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ORIGINAL ARTICLE

Genetic Alterations in Hungarian Patients with Papillary Thyroid Cancer

Bálint Tobiás[1] · Csaba Halászlaki[1] · Bernadett Balla[1] · János P. Kósa[1,5] · Kristóf Árvai[5] · Péter Horváth[1] · István Takács[1] · Zsolt Nagy[1] · Evelin Horváth[1] · János Horányi[2] · Balázs Járay[3] · Eszter Székely[3] · Tamás Székely[3] · Gabriella Győri[4] · Zsuzsanna Putz[1] · Magdolna Dank[1] · Zsuzsanna Valkusz[6] · Béla Vasas[7] · Béla Iványi[7] · Péter Lakatos[1]

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Abstract The incidence of thyroid cancers is increasing worldwide. Some somatic oncogene mutations (*BRAF*, *NRAS*, *HRAS*, *KRAS*) as well as gene translocations (*RET*/*PTC*, *PAX8*/*PPAR-gamma*) have been associated with the development of thyroid cancer. In our study, we analyzed these genetic alterations in 394 thyroid tissue samples (197 papillary carcinomas and 197 healthy). The somatic mutations and translocations were detected by Light Cycler melting method and Real-Time Polymerase Chain Reaction techniques, respectively. In tumorous samples, 86 *BRAF* (44.2 %), 5 *NRAS* (3.1 %), 2 *HRAS* (1.0 %) and 1 *KRAS* (0.5 %) mutations were found, as well as 9 *RET*/*PTC1* (4.6 %) and 1 *RET*/*PTC3* (0.5 %) translocations. No genetic alteration was seen in the non tumorous control thyroid tissues. No correlation was detected between the genetic variants and the pathological subtypes of papillary cancer as well as the severity of the disease. Our results are only partly concordant with the data found in the literature.

Keywords Papillary thyroid cancer · *BRAF* · RAS · *RET*/*PTC* · Mutation

Abbreviations

BRAF	v-raf murine sarcoma viral oncogene homolog B1
EGFR	Epidermal growth factor receptor
FNA	Fine needle aspiration
FNAB	Fine needle aspiration biopsy
FVPTC	Follicular variant papillary carcinoma
HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
MAPK	Mitogen-activated protein kinase
PAX8/PPAR-gamma	Paired box8/peroxisome proliferator-activated receptor gamma
PTC	Papillary thyroid carcinoma
PTMC	Papillary thyroid microcarcinoma
RAS	Rat sarcoma viral oncogene homolog
RET/*PTC*	RET tyrosine-kinase proto-oncogene/ papillary thyroid carcinoma

Introduction

Thyroid nodules are very common in clinical practice. Although these nodules are benign in most of the cases, approximately 5 % are malignant. The number of detected thyroid cancers has dramatically increased during the last decades. Their incidence highly depends on the diagnostic

Bálint Tobiás and Csaba Halászlaki equally contributed to this work.

✉ Bálint Tobiás
tobias.balint@med.semmelweis-univ.hu

[1] 1st Department of Internal Medicine, Semmelweis University, Korányi S. Str. 2a, Budapest 1083, Hungary

[2] 1st Department of Surgery, Semmelweis University, Budapest, Hungary

[3] 2nd Department of Pathology, Semmelweis University, Budapest, Hungary

[4] Department of Radiology and Oncotherapy, Semmelweis University, Budapest, Hungary

[5] PentaCore Laboratory, Budapest, Hungary

[6] Department of Medicine, University of Szeged, Szeged, Hungary

[7] Department of Pathology, University of Szeged, Szeged, Hungary

methods, as well. In Hungary (population is 10 millions), approximately 750 new thyroid cancer cases are recorded annually by National Cancer Registry.

Thyroid epithelial tumors exhibit a broad spectrum of neoplastic pathology varying from well-differentiated benign tumors to highly malignant anaplastic carcinomas. The geographical differences in the incidence of thyroid carcinomas are partly related to the variation in dietary iodine intake. In iodine-deficient areas, the rate of malignant papillary transformation is higher compared to the follicular ones [1, 2]. The incidence of undifferentiated cancer is also higher in iodine-deficient areas [3, 4] Hungary is generally considered as a heavily iodine-deficient country, such as the UK, Belgium and Poland [3, 5–8].

Papillary cancer (PTC) is the most common histological type of all thyroid malignancies (60–80 %). Somatic mutations are found in more than 40–70 % of papillary carcinoma cases. The frequencies of *BRAF* (v-raf murine sarcoma viral oncogene homolog B1) mutation are 40–45 %, RAS (rat sarcoma viral oncogene homolog) mutations are 10–20 %, *RET*/*PTC* (RET tyrosine-kinase proto-oncogene/papillary thyroid carcinoma) rearrangements are 10–20 % in case of PTC [1, 9–11].

Recent advances in molecular genetics of thyroid cancer can be applied to develop new diagnostic markers for fine needle aspiration (FNA) samples [12]. PTC frequently carries *BRAF* and/or RAS mutations as well as *RET*/*PTC* rearrangements and some of them are associated with unfavorable outcome [12–16]. *BRAF* mutation has been associated with more aggressive tumor behavior, such as extrathyroidal extension, lymph node involvement, resistance to radioactive iodine, and tumor recurrence [11, 15–19].

In the present study, we planned to investigate the frequency of somatic mutations of *BRAF*, *HRAS*, *NRAS* and *KRAS* genes and the rearrangements of *RET*/*PTC1*, *RET*/*PTC3* and PAX8/PPAR-gamma both in cancerous and tumor free control thyroid tissues of Hungarian subjects. We also aimed to examine the correlation between the genetic variants and the subtypes of PTC as well as the severity of the disease.

Materials and Methods

Thyroid Tissue Samples

We obtained two types of samples: intraoperative fresh frozen and formalin-fixed paraffin-embedded samples. The intraoperative samples were collected from consecutive patients at the 1st Department of Surgery, Semmelweis University, between 2010 and 2014. The paraffin-embedded tissue blocks were received from the archives of the 2nd Department of Pathology, Semmelweis University as well as the Department of Pathology, University of Szeged and the National Institute of Oncology. Altogether, we examined 394 thyroid tissue samples (197 malignant and 197 control of the same subjects). The histological results were confirmed by two independent pathologist, who selected the tumorous and tumor free place in each samples for further analysis for us. The study protocol was reviewed and approved by the Ethic Committee (ETT-TUKEB 1160–0/2010-1018EKU). Patients gave informed consent.

Nucleic Acid Isolation

The thyroid tissues were stored in −72 °C after surgery until processing or were paraffin-embedded. The first step was comminution in phosphate-buffered saline (PBS) with Fisher Scientific PowerGen 125 tissue grinder (Fisher Scientific GmbH, Germany) when processing the intraoperative tissue samples. Genomic DNA was isolated using Roche High Pure PCR template Preparation Kit (Roche, Indianapolis, IN, USA). Total RNA was separated by Roche High Pure RNA Isolation Kit (Roche) from intraoperative tissue samples. From paraffin-embedded tissue samples, genomic DNA was obtained by Roche High Pure PCR template Preparation Kit (Roche), while total RNA was isolated by Roche High Pure RNA Paraffin Kit (Roche, Indianapolis, IN, USA). Quantification of isolated DNA and RNA was assessed by NanoDrop spectrophotometer (Nanodrop Technologies, Montchanin, DE, USA). DNA and RNA isolation was successful from all samples.

Detection of Point Mutations

We used a slightly modified protocol described by Nikiforov et al. [12] for the detection of genetic alterations. Briefly, the genomic DNA was tested for *BRAF* codon 600 (rs113488022), *NRAS* codon 61 (rs79057879), *HRAS* codon 61 (rs28933406), *KRAS* codons 12 and 13 (rs121913535) point mutations using real-time PCR and fluorescence melting curve analysis (Roche Light Cycler 2.0 Instrument, Roche Instrument Center AG, Rotkreuz, Switzerland). Amplification was performed using 20–50 ng of genomic DNA, 40–40 pmol of each primer (TIB MOLBIOL, Berlin, Germany), 2–2 pmol of each hybridization probe (TIB MOLBIOL), 1.5 μl water, 0.5 μl bovine serum albumin (10 mg/ml, Sigma-Aldrich, St. Louis, MO, USA) and 5 μl JumpStartTaq ReadyMix PCR polymerase (Sigma-Aldrich). The reaction mixture was subjected to 60 cycles of PCR amplification consisting of denaturation at 95 °C for 5 s, annealing at 54 °C for 20 s, and extension at 72 °C for 12 s. Post-amplification fluorescence melting curve analysis was performed by gradual heating of samples at a rate of 0.1 °C/s from 45 °C to 95 °C. Fluorescence melting peaks were built by plotting of the negative derivative of fluorescent signal corresponding to the temperature (−dF/dT). The sensitivity

of mutation detection by melting curve analysis was 10 % of cells with a mutant allele in the background of normal cells, as established by serial dilutions of the positive controls (Fig. 1).

Detection of Rearrangements

PAX8ex7 and PAX8ex9/PPAR-gamma, *RET*/*PTC1* and *RET*/*PTC3* rearrangements were detected on RNA by RT-PCR ABI Prism 7500 (LT, Foster City, CA, USA) with primers designed to flank the respective point. Reverse transcription was performed using 200 U SuperScriptIII RNase H-reverse transcriptase (Invitrogen Life Technologies, Carlsbad, California, USA), 40 U RNaseOUT Ribonuclease Inhibitor (Invitrogen Life Technologies) and 2 μl random primer (Promega, Madison, WI, USA) and 250–300 ng RNA at 37 °C for 1 h. The PCR reactions were amplified in 20 μl volume using 2 μl cDNA, 10 μl TaqMan 2× Universal PCR Master Mix NoAmpErase UNG (Life Technologies), 40 pmol

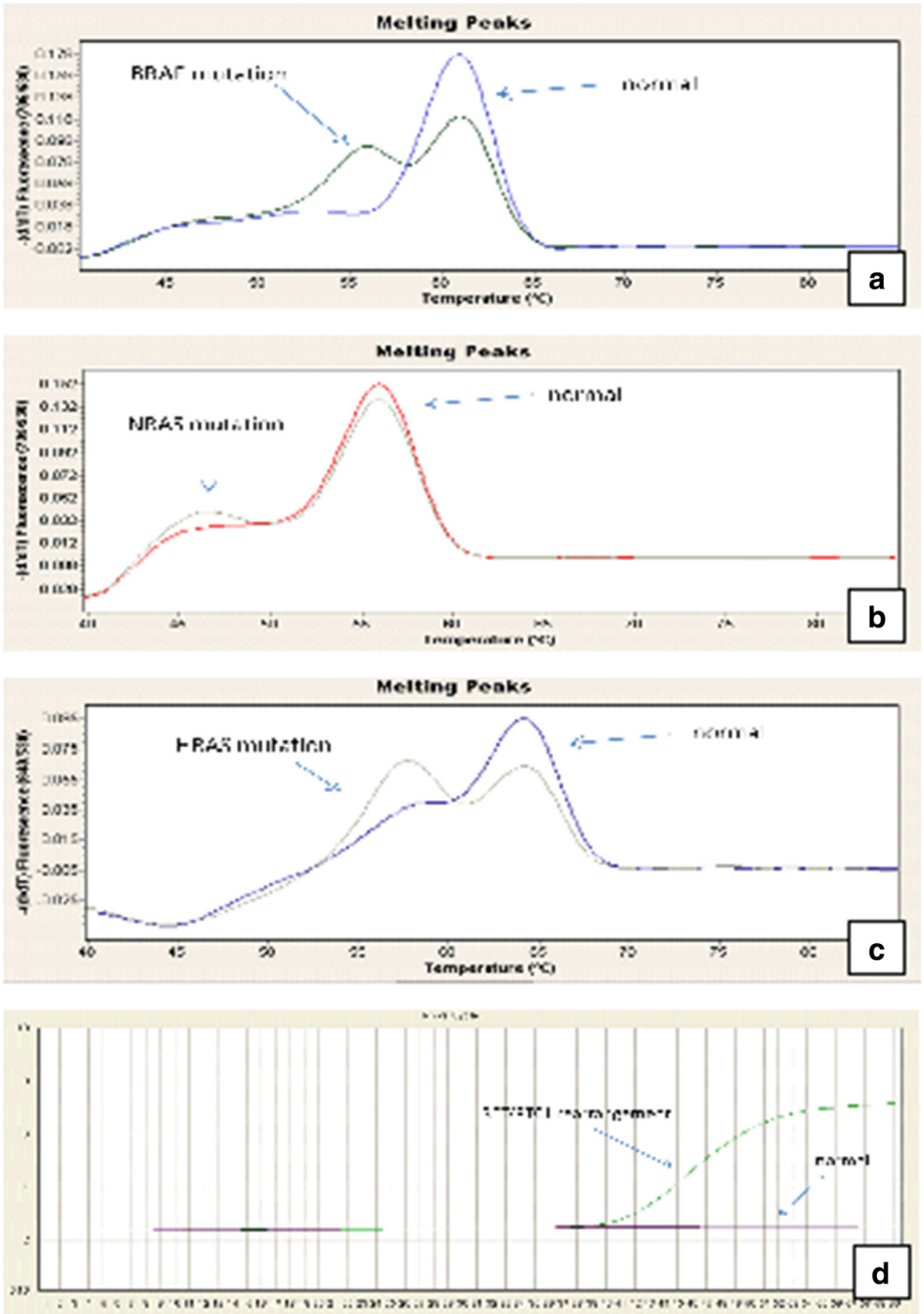

Fig. 1 Melting curve of pathological (**a**) BRAF mutation (rs113488022), (**b**) NRAS mutation (rs79057879), (**c**) HRAS mutation (rs28933406) and mutation negative controls. Expression curves of (**d**) RET and CCDC6 (RET/PTC1) gene rearrangement in normal and pathological thyroid tissue samples

Table 1 Distribution of genetic alterations in thyroid tissue samples

	Number of DNA samples	*BRAF*	*HRAS*	*KRAS*	*NRAS*	*RET/PTC3*	*RET/PTC1*
Papillary cc.	197	87 (44.2 %)	2 (1.0 %)	1 (0.5 %)	6 (3.1 %)	1 (0.5 %)	9 (4.6 %)
Normal tissue	197	0	0	0	0	0	0
Sum	394						

of each primer, 2 pmol each hybridization TaqMan probe 20× (LT) and 7.5 μl water. Every gene rearrangement was examined in 2–2 parallel measurements in a 96-well plate. The RT-PCR reaction was carried out at 50 °C for 2 min and denaturation at 95 °C for 10 min, followed by 60-cycle PCR amplification: denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 60 s (Fig. 1).

Statistical Analysis

We created three different groups of patients by their clinical and histological data. The relationship between these data and the presence of genetic variants was analyzed. Group A was composed by patients who did not have thyroid cancer metastasis, vascular invasion and the tumor size was 10 mm or less. In group B, tumor metastasis or vascular invasion were not detected and the tumor diameter was more than 10 mm. Group C was composed of patients with metastasis, vascular invasion and the diameter of the nodule was more than 10 mm. We applied Chi-square test with SPSS Statistics 20 program was applied for statistical analysis.

The correlation between the distribution of genetic alterations and the subtypes of PTC was also examined. We applied linear regression test from SPSS Statistic 20 program for statistical evaluation.

Results

The 197 patients with PTC included 63 men (age: 49.9 ± 14.6) and 134 women (age: 47.7 ± 16.2). We found double genetic alterations in 7 cases, and single variants in 92 cancer samples. No genetic alteration of the examined genes was detected in 98 samples. The distribution of genetic variants can be seen in Table 1. The distribution of mutation frequency in subtypes of PTC is shown in Table 2. Altogether, 53.9 % of PTC samples contained one or two genetic alterations (48.8 % mutations and 5.1 % rearrangements). None of the above genetic alterations was identified in the corresponding normal thyroid tissues.

We analyzed mutation and expression changes separately in formalin-fixed paraffin embedded (FFPE) and fresh frozen (FFS) tissues. We could not find significant differences in genetic data in the two sample types.

Correlation was not detected between the genetic data and the severity of the disease in PTC patients. There was a tendency for increased frequency for *BRAF* mutation in the tall cell variant, however, no significant relationship could be demonstrated between genetic variants and the subtypes of PTC.

Discussion

The most common genetic alterations in PTC are point mutations in the *BRAF* and RAS genes followed by *RET*/*PTC* rearrangements [2]. The demonstration of the occurrence of these changes in PTC has marked conceptual implications for thyroid carcinogenesis as well as clinicopathological significance. These oncogenes might initiate PTC tumorgenesis or, instead, they might occur after the development of a thyroid tumor [20].

The presence of a *BRAF* mutation may preoperatively predict the behavior of microscopic PTC, suggesting a more aggressive surgical approach [21–23]. The incidence of *BRAF* mutation in PTC has been reported in 40–45 % of the cases [2, 24, 25]. Reviewing the incidence of genetic alterations in thyroid cancers from different countries, Soares and co-workers

Table 2 Distribution of genetic alterations in the subtypes of PTC

Subtype of PTC	Number of samples	*BRAF*	*KRAS*	*HRAS*	*NRAS*	*RET/PTC3*	*RET/PTC1*
Classical	137	61 (44.5 %)	1 (0.7 %)	2 (1.5 %)	3 (2.2 %)	1 (0.7 %)	8 (5.8 %)
Follicular variant	17	7 (41.2 %)	0	0	1 (5.9 %)	0	0
Encapsulated	9	5 (55.6 %)	0	0	0	0	0
Microcarcinoma	19	7 (36.8 %)	0	0	0	0	1 (5.3 %)
Tall cell	7	5 (71.4 %)	0	0	2 (28.6 %)	0	0
Hürthle cell	8	2 (25.0 %)	0	0	0	0	0

[26] reported *BRAF* mutations in 56 % of the cases. In our study, *BRAF* mutations were present with similar frequency in PTC tissues while none was seen in the normal thyroid. Similarly to Bernstein and co-workers [27], we found *BRAF* mutations relatively more frequently (5/7) in tall cell variants of PTC, however, it did not reach significance, perhaps, due to the low number of this subtype present in our study. Two samples of this subtype carried both *BRAF* and *NRAS* simultaneously. *BRAF* mutation frequency found in our microcarcinoma subset is similar to that of shown by Zheng and co-workers [28], who analyzed a large number of cancers and detected *BRAF* mutations in 40.1 %.

There is evidence that RAS mutations, although more frequent in follicular cancer, are also present in a rather high percentage of PTC, especially in follicular variants and encapsulated forms [2]. The frequency of RAS mutations in PTC is varying, ranging 0–16 % [26, 29]. We could not show high frequency in our Hungarian samples. Our occurrence rate was much lower (4.1 %) than in the US data demonstrating a 10–20 % RAS mutation frequency in PTC samples [11, 12]. In contrast, Di Cristofaro at al. [29, 30] did not find RAS mutations in PTC in a French population.

In our study, we detected *RET*/*PTC* rearrangements also with lower frequency than others [25, 26, 31]. While those authors reported a 10–40 % frequency of *RET*/*PTC* rearrangements in PTC, we could only show a 5.1 % in our patients. The presence of *RET*/*PTC3* results in a very aggressive growth of papillary cancer [11, 25, 32–34], thus, it carries a prognostic value. Leeman-Neill et al. [35] found higher frequency of *RET*/*PTC* (35 %) in 62 post-Chernobyl PTCs samples.

Surprisingly, we did not find PAX8/PPAR-gamma rearrangement in our samples. The frequency of PAX8/PPAR-gamma rearrangement in PTC remains controversial. Armstrong at al. [36] reported PAX8/PPAR-gamma rearrangement in the follicular variant of PTC with low frequency (1–5 %). However, a much higher prevalence of PAX8/PPAR-gamma rearrangement (37 %) was reported in the follicular variant by Castro et al. [37]. Klemke et al. [38] and Soares at al. [26] reported that this type of genetic alteration could not be found in theirs samples. Eszlinger et al. [39] investigated 310 FNA samples and they found PAX8/PPAR-gamma rearrangement in 8 of 310 samples and only 4 were associated with cancer. The observed geographical difference in the occurrence of this rearrangement needs further investigations.

According to some authors, a correlation appears to exist between the genetic alterations, especially *BRAF* mutations and the severity of the disease in PTC patients [23, 40–44]. However, others could not corroborate these findings, and the *BRAF* status did not seem to affect the metastatic behavior of PTC and – according to these authors - it should not be considered as a negative determinant in predicting patients outcome [34, 41, 45–50]. Our data support this latter notion since we could show no effect of genetic alterations including *BRAF* status on disease severity.

The different frequency of genetic variants in our study compared to others might be due to the different iodine intake in Hungary. The US is a high iodine intake area in contrast to Europe including Hungary which is known of low dietary iodine intake of the population [51–53]. Guan et al. [24] reported, that high iodine intake seems to be a significant risk factor for the occurrence of *BRAF* mutation in the thyroid gland and may therefore be a risk factor for the development of PTC in Chinese population. In Austria, the change in iodine supply over the last 40 years led to a change in histologic type of thyroid cancer, i.e. the ration of papillary cancer increased [54]. The geographic differences in the incidence of thyroid carcinomas are – at least partly - related to the variations in dietary iodine intake [2, 3, 55]. In an earlier study, the relationship between iodine intake and oncogene RAS mutations in thyroid cancer was examined by a Canadian and a Hungarian research group. The comparison between one of the highest (Canada) and lowest (Hungary) dietary iodine intake area could not identify any RAS mutations in 22 specimens with PTC histology [5]. Another study from Serbia [34], also known as a similarly low iodine intake area as Hungary, found similarly low RAS mutation frequency in PTC as we did in our samples.

It is evident that numerous molecular, clinical and pathological features of PTC have changed over the last decades [2], including a range of genetic alterations in various subpopulations and their role in the disease prognosis. The BRAF and RAS mutation panel is expected to determine the therapeutic treatment of papillary thyroid cancer soon. In addition, local differences, such as iodine intake, may also influence the occurrence of genetic variants in PTC which should be taken into account when evaluating their importance. Our Hungarian data add to the variety of knowledge of this area.

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