Extracellular calcium sensing under normal and pathological conditions
Investigation of *CaSR* gene mutations in Hungarian patients

PhD theses

Judit Tőke, M. D.
Semmelweis University,
Doctoral School of Clinical Medicine

Program leader: Prof. Károly Rácz, Full Professor, M.D., Ph.D., D.Sc.
Supervisor: Miklós Tóth, Associate Professor, M.D., Ph.D.

Critical examiners: László Mátyus, Full Professor, M.D., Ph.D., D.Sc.
Miklós Garami, Associate Professor, M.D., Ph.D.

President of Defence Board: Prof. Ilona Kovalszky, Full Professor M.D., Ph.D., D.Sc.
Members of Defence Board: István Takács, Lecturer, M.D., Ph.D.
László Kovács, M.D., Ph.D.

Budapest
2009

I. INTRODUCTION

The stability of the extra-, and intracellular calcium levels plays a key role in maintaining the homeostasis of living organs. Ionic calcium has been known to function as an important intracellular second messenger for many decades. In addition, a whole series of experimental and clinical studies from the past thirty years provided evidences that extracellular ionic calcium itself is also a first messenger, since it is the ligand of a cell surface G protein-coupled receptor, called calcium-sensing receptor (CaSR). Several years after cloning the human receptor it was proved that inactivating mutations of the *CaSR* gene are responsible for some inherited disorders characterized by defective calcium sensing. The heterozygous inactivating mutations cause symptom-free familial hypocalciuric hypercalcemia (FHH), while the homozygous inactivating mutations result in the life-threatening neonatal severe hyperparathyroidism (NSHPT). The pathogenesis and the severity of the symptoms of NSHPT may differ according to the genotype of the *CaSR* gene. If a gene mutation affects only the paternal allele, the neonatal severe disorder may revert to a life-long benign state with symptomless hypercalcemia resembling to FHH. These diseases are rare, and the data are

limited about their prevalence. The differentiation of these patients from the others with parathyroid hormone (PTH)-dependent hypercalcemia is essential for choosing the adequate therapy. There are few guidelines on clinical investigation of the patients with altered calcium sensing.

The patients with disorders of CaSR, diagnosed at the 2nd Department of Medicine of Semmelweis University, were in the focus of my doctoral investigations. Summarizing the molecular biological data and the clinical experience about these patients, we developed a diagnostic algorithm for PTH-dependent and -independent hypercalcemia, by which these often symptom-free diseases may be recognized more efficiently.

II. OBJECTIVES

The patients of 2nd Department of Medicine of Semmelweis University diagnosed with PTH-dependent hypercalcemia between 1997-2007 were analysed in my work.

1. The first objective of my studies was to summarize the clinical features of our patients with familial hypocalciuric

hypercalcemia (FHH). I intended to perform the mutational analysis of the *CaSR* genes of the patients with clinically diagnosed FHH.

2. My second objective was to analyze the disease course, the clinical and laboratory data of a Hungarian patient with neonatal hyperparathyroidism and to determine the genotype of the *CaSR* gene of the index patient and his relatives, applying molecular genetic methods.

3. I also wanted to investigate the effects of the two *CaSR* point mutations identified in a patient with neonatal hyperparathyroidism on the calcium sensing receptors expressed heterologously in HEK-293 cells. I was also curious about the cell surface expression of the double mutated CaSR in HEK-293 cells.

4. It was also aimed to determine of the relative frequency of the PTH-dependent hypercalcemic disorders, diagnosed in our endocrine department between 1997 and 2007. I would have liked to investigate the possible differences of the clinical and laboratory data between the patients with diseases of different etiology.

III. PATIENTS AND METHODS

Patients

Between 1997 and 2007, 8 patients were diagnosed with FHH based on clinical investigations. The clinical data of a patient with unusual clinical course of NSHPT was investigated in detail. Molecular biological examinations were performed on DNA prepared from peripheral leukocytes of the index patients and his relatives. The index patient was the second child of the family, born in 1995 after an uneventful 41-week gestation to healthy parents with normal birth weight and normal Apgar scores. At the 2nd day of life, the patient developed respiratory distress and generalized hypotonia, and was referred to a perinatal intensive care unit. On admission moderate hypercalcemia with marked hypocalciuria, hypophosphatemia and extremely elevated serum alkaline phosphatase concentrations were detected while the PTH level was in the normal range. X-ray studies indicated generalized skeletal undermineralization. During a 30-day course of intensive conservative medical therapy, his general clinical state gradually improved, and he was mentally and physically completely symptomless during later development. The patient

came to clinical attention again at the age of 6 years, when the boy was referred to a pediatric endocrinologist for evaluation of obesity. Routine laboratory data indicated persisting hypercalcemia while urinary calcium to creatinine clearance ratio (CCCR) was very low (0·0073). X-ray studies showed normal skeletal morphology Neck ultrasonography failed to demonstrate enlarged parathyroid glands. No evidence of renal stones or nephrocalcinosis were detected.

In order to establish the relative frequencies of disorders related to inactivating CaSR gene mutations in our department, clinical and laboratory data of 141 consecutive patients diagnosed with PTH-dependent hypercalcemia were retrospectively reviewed and analyzed.

Clinical examinations

The diagnosis of disorders related to CaSR was based on routine laboratory examinations performed with standard methods. The urinary calcium excretion was determined on a 24-h urine sample and CCCR was calculated with the following formula: calcium concentration of 24-hour collected urinary sample/serum total calcium concentration) / (creatinine concentration of 24-hour collected urinary sample/serum creatinine concentration. CCCR less than 0.01 was considered

to be highly suspicious to FHH. The bone metabolism was characterized with serum alkaline phosphatase (ALP), osteocalcin (OC) and beta-CrossLaps (βCL) levels. The parathyroid function was measured with serum PTH. Osteodensitometric measurements were also performed using dual x-ray absorptiometry on Hologic QDR 4500C equipment. The bone mineral density (BMD) and the BMD z-score values of lumbar spines and of femoral neck. were analysed.

Molecular biological methods

The mutation analyses of CaSR gene were performed at the Molecular Biological Laboratory of 2nd Department of Medicine, Semmelweis University. Genomic DNA was extracted from peripheral blood leucocytes using the DNA isolation kit for mammalian blood. The entire coding region (exons 2–7) of the *CaSR* gene was amplified with polimerase chain reaction (PCR) as described previously. For direct sequencing of amplified exons, standard sequence analysis was performed with LiCOR IR2 and ABI Prism 310 sequencer.

The allelic localisation of *CaSR* gene mutations identified in our NSHPT patient and the cell culture studies were performed

in laboratory of professor Péter Enyedi (Department of Physiology, Semmelweis University).
Total RNA was prepared from peripheral blood leucocytes of the index patient using the guanidium-isothiocyanate, phenol-chloroform method. MMLV reverse transcriptase and random hexamers were used for the reverse transcription of 1 μg of total RNA. The cDNA fragments were amplified with two consecutive PCRs. The purified PCR product was cloned into pCR4-TOPO vector using TOPO TA Cloning Kit and sequenced.

In order to analyse the functional consequences of CaSR gene mutations identified in our NSHPT patient, HEK293 cells were transiently transfected with wild type and mutant CaSR cDNA. Reconstructed human *CaSR* coding sequence (rHuPCaR4·0 in pcDNA3 vector) was provided by Dr Edward M. Brown. The R551K and the A986S point mutations were performed by site-directed mutagenesis with QuickChange kit and PfuTurbo DNA polymerase. The point mutations were verified by DNA sequencing.

The cell surface expression of CaSR bearing point mutations identified in our NSHPT patient were analysed in HEK293

cells. In order to visualise the CaSRs, the receptor was fusioned to red fluorescent protein (RFP). The R551K and the A986S point mutations were performed by site-directed mutagenesis of the reconstructed wild type CaSR-RFP vector with QuickChange kit and PfuTurbo DNA polymerase as described above. The point mutations were verified by sequencing.

Cell culture, transfection

The HEK293 cells were maintained in „Dulbecco's modified Eagle's" medium containing 10% fetal calf serum in 37° C, 5% CO2 thermostate.

The expression of CaSRs in HEK293 cells were analysed with fluorimetry, 48 hours following the transient infection.

Measurement of cytoplasmic calcium ion concentrations of HEK293 cells transfected with CaSR constructs by fluorimetry

Test solutions contained increasing concentrations of CaCl from 0.5 mM to 32 mM. HEK293 cells were loaded with 1 μM fura-PE3 AM intracellular calcium indicator dye at 37 °C. Wild type and mutant CaSR function was characterized by measuring intracellular 340/380-nm fluorescence ratios of fura-PE3. Fluorescence images were acquired by an inverted

microscope with Xenon arc lamp and an intensified CCD camera at 37 °C. The images were analysed by Imagemaster software. Each bath solution was applied for 60 s and the time-averaged fluorescence ratio was calculated. The bath solutions were added sequentially without washout period.

Confocal microscopy

In order to visualize the localization of the wild type and the mutated CaSRs identified in our NSHPT patients, HEK-293 cells transiently transfected with wild type CaSR-RFP and dmCaSR-RFP constructs were analysed using a Zeiss LSM 510 confocal microscope (x63 objective).

Statistical methods

EC50 values (the extracellular Ca2+ concentration which produces 50% of the maximum intracellular calcium response) were determined after sigmoidal curve fitting using Origin 6·0 software (OriginLab, Northampton, MA). Comparison of EC50 values was performed using one-way ANOVA and Tukey's post hoc analysis ($P < 0.05$) with an SPSS software package.

IV. RESULTS

1. **Eight** patients were diagnosed with FHH. In these patients and in 2 of their relatives symptomless hypercalcemia (serum total calcium: 2.84±0.26 mmol/l; mean±SD; normal value: 2.25-2.61 mmol/l) and decreased CCCR values (CCCR: 0.006±0.004) were demonstrated. Mean serum PTH concentration was in the upper normal range (64.8±32.8 pg/ml; normal range: 11-64 pg/ml). BMD Z-scores were in normal range in all examined regions. Serum bone markers were also in the reference range. *CaSR* gene mutations were identified in 2 of the 8 patients (25%). These point mutations are proved to be gene defects causing FHH (P55L és C582Y). These point mutations were also detected in father of one patient (P55L) and in mother of an other patient (C582Y). Known CaSR gene polymorphisms were detected in other 2 patients (R990G, Q1011E). Mutation analysis showed normal CaSR gene sequence in 4 patients.

2. The disease course and clinical parameters of our NSHPT patient confirmed that NSHPT may revert spontaneously to symptomless hypercalcemia that does not disturb normal

physical and mental development. Using DNA sample derived from periferal leukocytes of our NSHPT patient, a novel heterozygous point mutation was identified on codon 551 of the *CaSR* gene (AGG→AAG; Accession no. *Hm040041*) resulting in a conservative arginine→lysine amino acid change in the CaSR protein. This point mutation was absent in patient's relatives. A known heterozygous polymorphism (A986S) was detected on exon 7 in the NSHPT patient and in his father. The known heterozygous R990G polymorphism was detected in his mother. The sister of our patient had no genetic alteration in the CaSR gene. With allele sequence analysis, I demonstrated that the R551K and the A986S point mutations are in the paternal allele in index patient, while the maternal allele contained the normal CaSR gene sequence. Our patient represents the first case of NSHPT with *de novo* heterozygous CaSR gene mutation who was cured without parathyroid surgery.

3. The HEK293 cells expressing wild type CaSR gave an EC_{50} for extracellular calcium concentration of 3.38 ± 0.62 mmol/l (n= 13), while the EC_{50} value of CaSR with the mutation R551K was significantly higher (6·10 ± 0·83

mmol/l; $n = 13$, $P = 0{\cdot}038$). The R551K point mutation was shown to have an inactivating effect for the CaSR resulting in a decreased calcium sensitivity. This effect of R551K mutation was not altered when A986S polymorphism was simultaneously present in the expressed CaSR as the EC_{50} value of double mutated CaSRs was 6.64 ± 1.01 mmol/l (n=11). The calcium sensitivity of wild type and double point mutated receptors coexpressed in HEK-293 cells was similar to those that expressed the wild type receptor alone (EC50 = 3.02 ± 0.25 mmol/l; n=14). This might be explained by the high expression level of CaSRs on HEK-293 cell surfaces compared to normal cells. Confocal images revealed that the double mutant CaSR was targeted predominantly to the cell surface, similarly to the wild type receptors.

4. 141 PTH-dependent hypercalcemic patients were examined. 123 (87.2%) patients presented with sporadic primary hyperparathyroidism (PHPT), 15 (10.6%) patients with familiar PHPT: 11 patients had MEN1 syndrome, 2 patients had MEN2A syndrome. One patient was diagnosed with familiar isolated hyperparathyroidism and another with hyperparathyridism-jaw tumor syndrome. In 3 (2.2%)

patients CaSR gene mutations were detected; in two of them FHH, and in the third one NHPT were diagnosed. The relative frequencies of the these diseases corresponded well to the data in the literature and the indirect estimations. In the patients of less than 30 years of age, only familiar PHPT or diseases related to the deficient function of CaSR were verified. The plasma PTH concentration was elevated in both the sporadic and familiar PHPT cases (in the sporadic cases 137; 69-1301 pg/ml, whereas in the familiar PHPT cases 174.9; 83.8-414.0 pg/ml (median; min-max, respectively). However, the plasma PTH concentration was normal (43.5; 34.1-56 pg/ml) in the patients with mutated *CaSR* gene. Among the complications of PHPT, the frequency of kidney stones and osteoporosis were similar between the sporadic and familiar cases. Our results also confirm that the genetic examinations included in the diagnostic algorithm of PTH-dependent hypercalcemic conditions substantially promote the differential diagnosis of the diseases with hypercalcemia, and may also contribute to the establishment of the final diagnosis and appropriate therapy.

ARTICLES DIRECTLY RELATED TO THE DOCTORAL THESIS

1. **Toke J,** Czirjak G, Patocs A, Enyedi B, Gergics P, Csakvary V, Enyedi P, Toth M: Neonatal severe hyperparathyroidism associated with a novel de novo heterozygous R551K inactivating mutation and a heterozygous A986S polymorphism of the calcium-sensing receptor gene.
   Clinical Endocrinology 67:385-392, 2007
   IF: 3.370

2. **Toke J**, Patocs A, Balogh K, Gergics P, Stenczer B, Racz K, Toth M: Parathyroid hormone-dependent hypercalcemia.
   Wiener Klinische Wochenschrift 121:236-245, 2009
   IF: 0.885

3. Tóth M, **Tőke J,** Horányi J, Stenczer B, Patócs A, Balogh K, Jakab Zs, Szücs N, Gergics P, Varga I, Rácz K, Tulassay Zs: A sporadikus és familiáris primer hyperparathyreosis klinikai és laboratóriumi jellemzői.
   Magyar Belorvosi Archivum 60:154-159, 2007

4. **Tőke J**, Tóth M, Patócs A, Bertalan R, Gergics P, Stenczer B, Speer G, Lakatos P, Rácz K, Tulassay Zs: A kalcium-szenzor gén mutációvizsgálatának hazai tapasztalatai.
   Magyar Belorvosi Archívum 60: 451–454, 2007

5. **Tőke J**, Patócs A, Gergics P, Bertalan R, Tóth M, Rácz K, Tulassay Zs: Az extracelluláris kalcium koncentráció érzékelése egészséges és kóros állapotokban.
   Orvosi Hetilap 150: 781-790, 2009

ARTICLES NOT RELATED DIRECTLY TO THE DOCTORAL THESIS

1. Tóth M, **Tőke J**, Kiss E, Bernád I, Miheller P, Szücs N, Rácz K: Acromegalia McCune-Albright szindrómában.
   Orvosi Hetilap 143:1070-1073, 2002

2. Balogh K, Hunyady L, Patócs A, Valkusz Zs, Bertalan R, Gergics P, Majnik J, **Tőke J**, Tóth M, Szücs N, Gláz E, Fűtő L, Horányi J, Rácz K, Tulassay Zs: Az 1-es típusú multiplex endocrin neoplasia klinikai tünetei, diagnózisa és kezelése. A genetikai vizsgálatok hazai tapasztalatai.
   Orvosi Hetilap 146:2191-2197, 2005

3. Bertalan R, Patócs A, Balogh K, **Tőke J**, Boyle B, Tóth M, Kiss R, Varga I, Gláz E, Rácz K, Tulassay Zs: A phaeochromocytoma örökletes formáinak klinikai és genetikai szűrése.
   Magyar Belorvosi Archivum 59:103-108, 2006

4. Tóth M, **Tőke J**, Fütő L, Varga I, Kiss R, Pusztai P, Szücs N, Adler I, Karlinger K, Horányi J, Czirják S, Pásztor E, Gláz E, Rácz K, Tulassay Zs: A Cushing-szindróma kezelésének eredményei 115 beteg adatainak retrospektív elemzése alapján.
   Magyar Belorvosi Archivum 59:131-138, 2006

5. Majnik J, Patócs A, Balogh K, Luczay A, Török D, Szabó V, Borgulya G, Gergics P, Szappanos Á, Bertalan R, Belema B, **Tőke J**, Sereg M, Nagy ZZ, Sólyom J, Tóth M, Gláz E, Rácz K, Németh J, Fekete Gy, Tulassay Z: Glükokortikoidreceptor gén szekvenciavariánsai és jelentőségük a glükokortikoidok iránti érzékenység meghatározásában.
   Orvosi Hetilap, 147:2107-2115, 2006

6. Halasz Z, **Toke J**, Patocs A, Bertalan R, Tombol Z, Sallai A, Hosszu E, Muzsnai A, Kovacs L, Solyom L, Fekete G, Racz K: High Prevalence of PROP1 Gene Mutations in Hungarian

Patients with Childhood-Onset Combined Anterior Pituitary Hormone Deficiency.
Endocrine 30:255-260, 2006
IF:1.805

7. Tóth M, Vajda Zs, Görömbey Z, Molnár F, Major L, **Tőke J**, Szabolcs I, Szücs N, Kovács L, Kiss R, Czirják S, Fütő L, Gláz E, Góth M, Rácz K: Sinus petrosus inferior katéterezés értékelése az adrenokortikotrop hormon dependens Cushing-szindróma elkülönítő diagnosztikájában
Orvosi Hetilap 148:843-847, 2007

8. **Tőke J**, Tóth M, Tulassay Zs: A csontanyagcsere jellegzetességei a cukorbetegség különböző formáiban.
Magyar Belorvosi Archivum 60:437-442, 2007

9. Pusztai P, **Toke J**, Somogyi A, Ruzicska E, Sarman B, Racz K, Tulassay Z: Plasma ghrelin response to an oral glucose load in growth hormone-deficient adults treated with growth hormone.
Wiener Kliniche Wochenschrift 119:99-103, 2007
IF: 0.885

10. Halasz Z, Bertalan R, **Toke J**, Patocs A, Toth M, Glaz E, Fekete G, Racz K: Laterality disturbance and hypopituitarism. A case report of co-existing situs inversus totalis and combined pituitary hormone deficiency.
Journal of Endocrinological Investigation 31:74-78, 2008
IF: 2.021

11. Fütő L, **Toke J**, Patócs A, Szappanos Á, Varga I, Gláz E, Tulassay Z, Rácz K, Tóth M: Skeletal differences in bone mineral area and content before and after cure of endogenous Cushing's syndrome.
Osteoporosis International 19:941-949, 2008
IF: 3.893

12. Pusztai P, Sarman B, Ruzicska E, **Toke J**, Racz K, Somogyi A, Tulassay Zs: Ghrelin: a new peptide regulating the

neurohormonal system, energy homeostasis and glucose metabolism.
Diabetes Metababolism Research and Reviews 24:343-352, 2008
IF: 3.087

13. Bertalan R, Patocs A, Vasarhelyi B, Treszl A, Varga I, Szabo E, Tamas J, **Toke J**, Boyle B, Nobilis A, Rigo J Jr, Racz K:
Association between birth weight in preterm neonates and the BclI polymorphism of the glucocorticoid receptor gene.
Journal of Steroid Biochemistry and Molecular Biology 111:91-94, 2008
IF: 2.799

14. Sereg M, Szappanos A, **Toke J**, Karlinger K, Feldman K, Kaszper E, Varga I, Glaz E, Racz K, Toth M: Atherosclerotic risk factors and complications in patients with non-functioning adrenal adenomas treated with or without adrenalectomy. A long-term follow-up study.
European Journal of Endocrinology 160:647-655, 2009
IF: 3.239

15. Szappanos A, Patócs A, **Tőke J**, Boyle B, Sereg M, Majnik J, Borgulya G, Varga I, Likó I, Rácz K, Tóth M:
BclI polymorphism of the glucocorticoid receptor gene is associated with decreased bone mineral density in patients with endogenous hypercortisolism.
Clin Endocrinol (Oxf). 2009 Jan 22. [Epub ahead of print]
DOI: DOI: 10.1111/j.1365-2265.2009.03528
IF: 3.373