Examination of molecular markers in differential thyroid cancers

Ph.D. Thesis

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Introduction

The thyroid gland fills in an essential role in the human body. Its main effect is to alter the speed of the mental and metabolic processes to reflect the changes of the environment. If this balance gets corrupted then we can talk about an illness. In our country thyroid gland diseases are very common. The thyroid gland of Hungarians is larger at birth than the thyroid glands of people with adequate iodine levels. Observations suggest that the occurrence of these diseases is much higher in women than in men.

We have evidence of some kind of tumorous diseases in all our glands. In recent years the thyroid gland is getting more and more attention, which is partially due to the increasing presence of molecular biology in the field of medicine. After the sequencing of the human genome the field of medicine went through an enormous change and the approach towards diseases changed also. Thanks to new technologies like next-generation sequencing (NGS) the flow of information increased, the cost of genetic tests decreased, and genetics is becoming a part of our every day life. The formerly canonical method of detecting single nucleotide polymorphisms (SNPs) is displaced by robust gene panels.

Aims of this study

We wanted to examine the following questions:

1.) Analysis the ratio of somatic oncogene BRAF mutation in Hungarian patients with differential thyroid cancer and examination of this mutation in the respect of the mutation positivity and tumor agressivity.

2.) Analysis the ratio of somatic oncogene RAS group (NRAS, HRAS, KRAS) mutations in Hungarian patients with differential thyroid cancer and examination of these mutations in the respect of the mutation positivity and tumor agressivity.

3.) Analysis the ratio of somatic oncogene RET/PTC in Hungarian patients with differential thyroid cancer and examination of this rearrangement the respect of the mutation positivity and tumor agressivity.

4.) Analysis the ratio of somatic oncogene PAX/PPAR/gamma in Hungarian patients with differential thyroid cancer and examination of this rearrangement the respect of the mutation positivity and tumor aggressivity.

5.) Examination of SFN, HMGA2 and MRC2 gene expressions in Hungarian patients with papillary thyroid cancer.

6.) Analysis of CYP24A1 and CYP27B2 gene expression in papillary thyroid cancer and examination of these changes in expression in the respect of tumors aggressivity.

7.) Examination of SFN, HMGA2, MRC2 and CYP24A1 gene expressions in Hungarian patients with papillary thyroid cancers.

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 2010-2014. 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 436 thyroid tissue samples (218 malignant and 218 control of the same subjects). The study protocol was reviewed and approved by the Ethic Committee (ETT-TUKEB 1160-0/2010-1018EKU). Patients gave informed consent.

Nucleic acid (DNA, RNA) isolation

The thyroid tissues were stored in -80 ^{o}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

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). 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.

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.). Every set contained gene-specific forward and reverse primers and fluorescence-labelled probes. Probes span an exon junction and do not detect genomic DNA.

The "three-genes" (SFN, MRC2, HMGA2) expressions

The expression differences of selected genes (*ID CYP24A1: Hs00167999_m1, Applied Biosystems*) (*ID SFN: Hs.PT.51.20789121.g, ID MRC2: Hs.PT.51.20692535, ID HMGA2: Hs.PT.51.2803297, Integrated DNA Technologies*) were analyzed by Taqman probe-based quantitative real-time PCR. GAPDH was used as endogenous control for data normalization. Relative quantification was carried out from collected data (threshold cycle numbers) by Applied Biosystems 7500 System SDS software 1.3.

Examination of CYP24A1 és CYP27B1 gene expressions by real-time-PCR

The expression differences of selected genes (ID *CYP24A1*: Hs00167999_m1, ID *CYP27B1*: Hs00168017_m1) were analyzed by predesigned and validated gene-specific TaqMan probe-based quantitative real-time RT-PCR technique from Applied Biosystems (Foster City, CA, USA). Every set contained gene-specific forward and reverse primers and fluorescence-labelled probes. Probes span an exon junction and do not detect genomic DNA.

General housekeeping gene *GAPDH* (ID: Hs99999905_m1) was used as an endogenous control for data normalization. Relative quantification studies were made from collected data (threshold cycle numbers, referred to as Ct) with 7500 System SDS software 1.3 (Applied Biosystems). The relative quantity (RQ) of the gene-specific mRNA was calculated from the averaged value of dCt-s (target gene Ct – endogenous control gene Ct) and analyzed with 7500 System SDS software 1.3 from Applied Biosystems according to the manufacturer's recommendations. Two fold or more changes of relative gene expression in tumor tissue compared to non-cancerous control were considered as cutoff for the difference.

Immunohistochemistry

Immunohistochemistry

Immunohistochemical detection of CYP24A1 protein expression was analyzed on formalin-fixed and paraffin-embedded tissue samples of five representative cases, applying an affinity purified anti-human CYP24A1 rabbit polyclonal antibody (Prestige Antibodies, Sigma-Aldrich; 1:200) as primary antibody. Briefly, 2 μm-thick paraffin sections were de-waxed, blocked for endogenic peroxidase activities in ethanol containing 1.5% (v/v) H2O2, and heat-treated in 10 mmol/L (pH10.5) TRIS buffer solution for antigen retrieval using a household electronic pressure cooker (Avair IDA). After protein blocking in TRIS-buffered saline (TBS, pH 7.4) containing 5% (w/v) low fat milk powder, the sections were incubated with the primary antibody at room temperature for 70 min. Detection was performed using Novolink polymer kit (Leica Biosystems/Novocastra), and nuclear staining was carried out with Mayer's hematoxylin. The immunohistochemical stainings were executed in a 4-channel Freedom Evo liquid handling platform (TECAN, Mannedorf, Switzerland).

Statistical analysis

- **Examination of somatic oncogene alterations in differential thyroid cancer**

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.

1. group was composed by patients who did not have thyroid cancer metastasis, vascular invasion and the tumor size was 10 mm or less.
2. group B, tumor metastasis or vascular invasion were not detected and the tumor diameter was more than 10 mm.
3. 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.

- **Examination of gene expressions in papillary thyroid cancers**

We tested the significance level of relative changes in *CYP24A1* and *CYP27B1* genes expressions in 100 human papillary tumors vs. their own control thyroid tissues

with a nonparametric method, Mann-Whitney U-test. Results with a p value of 0.05 or lower were considered statistically significant. Pearson's correlation coefficient was used to measure correlation between continuous variables (in our work between *CYP24A1* expression and age at the time of PTC diagnosis).

Four functional subsets of PTC samples have been created for evaluating the rate of *CYP24A1* overexpressed, down-regulated and unchanged tumors. The subgroups were defined by the following criteria: 1, presence of somatic oncogene mutation (*BRAF*, *HRAS*, *KRAS*, *NRAS*) and/or rearrangement (*ELE1/RET*, *CCDC6/RET*); 2, conventional PTC or other PTC histological variants (follicular, hürthle-cell, tall-cell, encapsulated and micro carcinomas); 3, other thyroid disease is present in addition to PTC (Hashimoto, hypothyroidism, hyperthyroidism); 4, lymph node metastasis and/or vascular invasion are confirmed. The distribution of tumor samples exhibiting increased, decreased or unchanged *CYP24A1* expression levels in the four different subsets was analyzed using chi-square test.

The univariate Mann-Whitney U-test cannot fully recover the information hidden in the data, and more exhaustive multivariate procedures were called for.

- **Principal Components Analysis (PCA)**

We used centered principal components analysis to summarize multivariate data structure in terms of a few important and uncorrelated dimensions, called the components. Each component is extracted such that its share from the total variance is maximized. Coordinates of observations are obtained from the eigenvectors in order to show the positions in the component space, whereas coordinates of variables are their correlations with components, multiplied by an arbitrary scale factor to enhance visibility in the diagram. In the graphical display the variables are emphasized by lines pointing to their positions. The directions and lengths of lines pointing to positions representing the variables in the diagram are informative on the correlations and relative importance of variables, respectively. The overall picture provided by PCA is also useful in selecting variables that best reflect trends and differences among patient groups. Computations were performed by the SYNTAX 2000 program package

Results

- **Examination of somatic oncogene BRAF mutation in differential thyroid cancer**

The 218 patients with differencial thyroid cancer included 70 men (age: 50.5 ± 12.2) and 148 women (age: 48.6 ± 16.5). We found double genetic alterations in 7 cases in all differenciatid thyroid cancer samples (1 KRAS, 3 NRAS mutations and 3 RET/PTC rearrangements), and single variants in 91 cancer samples. No genetic alteration of the examined genes was detected in 127 samples. Altogether, 41.7% of all samples contained one or two genetic alterations. We found 4 follicular thyroid cancer samples with BRAF mutations. None of the above genetic alterations was identified in the corresponding normal thyroid tissues.

Correlation was not detected between the genetic data and the severity of the disease in 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.

- **Examination of somatic oncogene RAS group (NRAS, HRAS, KRAS) mutation in differential thyroid cancer**

The 218 patients with differencial thyroid cancer included 70 men (age: 50.5 ± 12.2) and 148 women (age: 48.6 ± 16.5). We found 8 mutations (4.1%) in PTC and 6 mutations (28.6%) in FTC. We found double genetic alterations in 7 cases in all differenciatid thyroid cancer samples (1 KRAS, 3 BRAF mutations and 3 RET/PTC rearrangements). We detected 2 HRAS, 1 KRAS and 5 NRAS mutations in PTC and 1 HRAS and 5 NRAS in FTC samples.

- **Examination of somatic oncogene RET/PTC rearrangement in differential thyroid cancer**

The 218 patients with differencial thyroid cancer included 70 men (age: 50.5 ± 12.2) and 148 women (age: 48.6 ± 16.5). We found 10 rearrangements (5.1%) in PTC and 1 rearrangement (4.8%) in FTC. We found double genetic alterations in 4 cases in all differenciatid thyroid cancer samples (3 BRAF with RET/PTC1 and 1 NRAS with RET/PTC1).

- **Examination of somatic oncogene PAX/PPARgamma rearrangement in differential thyroid cancer**

The 218 patients with differencial thyroid cancer included 70 men (age: 50.5 ± 12.2) and 148 women (age: 48.6 ± 16.5). We did not find PAX8/PPAR-gamma rearrangement in our samples.

- **Differences in the expression of SFN, MRC2, HMGA2 on Hungarian PTC samples**

The study of the „3-gene model" was carried out on 58 tumor samples and on the surrounding intact tissues. 23 samples were acquired from men and 35 from women (Age was between 18 and 81, average: 47.7 years ± 12.4 years).

When we compared the samples with somatic mutations to the ones without it we found that the mutated samples showed higher expression of SFN (69.7% vs. 57.1%), MRC2 (59.4% vs. 45.0%) and HMGA2 (60.6% vs. 42.9%), however the difference was not statistically significant.

Different subtypes of papillary thyroid carcinoma (follicular, Hürthle-cell, tall cell, encapsulated and microcarcinomas) showed a higher expression of SFN (65.2% vs 64.5%) and HMGA2 (56.5% vs. 51.6%) than the classic variant of PTC. These differences were not statistically significant.

We also compared simple cases of PTC with PTC patients with other thyroid related comorbidities (Hashimoto-thyreoiditis, hypothyreosis, hyperthyreosis). The expressions of HMGA2 (75.0% vs. 44.7%) and MRC2 (57.1% vs 52.6%) were increased in the group of patients with comorbidities, however the expression of SFN (56.3% vs. 68.4%) showed an increase in patients with only PTC. These results did not reach statistical significance.

We also compared samples with aggressive attributes to samples without these features. We put the samples with lymph node metastases and vascular invasion in the group with aggressive features. All other tumors were sorted into the low-aggressivity group. We observed that the expression of SFN gene (82.6% vs 51.6%) and HMGA gene (65.2% vs. 45.2%) was substantially increased in the aggressive tumors. The expression values did not reach statistical significance.

- **Differences in the expression of CYP24A1 and CYP27B1 genes expression in Hungarian papillary thyroid cancers**

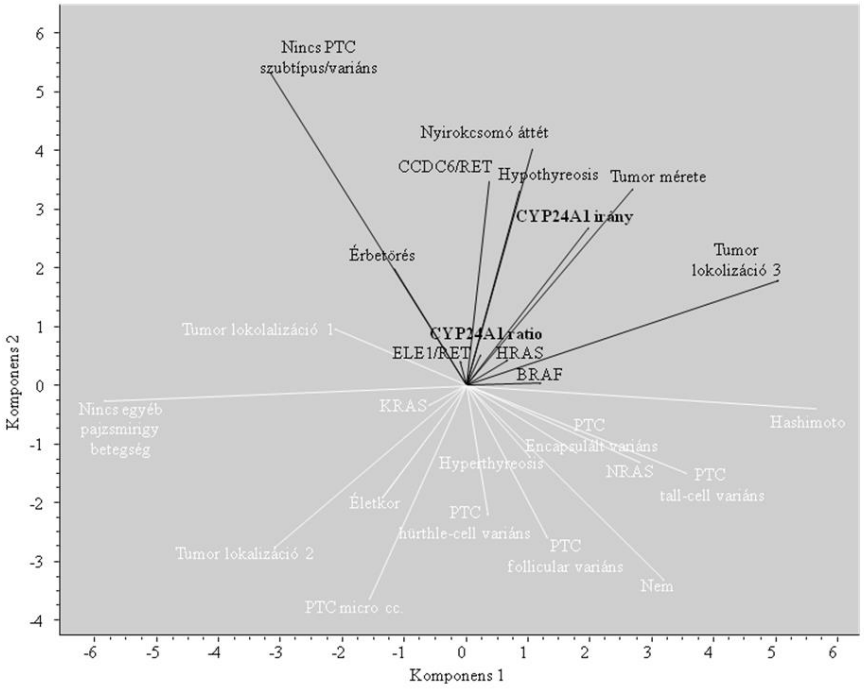
Thirty one males and sixty nine females participated in the study. The mean age was 49.1 years (range: 13 - 85 years) and 42% of patient were under 45 years.

CYP24A1 mRNA expression was markedly increased in 52 cases of the examined papillary cancers compared with that of normal/tumor-free thyroid tissue, sometimes reaching over 1000-fold elevation. In these samples the elevation of relative quantity of *CYP24A1* specific mRNA normalized to *GAPDH* was statistically significant whit $p<0.00001$. We measured lower *CYP24A1* transcription level in 24 malignant tissues. There was no variation in *CYP24A1* gene expression levels between tumorous and healthy control tissues in 13 subjects.

No significant alteration was seen in *CYP27B1* gene expression between neoplastic and normal tissues in any of the sample pairs (Figure 3). In more than 90% of the samples, the relative changes of *CYP27B1* gene expression did not reach the cut-off value (two fold or more, mean fold change: 0.88±0.44).

Immunostaining was applied to demonstrate the quantitative real-time PCR-detected changes of *CYP24A1*-specific mRNA in protein level. Immunohistochemistry results were consistent with the qPCR results, elevation of *CYP24A1* gene expression was always accompanied with increased protein level.

There was a tendency toward difference in the distribution of high-level *CYP24A1* in the PTC subsets, however, it did not reach statistical significance applying chi-square test. Higher rate of elevated *CYP24A1* activity was seen if somatic oncogene mutations were detected in tumor tissues (60.7% vs. 54.5%). *CYP24A1* expression appeared to be increased in conventional PTC samples compared to PTC subtype variants (65.3% vs. 50.0%). PTC samples without any other thyroid disease exhibited a trend toward higher *CYP24A1* expression (59.4% vs. 56.0%). Also, the presence of *CYP24A1* overexpressed cancers tended to be elevated in PTCs with regional lymph node metastasis and/or vascular invasion (65.7% vs. 52.9%).



Fugure 1. Ordination diagram showing correlations of variables along components 1 and 2

The PCA ordination diagram shows the relationships among the analyzed 26 demographic, clinical, histological and genetic variables measures in 89 PTC samples. The first two eigenvalues explain 10.4% and 9% of the total variation, respectively. There was positive correlation between increased *CYP24A1* expression rate (*CYP24A1* direction) and a group of variables reflecting tumor malignity (mainly vascular invasion, lymph node metastasis, tumor size, hypothyreosis) as explained mostly by axis 2. We found a correlation between higher *CYP24A1* expression rate and the occurrence of point mutations in oncogenic tumor markers (*BRAF, HRAS, CCDC6/RET*) as well as a group of variables including tumor malignity in a multiparametric PCA statistical method. (Figure 1)

- **Differences in the expression of SFN, MRC2, HMGA2 and CYP24A1 on Hungarian PTC samples**

We compared the CYP24A1 and " 3-gene model" expression based on the presence of somatic oncogene mutations (BRAF, NRAS, HRAS, KRAS and RET/PTC gene rearrangement). In case of CYP24A1 there was no change in expression, the "3-gene model" showed a slight elevation in expression.

Different subtypes of papillary thyroid carcinoma (follicular, Hürthle-cell, tall cell, encapsulated and microcarcinomas) showed a higher expression of SFN (65.2% vs 64.5%) and HMGA2 (56.5% vs. 51.6%) than the classic variant of PTC. However CYP24A1 expression (66.7% vs. 50.0%) and MRC2 expression (60.0% vs. 45.5%) was increased in the classic PTC variants. These differences were not statistically significant.

We also compared simple cases of PTC with PTC patients with other thyroid related comorbidities (Hashimoto-thyreoiditis, hypothyreosis, hyperthyreosis). The expressions of HMGA2 (75.0% vs. 44.7%) and MRC2 (57.1% vs 52.6%) were increased in the group of patients with comorbidities, however the expression of SFN (56.3% vs. 68.4%) and CYP24A1 (65.8% vs. 47.0%) showed an increase in patients with only PTC. These results did not reach statistical significance.

We also compared samples with aggressive attributes to samples without these features. We put the samples with lymph node metastases and vascular invasion in the group with aggressive features. All other tumors were sorted into the low-aggressivity group. We observed that the expression of SFN gene (82.6% vs 51.6%) was substantially increased the expression of HMGA gene (65.2% vs. 45.2%) and CYP24A1 (64.0% vs 45.2%) slightly increased in the aggressive tumors. The samples with low malignancy showed a decreased expression of all these genes. The expression values did not reach statistical significance.

Conclusion

Through our study the viewer can have an overview of the genetic diversity of Hungarian PTC samples. Apart from a few exceptions Hungarian data is in accordance with the international literature. The proportion of RAS mutations is well-below the international average. The biggest surprise is that the PAX/PPARgamma rearrangement is not typical in follicular thyroid cancer. We do not know the exact reason behind these phenomena, but we suppose it might be related to the lower iodine levels of Hungary.

Furthermore the complex gene-expression studies confirmed that the vitamin D neutralizing CYP24A1 is associated with more aggressive forms of PTC (lymph node metastases and vascular infiltration) and shows a positive correlation with other somatic gene mutations and tumor size. In our relatively large sample size the activity of CYP24A1 is substantially increased compared to the healthy control tissue. The PCA analysis shows a positive correlation between CYP24A1 expression and the presence of point mutations (BRAF, HRAS), gene rearrangements (RET/PTC1) and the negative properties of the samples. This presents the idea that CYP24A1 is directly involved in the tumorgenesis of thyroid gland cancers.

A significant percent of thyroid cancers is differentiated thyroid cancer. Thanks to genetic studies we recognize more and more genetic factors in the background of tumorgenesis. These new findings could form the basis of new diagnostic methods and the development of new drug molecules.

The routine fine-needle aspiration biopsy of thyroid gland nodules could be extended with molecular genetic tests. This could further decrease the number of inconclusive biopsies and it would be possible to surgically remove nodules that present themselves as benign under the microscope, but already show malignant genetic alterations.

The protocols set during this work also peoduced the basis for the introduction of these tests as routine diagnostics.

The oncogenes we studied play an important role in the MAP-kinase pathway, therefore they influence the differentiation, division and apoptosis of cells. Thanks to the rapid development of pharmacogenomics personal medicine is becoming more and more important in the treatment of diseases and tumors. The introduction of cetuximab, an EGFR inhibitor, to treat metastatic colon carcinoma was a major achievement of

pharmacogenomics. The BRAF inhibitor vemurafenib which is very promising in the treatment of BRAF positive melanomas is not in use for the treatment of PTCs yet. The fact that we know which oncogene somatic mutation is typical for which tumor can form the basis for the development of new drug molecules. It would worth the effort to develop a molecule for the inhibition of CYP24A1 to further facilitate the anti-tumor effect of calcitriol.

Summary

There was a dramatic increase in diagnosed thyroid cancers in the last decade. The parameters allowing an early detection, like genetic alterations became essential.
Our aim here was to test the proportion of somatic oncogene mutations (BRAF, NRAS, KRAS, HRAS) and gene rearrangements (RET/PTC, PAX/PPARgamma) in thyroid gland tumors. We also tested the expression patterns of CYP24A1 and CYP27B1, and the expression patterns of the "3-gene model" (SFN? MRC2, HMGA2) in papillary thyroid carcinomas.

We could not find a statistically significant relationship between thw somatic oncogenic mutations, gene rearrangements and the aggressivity of the tumor by Chi-test. We divided our study group into four subgroups based on the clinical and histological properties. In case of the "3-gene model" we studied the change in expression (increased, decreased, did not change) in the percentage of samples in the four subgroups. Negative outcomes and genetic alterations were usually associated with increased expression. We used Chi-tests to study the change in CYP24A1 expression, but we could not find a significant change, we could only observe some trends. The result of the principal component analysis shows that the change in expression of CYP24A1 is positively correlated with the features associated with malignancy (lymph node metastases, tumor size, vascular infiltration, somatic oncogene mutations and/or gene-rearrangements).

Our data implies that the studied genetic alterations could play a role in tumorgenesis, therefore their detection could influence the post-surgical therapy of patients. Our results further enforce the idea that genetic testing has a place in the routine diagnostics of thyroid gland cold nodules as a predictive method of the nodule's susceptibility to malignant transformation and as a determinant of further therapy (radioiodine therapy, BRAF and NRAS inhibitors). Furthermore CYP24A1 inhibitors could be the next target of anticancer drugs to facilitate the anti-tumor effect of vitamin D.

PUBLICATIONS

Candidate's publications Related to the Thesis (5 publications):

Balla, B., J.P. Kosa, **B. Tobias**, C. Halaszlaki, I. Takacs, H. Horvath, G. Speer, Z. Nagy, J. Horanyi, B. Jaray, E. Szekely, and P. Lakatos (2011) *Marked increase in CYP24A1 gene expression in human papillary thyroid cancer.* **Thyroid**. **21**(4): p. 459-60.

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(*=authors equally contributed)

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