The benefits of molecular genetic analysis in the prevention of certain endocrine tumors

Ph.D. Thesis

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INTRODUCTION

Prevention is an important part of pediatrics practice that may be improved with the use of novel molecular biological techniques. In my work I studied three endocrine tumors: gonadoblastoma (GB), hereditary form of medullary thyroid cancer (MTC) and multiple thyroid adenomas associated with activating germline mutations of the thyrotropin receptor (*TSHR*) gene.

The presence of Y-chromosome material in patients with Turner syndrome (TS) is a risk factor for the development of GB. If Y-chromosome sequences are present in only a few cells, they may be missed by routine cytogenetic analysis. The use of molecular techniques to detect the presence of Y-chromosome fragments in such patients is becoming increasingly important. In my work I analyzed cryptic Y-chromosome derivatives in Hungarian TS patients using a novel real-time PCR (RT-PCR) developed by our group.

In the second part of my work I demonstrate the importance of clinical signs and symptoms and application of prevention tools in an aggressive cancer syndrome, the hereditary form of MTC, by presenting data obtained from multiple generations of an affected family. Multiple endocrine neoplasia type 2 (MEN2) is an inherited disease caused by germline mutations of the *RET* proto-oncogene. Mutation screening in patients with MEN2 is available in Hungary, and genetic screening in affected families makes it possible to identify mutation-carriers and offer them prophylactic thyroidectomy. Prophylactic thyroidectomy before the development of MTC in mutation carriers substantially reduces the risk of development of MTC.

The most aggressive MTC is found in MEN type 2B. Patients with MEN2B often have *de novo* mutations of the *RET* proto-oncogene. This form of MEN2 syndrome presents with typical dysmorphic features, mucosal neuromas, ganglioneuromatosis, MTC and phaeochromocytoma. Prevention of MTC requires

early diagnosis but, according to the literature, the diagnosis of MEN2B in several patients is delayed despite the typical phenotype. I emphasize the importance of the recognition and assessment of the phenotype by presenting the medical history of two unrelated young patients with MEN2B syndrome.

Activating germline mutations of the *TSHR* gene have been reported as a cause of sporadic and familial non-autoimmune thyrotoxicosis since 1994. In affected patients multiple thyroid adenomas and possibly thyroid cancer may develop. In the third part of my thesis I present the history of a young patient with sporadic non-autoimmune primary hyperthyroidism and report the results of molecular genetic studies using peripheral blood leukocytes and paraffin embedded thyroid tissues obtained from the patient. Because of the rarity of this disease, I collected and analyzed the main clinical features of patients previously reported in the international literature.

AIMS

In my work I studied endocrine tumors which can be prevented by prophylactic surgery. The objective of my work is presented in the following 9 points.

According to the literature dysgenetic gonads with Y-chromosome significantly increase the risk of developing germ cell tumors. TS is the most frequent cause of gonadal dysgenesis. Therefore, in the first part of my work I wanted to answer to the following questions:

1. What is the frequency of detectable whole Y-chromosome or Y-chromosome fragments in Hungarian TS patient population diagnosed by conventional cytogenetic analysis?
2. Is the novel RT-PCR based method applying aspecific DNA binding fluorescent stain developed in collaboration with the Laboratory of Molecular Genetics, 2nd Department of Pediatrics, Semmelweis University, Budapest

and Test Tube Baby Foundation, Szeged suitable for replacement of FISH and conventional PCR used in the literature?

3. What is the frequency of detectable Y-chromosome sequences among Hungarian TS patients using molecular genetic methods for the detection of cryptic Y-chromosome material?

4. What is the frequency of detectable GB among Y-chromosome positive TS patients?

5. What is the percentage of Y positive cells in gonadal tissues of patients undergoing gonadectomy? Is there a difference in the incidence of Y-chromosome mosaicism between gonadal tissues and peripheral blood samples?

According to international studies, early prophylactic thyroidectomy in family members harboring disease-causing *RET* mutations substantially improves the life expectancy.

6. My aim was to demonstrate the task of the pediatrics endocrinologist in the prevention of hereditary MTC by presenting findings in a family with members of 4 generations who had codon 804 mutation of the *RET* proto-oncogene.

7. I wanted to draw attention to specific signs of a rare syndrome, MEN 2B, and emphasize the importance of medical history for early diagnosis in two unrelated patients evaluated in our department.

In the third part of my thesis I deal with current diagnostic and therapeutic modalities of non-autoimmune primary hyperthyroidism due to activating mutations of the *TSHR* gene. I present the medical history of a young patient with this rare disease whose clinical findings tempted me to pose the following questions:

8. Can we confirm the diagnosis of this hereditary disease using molecular biologic techniques in a young patient who had non-autoimmune primary hyperthyroidism since infancy?

9. Do sporadic and familial forms of non-autoimmune primary hyperthyroidism due to activating mutations of the *TSHR* gene differ in disease onset and severity, the age at the time of diagnosis and in the efficacy of various therapies?

SUBJECTS AND METHODS

TS patients

The study included 130 consecutive patients with TS referred to the Hungarian Pediatric Endocrinology Network. The age of patients was between 0.1 and 20 years. The clinical diagnosis of the patients was set upon medical history and clinical features. The diagnosis of TS was verified by cytogenetic analysis (standard karyotyping) in all cases. Exclusion criteria were ambiguous genitalia and enlarged clitoris. For the isolation of genomic DNA 2 ml of peripheral blood was collected from each patient. Written informed consent was obtained from the children older than ten years old and from the parents of the patients. The study was approved by the Regional and Institutional Committee of Science and Research Ethics, Semmelweis University (TUKEB number: 11/2009).

Children members of a family with *RET* codon 804 mutation

A six-year-old patient was referred for evaluation because of obesity. Family history was positive for MEN 2A. The pedigree indicated that 3 older members and one young adult member of the family had medullary thyroid carcinoma. Genetic analysis of members of the fourth generation (two boys and five girls, age: 2 months – 9.2 years) was initiated.

Patients with MEN type 2B

Patient 1. A 17-year-old boy was referred for genetic consultation to our department. He was considered as having Crohn disease because of abdominal pain and distension since the age of 6 years. He previously underwent two minor surgeries for a large tongue with neuromas and hypertrophic gums. MEN type 2B was considered because of his history, marfanoid phenotype (skeletal abnormalities such as pectus excavatum, slight kyphoscoliosis, arachnodactyly, massive eyebrows) and thick lips. His height was 176.5 cm (between 50th and 75th centiles) and his weight weight was 44 kg (-5.5 kg below 3rd centiles).

Patient 2. A 10-year-old girl presented with a solid mass in her neck. Her medical history was unremarkable, and she was in good health. Her height was 144.7 cm (between 75th and 90th centiles), and her weight was 35.6 kg (between 75th and 90th centiles). She had thick lips, neuromas of the tongue and a solitary thyroid nodule. Thyroid scan showed a cold nodule in the right lobe, and fine needle aspiration cytology indicated MTC.

Patient with non-autoimmune primary hyperthyroidism

A young boy born in 1991 presented with an unconscious state, exsiccation, wet skin, fever and tachycardia at the age of 10 months. Non-autoimmune primary hyperthyroidism was diagnosed. Continuous propylthiouracil treatment resulted in a prolonged clinical cure lasting for 10 years. At the age of 11 years and 5 months the patient underwent subtotal thyroidectomy because of symptoms of the trachea compression caused by a progressive multinodular goiter. Two months after surgery hormonal evaluation indicated again recurrent hyperthyroidism and the patient was treated with propylthiouracil during the next 4 years. At the age of 15 years the patient developed again symptoms of trachea compression. Radioiodine

treatment resulted in a regression of the recurrent goiter and a permanent cure of hyperthyroidism without relapse during the last three years of his follow up.

MOLECULAR GENETIC METHODS

For the detection of Y-chromosome fragments a novel RT-PCR based method applying aspecific DNA binding fluorescent paint was used, which was developed in a collaboration between the Laboratory of Molecular Genetics, 2nd Department of Pediatrics, Semmelweis University, Budapest and Test Tube Baby Foundation, Szeged. Genomic DNA was isolated from 2 ml of peripheral blood with a commercial DNA isolation kit (QIAamp DNA Blood Midi Kit, Qiagen Inc, Hilden, Germany). In 3 patients who underwent gonadectomy DNA was also extracted from paraffin embedded gonadal tissues using QIAmp Tissue Kit (Qiagen Inc.). Four Y-chromosome specific sequences, including *SRY*, *TSPY1* (short arm), *DDX3Y* and *HSFY1* (long arm) were amplified in the presence of a double stranded DNA binding dye (SYBRGreen). Specific primer binding was analyzed by BLAST search tool (http://www.ncbi.nlm.nih.gov/BLAST/). Y-chromosome specific PCR products were subsequently detected, and β-actin served as internal positive control. In order to determine the sensitivity of our assay the RT-PCR was carried out on a DNA sample from a male individual tested for whole Y-chromosome positivity by FISH. Descending dilution series were analyzed, in which the male DNA was diluted with female DNA to an end concentration of 50 ng/μL in a series from 100% to 0.01% of Y-chromosome containing DNA material.

RT-PCR reaction was carried out on LightCycler™ instrument (Roche Diagnostics GmbH, Mannhein, Germany). Specific PCR products were tested using cycle sequencing. DNA samples were amplified with the Y-chromosome specific primer-pairs and with the β-actin specific primers serving as positive control and calibrator for the semiquantitative analysis. The PCR reaction was carried out in a

final volume of 20 µL using 2 µL of SYBR containing master mix (LightCycler DNA Master SYBR Green I, Roche), 40 ng of extracted DNA and primers at a final concentration of 0.5 pM/µL.

To verify the specificity of our PCR products, bidirectional cycle sequencing analysis was carried out with each primer used for PCR. The PCR reactions were performed in a PE9600 Thermal Cycler (Perkin Elmer, Waltham, Mass., USA). PCR products were purified with QIAquick PCR Purification Kit (Qiagen Inc.). The cycle sequencing reaction was performed with BigDye® Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on a PE9600 Thermal Cycler (initial denaturation 96ºC for 1 min, 25 cycles with a denaturation step at 96ºC for 10 s, annealing and extension steps at 59ºC for 4 min). Products were detected on ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems).

Molecular genetic analysis of exons 14 and 16 of the *RET* proto-oncogene was carried out in the Laboratory of Molecular Genetics, 2nd Department of Medicine, Semmelweis University as previously reported. Genomic DNA was extracted from peripheral blood leukocytes using QIAamp Blood Kit (QIAGEN, GmbH, Germany).

Molecular genetic analysis of the *TSHR* gene was carried out in the Laboratory of Molecular Genetics, 2nd Department of Medicine, Semmelweis University. Genomic DNA was extracted from peripheral blood leukocytes from the patient and his parents using QIAamp Blood Kit (QIAGEN, GmbH, Germany). DNA was also obtained from paraffin embedded thyroid tissue specimens of the patient using QIAmp Tissue Kit (QIAGEN, GmbH, Germany). The mutation hotspot region located in exon 10 of the human *TSHR* gene was amplified with five sets of oligonucleotide primers. Oligonucleotide primers were designed using the

Primer3 software. Direct bidirectional sequencing was performed using ABI Genetic Analyser Model 3100 (Foster, California, USA).

RESULTS

Characteristics of the novel RT-PCR method for the detection of Y-chromosome material

RT-PCR proved to be a useful and reliable tool for the detection of small amounts of DNA. Real-time detection is as sensitive as conventional quantitative PCR techniques. Regarding its simplicity, rapidity and low cost, the method can be used for mass screening.

We were able to detect Y-specific products in male DNA samples diluted up to 1:1000 with female DNA. We concluded that this method is capable of detecting Y-chromosome fragments if at least 0.1% of the cells contain Y-chromosome material (5 pg DNA). Cycle sequencing proved that all of our amplified PCR product sequences matched the genomic sequences (*β-actin* GenBank Accession No.: NT_007819.16; *SRY*: GenBank Accession No.: NT_011896.9; *TSPY1* GenBank Accession No.: NT_011878.9; *DDX3Y* and *HSFY1* GenBank Accession No.: NT_011875.11).

Presence of Y-chromosome material in patients with TS

Conventional cytogenetic karyotyping showed mosaicism in 37 cases of the 130 TS patients and among them this method revealed 3 patients with Y-chromosome positivity. RT-PCR revealed further six patients with Y-chromosome material, who were initially considered as Y-negatives by standard karyotyping. Clinical features of Y-chromosome material positive patients were similar to other TS patients and they had no signs of virilisation. Prophylactic gonadectomy was carried out in each

of the 9 patients and one of them was diagnosed as having bilateral gonadoblastoma without clinical symptoms.

DNA from paraffin embedded gonadal tissue was available from 3 gonadectomized patients. RT-PCR analysis of the gonadal DNA of these 3 patients revealed that Y-chromosome material was present in 100%, 30% and 10% of cells, whereas corresponding percentages of Y positive cells in peripheral blood DNA samples of the same patients were 36%, 52% and 36%, respectively.

Molecular genetic analysis, hormonal tests and radiologic imaging findings of children members of a family with *RET* codon 804 mutation

Genetic analysis of members of the fourth generation of a family with MEN2 syndrome revealed 2 mutation carrier girls. Their hormonal tests and thyroid gland imaging were normal. Prophylactic thyroidectomy was performed. Histological examination showed normal thyroid gland in the 2.4-year-old girl while C-cell hyperplasia was verified in the 9.3-year-old girl.

Molecular genetic analysis, hormonal tests and radiologic imaging findings of patients with MEN2B

Genetic analysis of the two unrelated patients revealed a point mutation at codon 918 (M918T) of exon 16 of the *RET* proto-oncogene. Genetic analysis of their parents showed the absence of this mutation. Laboratory tests and imaging studies were consistent with MTC. Both patients underwent total thyroidectomy and lymph node dissection. Histological examination confirmed the diagnosis of MTC in both patients. In the first patient postoperative plasma calcitonin level remained high and PET-CT showed the presence of residual lymph nodes in the cervical region. After reoperation with a more extensive lymph node dissection the patient was doing well, but his plasma calcitonin level remained slightly increased. The second

patient had also a repeat neck surgery. Two years after an extensive cervical lymph node dissection she had again high plasma calcitonin level. Phaeochromocytoma was absent in both patients.

Molecular genetic analysis of the *TSHR* gene in a patient with non-autoimmune primary hyperthyroidism

Sequencing of exon 10 of the *TSHR* gene showed a heterozygous substitution of adenine to cytosine at nucleotide position 1888 in DNA samples obtained from both peripheral blood leukocytes and thyroid tissue specimens of the patient. This mutation results in a change of isoleucine to leucine at amino acid position 630. DNA sequencing proved the absence of mutation in parents, indicated that the mutation developed *de novo* in the patient.

Analysis of clinical characteristics of patients with non-autoimmune primary hyperthyroidism reported in the literature

With the analysis of the presentation and course of previously reported 42 cases with non-autoimmune primary hyperthyroidism due to germline activating mutations of the *TSHR* gene I showed that 14 probands had sporadic disease while familial forms occurred in 28 families. I noted that the clinical picture was heterogeneous regarding disease onset and severity as well as the age at the time of diagnosis. All sporadic cases had disease onset before the first year of age, while in several familial cases (18 of the 28 probands) the clinical signs of the disease developed after the first year of age ($p<0,001$, Chi square). I found that in several cases antithyroid drugs proved to be ineffective for controlling hyperthyroidism, and relapse was frequent after subtotal thyreodectomy. Therefore, the most appropriate treatment of affected patients seems to be the removal as much thyroid tissue as possible. Radioiodine therapy may be also applied after thyreodectomy.

CONCLUSIONS

1. I found that classical cytogenetic analysis indicated Y-chromosome mosaicism in three of the 130 Hungarian TS patients corresponding to 2.3% of all cases.

2. I showed that the novel RT-PCR based method applying aspecific DNA binding fluorescent paint that was developed in collaboration between the Laboratory of Molecular Genetics, 2nd Department of Pediatrics, Semmelweis University, Budapest and Test Tube Baby Foundation, Szeged has a specificity similar to conventional PCR method. However, the novel RT-PCR has several advantages over conventional methods and it may replace FISH and conventional PCR used for the detection of Y-chromosome material.

3. I concluded that the frequency of Y-chromosome sequences increased from 2.3% to 6.9% among the 130 TS patients when cryptic Y-chromosome material was detected with the novel RT-PCR method.

4. I showed that GB was present in one Y-chromosome positive TS patient (1/9).

5. With the use of DNA from paraffin embedded gonadal tissue available from 3 gonadectomized patients I showed that Y-chromosome material was present in 100%, 30% and 10% of cells, whereas corresponding percentages of Y positive cells in peripheral blood DNA samples of the same patients were 36%, 52% and 36%, respectively.

6. I demonstrated the task of the pediatrics endocrinologist in the prevention of hereditary MTC in the course of management of the fourth generation of a family with *RET* codon 804 mutation. I emphasize the importance of histological diagnosis when thyroid neoplasia is noted in family history. In two young mutation carrier members of this family prophylactic thyroidectomy was performed and histology revealed C-cell hyperplasia in one of the 2 patients. I

concluded that prophylactic thyroidectomy in mutation carriers improves life expectancy significantly.

7. In my work I drew attention to specific signs of a rare syndrome, MEN 2B and emphasize the importance of early diagnosis by presenting the medical history of two unrelated patients evaluated in our department. Marfanoid phenotype, oral lesions such as diffuse or nodular enlargement of lips, tongue, and buccal mucosa and the presence of gastrointestinal symptoms may support the suspicion of this syndrome. I showed that delayed diagnosis influences unfavorably the results of surgical treatment.

8. I report the first Hungarian patient with non-autoimmune primary hyperthyroidism who proved to have a disease-causing heterozygous activating germline mutation of the *TSHR* gene (I630L). Its absence in parents indicated that the mutation developed *de novo* in this patient. Because of the autosomal dominant inheritance of the disease, genetic counseling will be necessary in members of the next generations of the family.

9. With the analysis of the presentation and clinical course of our patient with non-autoimmune primary hyperthyroidism due to germline activating mutations of the *TSHR* gene and those reported in the international literature I concluded that the most appropriate treatment of affected patients is total thyroidectomy in an early age, which may be followed by radioiodine therapy.

LIST OF ORIGINAL ARTICLES

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Rita Bertalan and Ágnes Sallai contributed equally to this work.

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