

Examination of genetic factors in the development of thyroid cancer

Ph.D. Thesis

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1. Introduction

In recent decades, great attention is turned to the malignant thyroid diseases, because the number of new cases diagnosed annually has increased significantly. Its main reason may be that modern routine diagnostic uses more and more sophisticated methods and they are easier to access. Reasons can be found in the fact that the frequency shows geographical differences. After the Chernobyl nuclear disaster and the Fukushima nuclear disaster currently attention also focused on thyroid cancers. With regard to fact that differentiated thyroid is well-treatable, and that the number of these has been increasing, so it is very important the knowledge of the thyroid cancers for practitioners for early detection.

Due to the increasing expansion of molecular genetics our knowledge of molecular-level understanding of the formation of thyroid tumours has been rapidly developed. Ongoing revolution in molecular biology during the past decade helps to cure tumours. Modern medicine is also affected by the fact that through the knowledge of the entire sequence of human genome a new era has begun, the "post-genomic" era, which is characterized by large-scale data flow, interpretation of genetic information and dissemination of new scientific technologies. Bioinformatics has moved to the forefront, due to which researchers could find previously unsuspected correlations, and they did not examine the hypothetically suspected genes, but those which show deviation in the genetic profile of an a healthy and diseased cells.

Nowadays the most reliable test tool for the differential diagnosis of thyroid nodules is the fine needle biopsy, but in case of the 10-40% of the samples the existing malignancy cannot be clearly demonstrated. The research on genetic mutations is playing an increasing role in its remedy.

Preoperative classification could be carried out any carefully; there will be cases when the intervention subsequently turns out to be unnecessarily radical, or on the contrary: completing surgery will be required.

2. Aims of this study

Nowadays molecular genetics now is an essential diagnostic device for many clinical aspects. The last decade it succeeded to detect such mutations, of which occurrence is more frequent in certain thyroid tumours. We set as a target of our study to identify more genes and rearrangements in benign thyroid nodules as potential clinical markers among samples of the inland patient population. We intended to increase diagnostic accuracy by examining genetic factors considered important for the emergence of thyroid cancer. In addition, the presence of gene mutations in benign nodules may predict the possibility of malignant transformation, helping to determine that which “cold” nodule’s surgical removal is needed essentially. During our prospective Hungarian study on large number of cases we put the emphasis on the following aspects:

1. Examination of somatic BRAF gene mutation in benign thyroid nodules, and analysis of the relationship between BRAF mutation and the clinical outcome.
2. Examination of the mutation of the somatic RAS gene family (HRAS, NRAS, KRAS) in benign thyroid nodules, and analysis of the relationship between RAS mutation and the clinical outcome.
3. Examination of somatic RET/PTC gene rearrangement in benign thyroid nodules, and analysis of the relationship between RET/PTC rearrangement and the clinical outcome.
4. Examination of somatic PAX8/PPAR- γ gene rearrangement in benign thyroid nodules, and analysis of the relationship between PAX8/PPAR- γ rearrangement and the clinical outcome.
5. Bio-statistical analysis of genetic differences used as a diagnostic tool, based on several years of patient follow-up.

3. Methods

3.1 Examined patients

In the present study 824 thyroid nodules examination was carried out. Patients arrived from around the country to the common Aspiration Cytology Clinic of the Semmelweis University 1st Department of Internal Medicine, 1st Department of Surgery and 2nd Department of Pathology. From here those patients have been selected who were suffering from thyroid nodular disease based on the physical examination, laboratory tests and diagnostic imaging (ultrasound, scintigraphy and CT). The diagnosis was given in all cases in accordance with the professional protocols in force, in the possession of the results of the clinical symptoms, diagnostic imaging and hormone tests.

Test samples were obtained from the patients' thyroid "cold" nodules by fine needle biopsy. During the aspiration cytology test 779 (611 female and 168 male, average age 54.7 ± 15.3) "cold" nodules proved to be benign, which was supported by two independent and experienced pathologist specialists' opinion.

Nodules showing malignancy or inconclusive results in the cytological examination were removed in the 1st Department of Surgery. In cases of the non-diagnostic findings or unacceptable findings a further test takes place prior to enrolment, or the patient in question had will not be in the study.

The study was approved by the Committee of Science and Research Ethics of Medical Scientific Council (ETT-TUKEB 1160-0/2010-1018EKU). Prior to enrolment, all patients received detailed written and oral information, and they gave their written informed consent to participate. Our study included total of 779 samples taken from thyroid nodules. Simultaneously with the sample collection the observation and contact occurred annually from the end of 2010.

Follow-up of the changes related the health status of individuals under investigation was carried out in the form of regularly repeated telephone interviews and medical visits. The following questions were asked during the interviews:

1. Has there been a noticeable change or deterioration in his/her condition since the tissue biopsy (needle biopsy)?
2. Has he/she got thyroid surgery after the biopsy?
3. Was there a repeated or new biopsy? (since the date above)

Beside the responses we recorded the histologic or cytological findings and the method of follow up.

3.2 Nucleic acid isolation

The DNA isolation from the sample material was performed by using the Roche High Pure PCR template Preparation Kit (Roche, Indianapolis, United States of America). The whole RNA was extracted from the sample by Roche High Pure RNA Isolation Kit (Roche). In all cases, we use protocol required by the company. The quantity and quality of the isolated DNA and RNA was checked by NanoDrop spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA), in the wavelength range 260 to 280 nm.

3.3 Examination of somatic oncogene gene mutations

The single nucleotide polymorphism (SNP) of BRAF gene codon 600, rs113488022, the HRAS gene codon 61, rs28933406, the NRAS gene codon 61, rs79057879, the KRAS gene codon 12, rs118135424 and codon 13, rs121913535 was examined. A Roche LightCycler device (Roche LightCycler 2.0 Instrument) was used for the fluorescence detection of DNA mutations predesigned primer pairs and oligonucleotide probes were used for each mutation. 1 µl isolated DNA, 0.5-0.5 µl from both primer (TIB MOLBIOL Berlin), 0.5-0.5 µl from both hybridization probe (TIB MOLBIOL Berlin), 1.5 µl water, 0.5 µl bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, United States of America) and 5 µl JumpStartTaq ReadyMix PCR polymerase (Sigma-

Aldrich) solution were used for amplification. The study was performed according to the following protocol: denaturation at 95 ° C for five minutes; 60 cycles of 10 s 95 ° C, 10 s 54 ° C and 15 s 72 ° C, and then we analysed the melting curves detected by the device between 40-80 ° C with fluorescent signal. The melting curve was identified from the negative derivative of fluorescence over temperature (-dF/dT) by the software. The mutation sensitivity of the method was 10%, which means that at least 10% has to be reached the proportion of cells carrying the mutant allele in the sample. All of this was based on studies conducted during the dilution of positive controls.

3.4 Examination of somatic oncogene gene rearrangements

The RET/PTC1, RET/PTC3, PAX8ex7/PPAR- γ and PAX8ex9/PPAR- γ gene rearrangements from RNA were examined by real-time polymerase chain reaction (RT-PCR) technique on ABI Prism 7500 (Applied Biosystem by Life Technologies, Foster City, CA, United States of America) system. 10 μ l RNA (250-300 ng) per sample during reverse transcription turned cDNA by using 200 U SuperScriptIII RNase H reverse transcriptase (Invitrogen Life Technologies, Carlsbad, California, USA), 40 U RNaseOUT ribonuclease inhibitor (Invitrogen Life Technologies) and 2 μ l random primers (Promega, Madison, WI, United States of America). The reaction-mixture was incubated at 37 ° C for one hour. Gene-specific TaqMan probe-based gene expression method was used, where each gene-specific set contained a 5' direction and a 3' direction primer, and a probe with fluorescent marker-molecule. The polymerase chain reaction (PCR) was conducted in a final volume of 20 μ l, and it contained 2 μ l cDNA, 10 μ l TaqMan 2x Universal PCR Master Mix NoAmpErase UNG (Applied Biosystems by Life Technologies), 0.5 μ l validated gene-specific, concentration of 2 pmol hybridization TaqMan probes 20x (Applied Biosystems by Life Technologies), a concentration of 40 pmol primers, and 7.5 μ l Ultrapure water. Each genes were tested with parallel measurements in 96-well plates according to the following protocol: as a first step 2-minute incubation at 50 ° C, then 10-minute denaturation at 95 ° C, and after it PCR amplification which consists of 60 cycles. Each cycle consists of the following: 15 s denaturation at 95 ° C, 15 s annealing at 55 ° C and 1 minute synthesis at 60 ° C.

3.5 Statistics

Our study can be biased in two directions: by the false positive and false negative results. Accordingly, the validity has two components: sensitivity and specificity. The examination is sensitive if it identifies correctly the really positive patients, with little number of false-negative results. This means that the sensitivity of the examination is almost 100%. The test is specific if it identifies correctly the not-sick patients, with little number of false-positive results. In other words the specificity of the examination is almost 100%.

Beyond the sensitivity and specificity, the success rate of an examination is the predictive value of the examination.

The positive predictive value of the examination shows the probability of sickness in case of a person with positive result. The negative predictive value measures the probability of being disease-free in case of a person with negative result.

Therefore the accuracy of an examination is expressed by such a number which provides ratio of real (positive and negative) results compared to the total outcome.

MedCalc Statistical Software 11.5.0 software package was used (MedCalc Software BVBA, Ostend, Belgium) for the statistical analysis, the chosen level of significance in all cases was $p < 0.05$.

4. Results

4.1 Examination of BRAF gene mutation in benign thyroid nodules

Regarding the BRAF gene mutations in a total of 779 samples of benign thyroid nodule were examined, of which 611 came from female patients and 168 from male patients, average age of all patients was 54.7 ± 15.3 . 39 (5%) of the samples carried the BRAF defect. During aspiration cytology test carried out initially all of the “cold nodules” proved to be benign. During the patients’ one year follow-up 727 (93%) of the nodules were benign, of which in 17 (2.3%) cases were BRAF mutations detected. 52 (6.7%) of malignant or precancerous disease were confirmed on the basis of an aspiration cytology or biopsy. In these 52 samples sample 22 (42%) of the BRAF gene mutation were found. After two years of follow-up 12 (2.4%) of BRAF mutations have been discovered out of 504 samples. Out of 474 (94%) benign nodules 4 (<1%) were BRAF positive. 30 (6%) malignant tumour and follicular adenoma or follicular neoplasia were confirmed. There were positive genetic testing in 8 (26.7%) cases considering the BRAF mutation. During the three year follow-up of the patients out of 250 thyroid nodules 4 (1.6%) BRAF mutations were identified. 237 (94.8%) benign thyroid nodules didn’t shoe BRAF positivity, this implies that the 4 (30.8%) mutations were found in the remaining 13 (5.2%), thyroid carcinomas.

4.1.1 Analysis of the relationship between BRAF mutations and the clinical outcome

During the patients’ one year follow-up BRAF mutation was discovered in cases histologically proven papillary carcinoma (40 samples out of 52). Follicular carcinoma was confirmed for 2 (4%) patients and in 10 (19%) cases other (follicular adenoma, anaplastic and medullary carcinoma), but there weren’t any genetic alterations detected from these samples.

After two years of follow-up all of the 8 (30.8%) BRAF positivity could be detected in papillary tumours. In case of 1 (3.3%) patient follicular carcinoma was found and in 3 cases follicular adenoma or neoplasia.

During the patients' three-year follow-up 13 (100%) of papillary thyroid carcinomas were discovered, in these 4 (30.8%) BRAF mutations were detected.

4.2 Examination of somatic RAS gene family (HRAS, NRAS, KRAS) mutation in benign thyroid nodules

Regarding the mutations of RAS gene family in a total of 779 samples of benign thyroid nodule were examined, of which 611 came from female patients and 168 from male patients, average age of all patients was 54.7 ± 15.3 . A total of 9 KRAS, 23 NRAS and 1 HRAS mutations were found (Σ 4.2%). During aspiration cytology test carried out initially all of the "cold nodules" proved to be benign. During the patients' one year follow-up 727 (93%) of the nodules were benign, of which in 9 (1.2%) cases were HRAS, in 22 (3%) cases NRAS and in 1 (<1%) case KRAS mutations detected. Out 52 (6.7%) of malignant or precancerous disease 1 (1.9%) were carried the RAS – NRAS gene mutation. After two years of follow-up 7 HRAS, 5 NRAS and 1 KRAS mutations (Σ 2.6%) have been discovered out of 504 samples. These 13 (2.7%) RAS mutations could be detected out of 474 (94%) benign nodules. The 30 (6%) lesions which were malignant or suspicious for malignancy didn't carry this genetic mutation. During the patients' three-year follow-up 7 HRAS, 1 NRAS, 1 KRAS mutations (Σ 3.6%) were identified out of 250 thyroid nodules. All of the 9 RAS positivity was detected out of 237 (94.8%) benign thyroid nodules, this implies that there wasn't found RAS mutation in the remaining 13 (5.2%) thyroid carcinomas.

4.2.1 Analysis of the relationship between RAS mutation and the clinical outcome

During the patients' one year follow-up 1 (2.5%) NRAS mutation was confirmed out of 40 (77%) cases proved to be papillary carcinoma. According to the relevant literature the RAS mutation, which is frequent in follicular carcinoma (FTC),

couldn't be detected in any FTC or follicular adenoma. Neither in the patients followed-up for two years nor the patients followed-up for three years was confirmed the malignant thyroid tumour indicating RAS positivity.

4.3 Examination of somatic RET/PTC gene arrangement in benign thyroid nodules.

1 RET/PTC3 gene arrangement was confirmed out of the 779 samples of thyroid nodule (611 female and 168 male, average age 54.7 ± 15.3). During aspiration cytology test carried out initially all of the "cold nodules" proved to be benign. During the patients' one year follow-up 727 (93%) of the nodules were benign, of which RET/PTC rearrangement wasn't found in any cases. Out of 52 (6.7%) malignant or precancerous diseases 1 (1.9%) carried the gene rearrangement.

4.3.1 Analysis of the relationship between the RET/PTC rearrangement and the clinical outcome

1 (2,5%) RET/PTC3 gene rearrangement was detected out of 40 (77%) papillary thyroid carcinomas considering the whole follow-up.

4.4 Examination of somatic PAX8/PPAR- γ gene rearrangement in benign thyroid nodules.

In case of PAX8/PPAR- γ gene rearrangement 779 samples of thyroid nodule were examined, of which 611 came from female patients and 168 from male patients, average age of all patients was 54.7 ± 15.3 . PAX8/PPAR- γ gene rearrangement weren't detected in any samples.

4.5 Bio-statistical analysis of genetic differences used as a diagnostic tool, based on several years of patient follow-up

4.5.1 One-year follow-up

Out of the 779 samples of thyroid nodule a total of 73 (9.4%) genetic differences were found. During the patients' one-year follow-up 678 (87%) nodules were benign and they were all negative in terms of mutations. 52 (6.7%) malignant or precancerous diseases were confirmed on the basis of aspiration cytology and histology. Genetic alterations were detected in 24 (46.2%) cases out of the above cases. 49 (6.3%) genetically positive nodules showed no malignancy. The sensitivity of the examination corresponds to the mutation positivity of the tumours: 46.2 %. The specificity was 93.3%, the positive predictive value was 32.9% and the negative predictive value was 96%. The accuracy of molecular biological examination after one year: 90.1 %. Narrowing the circle, and examining solely the papillary carcinomas, the sensitivity of the examination increases up to 60%.

4.5.2 Two-year follow-up

26 (5.2%) genetic alterations have been discovered out of 504 samples.

After the patients' two-year follow-up 457 (90,7 %) tissues were benign, and in case of these the genetic examinations gave negative results. Out of 30 (6%) malignant tumours the genetic examinations were positive in 9 (30%) cases. Genetic alteration couldn't be detected in case of 21 (4.2%) cancerous nodules, while 17 (3.4%) positive samples did not become cancerous. Sensitivity 30%, specificity 96.4%, positive predictive value was 34.6% and the negative predictive value was 95.6%. The accuracy of molecular biological examination after two years: 92.5 %. The sensitivity in cases of papillary carcinomas: 34.6 %.

4.5.3 Three-year follow-up

14 (5.6%) mutations were identified out of 250 thyroid nodules.

During the patients three-year follow-up 228 (91.2%) nodules were found which were benign and negative in terms of mutation. There were genetic alterations in 5 (38.5%) cases out of 13 (5.2%) papillary carcinomas. In 8 (3.2%) cases could not be detected any genetic mutation, while in 9 (3.6%) cases the genetic examination was positive in the benign nodules. Sensitivity 38.5%, specificity 96.2%, positive predictive value was 35.7% and the negative predictive value 96.6%. The accuracy of molecular biological examination after three years: 93.2 %.

The following comparative figure illustrates the statistical characteristics of the molecular genetic analysis: (Figure I)

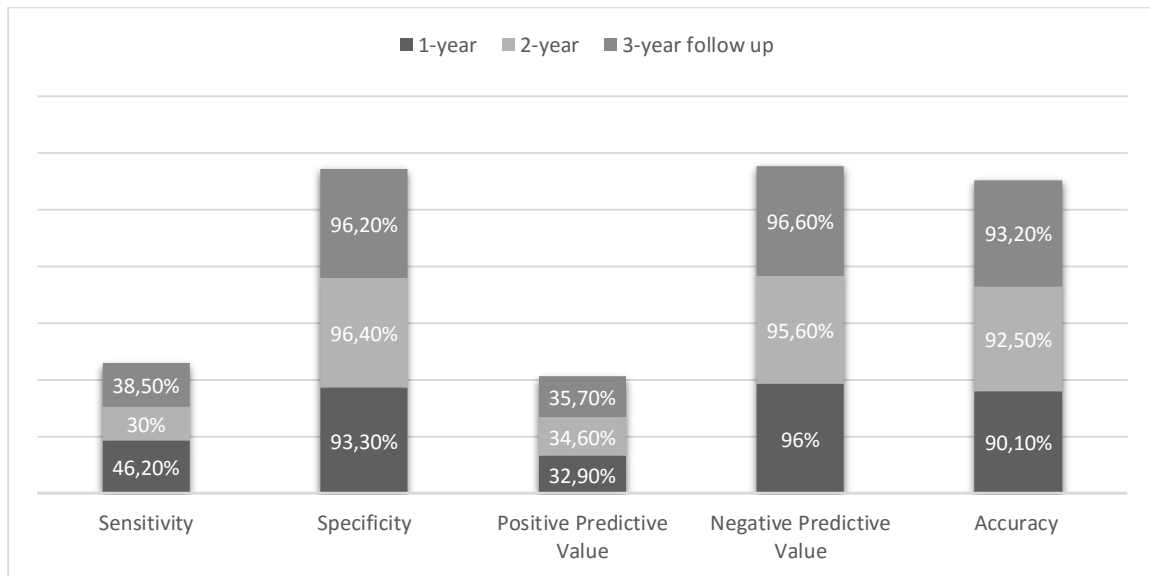


Figure I : Presentation of statistical characteristics based on the three-year follow-up

5. Conclusions

The following findings were made based on the results of longitudinal studies with large number of cases:

1. We explored among the first internationally the ratio of somatic mutations and gene rearrangements can be found in thyroid “cold” nodules taken during fine needle aspiration biopsy (FNAB).
2. We confirmed that gene differences, which play important role in the development of thyroid cancer, can be found in cytologically benign thyroid nodule samples.
3. We found that in Hungary, in the patient population examined due to thyroid nodule, the occurrence of genetic alterations is nearly identical to the data which is currently shown in related literature.
4. The presence of mutations in the benign lesions may indicate the possibility of malignant transformation, and it can serve as a basis for new diagnostic and treatment protocol concerning thyroid nodules.
5. The results of our study demonstrated high specificity and strong negative predictive value, so they confirm that genetic examination can increase the diagnostic accuracy.
6. In the routine diagnostics of thyroid nodules the outstanding ultrasound controlled aspiration cytology should be combined with the molecular genetic examination, thereby the number of doubtful results could be reduced.

5.1. New results

1. We have found that in the thyroid "cold" nodules samples taken by fine-needle biopsy can be detected the same gene arrangement (RET/PTC) and the same somatic mutations (BRAF, RAS) in our country as has been published in the international literature with the exception of the PAX8/PPAR- γ rearrangement.
2. We demonstrated that the malignant transformation of thyroid nodules can be anticipated by the examined genetic differences.
3. We confirmed based on the high specificity and a strong negative predictive value, that genetic examination improves diagnostic accuracy, and therefore can complement the thyroid aspiration cytology.
4. Based on our results the negative molecular test excludes the possibility of malignant transformation with high degree of certainty, the positive genetic examination might help to establish the surgical indication.
5. Our data supports the idea that genetic factors play a causative role in development of thyroid cancers.

6. Publications of the author

Publications related to the thesis:

Tobias B*, **Halaszlaki C***, Balla B, Kosa JP, Arvai K, Horvath P, Takacs I, Nagy Z, Horvath E, Horanyi J, Jaray B, Szekely E, Szekely T, Gyori G, Putz Z, Dank M, Valkusz Z, Vasas B, Ivanyi B, Lakatos P. (2015) Genetic Alterations in Hungarian Patients with Papillary Thyroid Cancer. Pathol Oncol Res, (2015.aug. 11., epub)

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