2$, 0.15 U Gold AmpliTaq Perkin Polymerase, 1×PCR Buffer II (10 mM Tris-HCl pH 8.3, 50 mM KCl) in a final volume of 15 μl. Cycling conditions were: denaturation step at 94˚C for 2 min and at 95˚C for 20 followed by 5 cycles of denaturation at 95˚C for 20, annealing at 62.5˚C for 30, primer extension at 72˚C for 1 min, 35 cycles of denaturation at 95˚C for 20, annealing at 57.5˚C for 30, primer extension at 72˚C for 1 min and one final run-off extension at 72˚C for 10 min. All PCR products were visualized by electrophoresis in 1.5% agarose gel in 0.5×TBE buffer. PCR products were purified using Agencourt AMPure (Beckman Coulter, Fullerton, CA, USA) and sequenced using the same forward primers of the PCR amplification and the DTCS Quick Start Master Mix (Beckman Coulter) by the DNA sequencing machine (CEQ8000; Beckman Coulter). The cyclesequencing conditions consisted of 30 cycles of 96˚C for 20 sec, 50˚C for 20 and 60˚C for 4 min.

Next-generation sequencing. The TruSight Cancer Panel (Illumina, San Diego, CA, USA) is an enrichment system targeted to more than 1,700 exons of 94 genes associated with various types of cancer (http://products.illumina.com/products/trusight_cancer.html). Libraries were generated using TruSight Rapid Capture along with the TruSight Cancer Sequencing Panel (Illumina) according to the manufacturer's sample preparation protocol. Briefly, 50 ng of each DNA sample was enzymatically fragmented and adapter sequences to the ends were added. The fragmentated DNA was purified from each patient's sample and barcodes and common adapters required for cluster generation and sequencing were added. The fragmented DNA was amplified by PCR followed by purification. Five hundred nanograms of each DNA library was pooled using up to six different barcodes into one pool. Then the libraries were twice hybridized to capture probes specific to the targeted region; the unhybridized material was washed away and the captured fragments were amplified using PCR followed by purification. The enriched libraries were quantified using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) and the quality was evaluated using a Bioanalyzer 2100 and High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA, USA). Libraries with a molarity 4 μM were subjected to cluster generation on flow cell and paired-end sequencing for 300 cycles by a MiSeq Reagent Kit v2. in a MiSeq sequencer platform (Illumina) was performed.

Bioinformatics. Sequence data were analyzed by the on-instrument software MiSeq Reporter v.2.4. (Illumina). The data were demultiplexed and FASTQ files were generated. Reads from pairend fragments were trimmed for low-quality and duplicate reads. The MiSeq Reporter software aligned the reads against the human reference sequence GRCH37.p5/hg19 using a Burrows-Wheeler Aligner (BWA). The variant calling for targeted regions were performed using a Genome Analysis Toolkit (GATK, Broad Institute, Cambridge, MA, USA).

Secondary analysis of the FASTQ data was performed by the NextGENe 2.3.4.3. (Softgenetics, State College, PA, USA). The NextGENe software uses a Preloaded Index Alignment algorithm. This algorithm employs the Burrows-Wheeler Transform (BWT). The first step was conversion of the MiSeq FASTQ files to the NextGENe-converted FASTA files. Additional filtres were not used. This means all sequencing data were included. The converted FASTA files were aligned by the NextGENe software to the reference sequence hg19 using paired reads and the following filters: matching requirement ≥12 bases and ≥85%; ambiguous mapping, detection of large indels were selected. The mutation report generated by the NextGENe software contains only variants from regions of interest.

Only variants detected by both MiSeq Reporter and NextGENe were considered. The resulting VCF files were analyzed using the Variant Studio Data Analysis v.2.2. software (Illumina). Only data from regions of genes of interest and with a coverage higher than 10 were annotated. The detected single-nucleotide variants (SNV) and small insertions/deletions (indels) were annotated and filtered for exonic non-synonymous variants, variants with a probable splice site or frameshift effect. The variants were annotated in genetic databases: the Single Nucleotide Polymorphism Database (dbSNP) (http://www.ncbi.nlm.nih.gov/ projects/ SNP/), Catalogue of Somatic Mutations in Cancer (COSMIC) (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/) and ClinVar database (http://www.ncbi.nlm.nih.gov/clinvar/) (19). The prediction of functional pathogenic effects of the missense variants to the protein structure and function were predicted in silico by PolyPhen-2 (20) and SIFT (21) and only variants in one of the software packages called as being damaged or deleterious were prioritized for further analysis. The missense variants called as both benign and tolerated in PolyPhen-2 and SIFT software were excluded, as well as variants having a frequency higher than 1% in all populations from the 1000 Genomes data project (22) and EVS (Exome Variant Server) from the NHLBI exome sequencing project (http://evs.gs.washington.edu/EVS/). The variants with altered allele depth ≤50 reads and a variant allelic frequency ≤5% were eliminated from the analysis. All variants were verified by visualization with an Integrative Genomics Viewer (23).

Results

The *BRAF* Val600Glu mutation was detected only in patient H; all other patients were *BRAF*-negative.

The TruSight Cancer Panel was applied to screen mutations in 94 cancer genes in our cohort of three PDTC and five ATC patients. In all samples, an adequate library for sequencing was obtained. Sequencing generated a minimum of 1.7×10^6 reads in all tumors with a median read length of 208 bp. Twenty-six genetic changes were identified applying the prioritization strategy described above. The majority (73%) of the identified somatic mutations was SNVs (15 missense, 3 nonsense variants and 1 splice acceptor variant), whereas indels were in a minority (27%) (Table I). The variants were found in 18 genes described in Table II. The average coverage of base site was 667, with a range from 172 to 2,580. The mean read depth of altered alleles was 338, ranging from 85 to 1,094.

Most of the variants (88%) were located in the conserved sequences. Fifty-two percent of SNVs were indexed in the dbSNP database, 36% were listed in the COSMIC catalog and 15% in the ClinVar database. Twenty-six percent of SNV were not reported in these databases. Fifty-seven percent of indels were listed in the COSMIC catalog.

Detected missense mutations and indel variants in each patient are summarized in Table I. All detected and filtered variants were unique and all patients had more than one genetic change in tumor tissue. Almost all, except two, patients had a mutation, insertion or deletion in the *TP53* gene.

Discussion

The genetic background of PDTC and ATC is very complicated and many genes are involved in the process of dedifferentiation of follicular cells, which it comprises activation of oncogenes, as well as inactivation of tumor-suppressor genes.

Some PDTCs and ATCs are suggested to dedifferentiate from DTC and these tumors still carry the genetic changes from the original PTC or FTC, such as, for example, the *BRAF* or *RAS* mutation. In our set of tumors, *BRAF* was mutated in one ATC patient and *HRAS* mutation was detected in another ATC patient. This suggests that these tumors may be derived from DTC by dedifferentiation. Because *BRAF* is not reported as being mutated in FTC (7), it is likely that the *BRAF*-mutated ATC was derived from *BRAF*-mutated PTC, although the specimen did not contain papillary components. The other genetic change in this *BRAF*-positive ATC is in the *EGFR* gene, which could be implied in the transformation to ATC. *EGFR* encodes a transmembrane receptor for epidermal growth factor. Strong *EGFR* over expression (24) and copy number gain in 46% of ATCs was reported (12). Mutations in *EGFR* are associated with various carcinoma types. Missense mutation Arg675Gln identified in this patient has been evaluated as deleterious and probably damaging in SIFT and PolyPhen, respectively.

In one ATC patient, the *HRAS* mutation we detected. *RAS* mutations are commonly found in ATC and it is assumed that they can predispose to dedifferentiation of DTC toward ATC (8). However, these mutations are also detected in follicular carcinoma or in follicular variant of PTC (25). Therefore, it is difficult to identify the origin of this ATC. A pathological evaluation did not find differentiated parts in the sample. Besides *HRAS* mutation, insertion of the *TP53* gene was revealed in this tumor, which probably contributed to dedifferentiation to ATC.

In total, NGS analysis after the stringent filter identified 26 variants in 18 genes. Some of the genes are known to be associated with PDTC or ATC. The most commonly mutated gene in our cohort was *TP53* (23% of all detected variants). In our set of tumors, two missense mutations, one splice site variant and three indels resulting in frameshift were detected in six out of eight patients (75% of samples). Except for Thr304LeufsTer41deletion and 673-2A>G splice site variant, all are located in conserved sequences. The missense mutations Arg282Gln and Pro250Leu and frameshift indels Thr304LeufsTer41 and Pro153AlafsTer28 are reported in the COSMIC database in various types of cancer. *TP53* encodes a tumor-suppressor gene involved in regulation of the cell cycle, DNA damage repair and apoptosis. Generally, *TP53* is the most frequently mutated gene in human cancer. *TP53* mutations are purported to be late genetic changes in thyroid cancer and probably contribute to the progression of DTC to PDTC and ATC. Overall, mutations in *TP53* are detected in about 26% of PDTCs and in more than 60% of ATCs (26-28).

Several variants in genes that normally play the role of tumor-suppressor were identified. These mutations could lead to loss of their controlled function. In patient A, an Asp805Asn mutation in the *CDH1* gene was found that is listed in the dbSNP database and in ClinVar with an uncertain significance. *CDH1* encodes adhesion protein cadherin 1, type 1, E-cadherin, and its reduced expression in PDTC and ATC compared to DTC was described (29, 30). Mutations in *CDH1* are detected in many cancer types and loss of function of *CDH1* is thought to increase proliferation, invasion and metastasis of cancer cells. The other detected tumor-suppressor gene, *PTEN,* encodes a phosphatidyl-inositol-3,4,5-trisphosphate- 3-phosphatase. It negatively regulates the intracellular level of phosphatidylinositol-3,4,5-trisphosphate in cells and, as a tumor suppressor, also negatively regulates the AKT/PKB signaling pathway. Mutations in *PTEN* were reported in 16% of ATCs (12). A Thr319Ter deletion and a Tyr16LeufsTer28 insertion in patients C and D were identified; both are listed in the COSMIC catalogue in other cancer types. In patient F, an Ala431Val variant in the *TSC2* tumor-suppressor gene was

found. It encodes a negative regulator of mTOR. A nonsense mutation was newly detected by whole exome sequencing in one ATC patient (31).

On the other hand, some genes and their activating mutations may promote tumorigenesis. *HNF1A* encodes a hepatocyte nuclear factor expressed mainly in liver cells. One study showed its high expression in ATC (32). An Ala161Thr substitution in patient D was detected. The *MET* protooncogene encodes the transmembrane tyrosine kinase receptor for hepatocyte growth factor HGF. *MET* is over expressed in thyroid carcinomas and is associated with a high risk for metastasis and recurrence in children and young adults with PTC (33). Copy number gain was observed in 12% of ATCs (12). Mutation Ser527Gly, called as tolerated in SIFT, but possibly damaging in PolyPhen, was found in patient D.

Other altered genes, *ATM*, *CHEK2* and *MUTYH*, have not yet been reported with PDTC or ATC but polymorphisms or aberrant expressions are associated with predisposition to DTC (34-37). All these genes are involved in DNA damage response and DNA repair. Nonsense and missense variants were found in our patients; all were reported in dbSNP, COSMIC or ClinVar in association with other cancer. Deletion Gly250GlufsTer4 in the *MSH2* gene (not reported in COSMIC) was detected in patient D. The gene encodes DNA mismatch repair protein. Expression of this gene was reported to be higher in malignant than in benign thyroid tumors (38). Glypican 3 (*GPC3*) has been reported to be up-regulated in thyroid cancer, mainly in FTC and PTC, but not expressed in ATC and, thus, it is supposed that *GPC3* expression is an early event in thyroid cancer (39). For this gene, the nonsense variant Glu54Ter was identified in patient B.

Furthermore, variants in other genes that are not presently associated with follicular cell-derived thyroid cancer were found. The *NF1* gene encodes a negative regulator of the RAS signaling pathway. A nonsense Gln2373Ter and a missense mutation Asp2632Gly in patients D and E were detected that have not been reported before. Genes *FANCD2* and *NBN* are involved in DNA repair. The detected missense mutation Ile935Leu for the *FANCD2* gene in patient A is reported in the dbSNP database. The variant Ile171Val for the *NBN* gene in patient E is reported additionally in ClinVar with controversial clinical impact. *RB1* encodes a negative regulator of the cell cycle when DNA is damaged. Deletion Asn290MetfsTer11 in patient D was found. The protein encoded by the *ADH1B* gene is an alcohol dehydrogenase. Missense variant His48Asn was detected in patient B. NGS is a high-throughput method that allows screening of a wide range of genes. In aggressive thyroid cancers it has been used only by Nikiforova *et al.*for target sequencing of 12 cancer genes and three gene fusions (40) and whole exome sequencing of one ATC patient by Wagle *et al.* (31). Using NGS, we were able to identify mutations that had not been reported previously: non-synonymous variants in genes *ADH1B, GPC3, MET, NF1* and deletions in the *MSH2* and *RB1* genes. All of them, except for the variant in the *ADH1B* gene, are located in conserved sequences. Other mutations have been listed in dbSNP databases, but with very rare frequency in COSMIC in other types of cancer or in ClinVar, mostly with an uncertain significance. Of course, this is not a complete list of all mutated genes because this panel tested 94 known cancer genes. We were also not able to detect larger gene or chromosomal changes, rearrangements and fused genes. Thus, we are not able to say if these described variants definitively represent all changes in the studied tumor tissues. We also cannot distinguish the detected genetic changes from germline mutations due to missing peripheral blood samples and the impossibility of obtaining them due to aggressive tumor behavior and death of patients from their disease. Nevertheless, using our very stringent bioinformatic pipeline, two software packages for variant analysis and predictive tools, false-positive calls of somatic mutations were most probably avoided. Additionally, any possible polymorphisms were excluded and only variants with a frequency less than 1% in the 1000 Genome project and EVS databases and possible benign mutations were retained when only non-synonymous variants or frameshift variants were prioritized.

To the best of our knowledge, this is the first comprehensive study of large targeted NGS analysis aimed at screening the mutational status of cancer-associated genes in PDTC and ATC. Our comprehensive genetic analysis revealed that each patient with these rare tumors has specific genetic changes, probably due to a different cause, different damage and/or different stage of dedifferentiation. These results could be added to the worldwide data on genetic biomarkers of PDTC and ATC and used in individualized molecular targeted therapy in the future.

Acknowledgements

This study was supported by the grant IGA MH CR NT/13901-4 and MH CZ-DRO (Institute of Endocrinology-EU, 00023761).

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Received December 15, 2014
Revised January 14, 2015
Accepted January 16, 2015