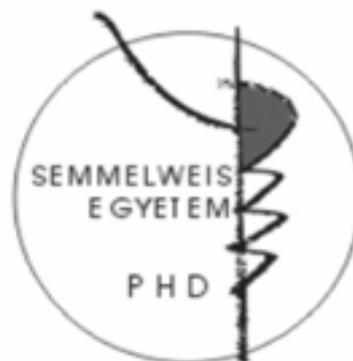


# **PATHOBIOCHEMISTRY OF PANCREATIC SECRETORY TRYPSIN INHIBITOR (SPINK1)**

Extract of PhD dissertation

**Orsolya Király**

Semmelweis University  
School of PhD Studies in Molecular Medicine  
Program: Pathobiochemistry



**Thesis advisor:**            **Dr. Miklós Sahin-Tóth**

Thesis committee:        Dr. Péter Gál  
   Dr. Attila Mócsai

Head of exam committee:    Dr. Péter Enyedi  
Exam committee:                Dr. Beáta Vértessy  
   Dr. Katalin Bartha

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## I. INTRODUCTION

Pancreatic secretory trypsin inhibitor (SPINK1) is secreted from pancreatic acinar cells together with pancreatic zymogens. The role of SPINK1 is to inhibit trypsin activity in the pancreas. Defects of this defense mechanism may result in pancreatic autodigestion and pancreatitis.

Pancreatitis is an acute or chronic inflammatory disease of the pancreas for which no specific therapy exists. Pancreatitis is caused by the premature activation of digestive zymogens within the pancreatic parenchyma which lead to autodigestion, tissue damage and an inflammatory response. Acute pancreatitis causes damage in the pancreas as well as other organs and can be fatal by causing multi-organ failure. Acute pancreatitis is most often caused by gallstones or alcohol. Chronic pancreatitis is a relapsing or continuing inflammation in the pancreas causing chronic pain and irreversible damage to exocrine and endocrine pancreatic functions. The most important predisposing factor to chronic pancreatitis is alcohol, causing 70% of cases in industrialized societies. The risk for pancreatic cancer is significantly increased in patients with chronic pancreatitis.

Generation of animal models of pancreatitis involves supraphysiological stimulation of pancreatic secretion and experimental pancreatitis elicited in these animals does not appropriately model the development of the human disease.

In about 20% of patients no etiological factor can be identified and these patients are diagnosed with idiopathic pancreatitis. In some cases several members of a family are affected and these cases are classified as either familial or hereditary pancreatitis. Hereditary pancreatitis is an autosomal dominant disease with several known genetic factors. Clinically, hereditary pancreatitis is indistinguishable from other types of the disease suggesting a common pathomechanism. Thus, the hereditary type can be an appropriate model for pancreatitis and elucidating its pathomechanism may help

in the prevention and treatment of all forms of the disease. Genetic factors have been implicated in other forms of pancreatitis suggesting a complex pathomechanism involving genetic and environmental factors.

Hereditary pancreatitis can be caused by mutations in human cationic trypsinogen which result in its increased autoactivation. This biochemical effect has been shown by several studies. Other predisposing factors are mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) which cause defects in transport function. These defects are assumed to indirectly increase autoactivation of trypsinogen. It has been shown that defects in CFTR function correlate with risk for pancreatitis.

Trypsin generated by autoactivation of trypsinogen can activate other pancreatic zymogens leading to autodigestion. Pancreatic secretory trypsin inhibitor (SPINK1) protects against autodigestion and pancreatitis by inhibiting trypsin activity in the pancreas. Several pancreatitis associated mutations have been identified in SPINK1 which probably increase susceptibility to pancreatitis by impairing SPINK1 function.

The most common mutation in SPINK1 is N34S which is an established risk factor for chronic pancreatitis. The N34S mutation is found in 12,6% of patients with chronic pancreatitis and 1,9% of the general population. Thus, mutations in SPINK1 are generally thought to be disease modifiers instead of disease-causing mutations.

Other known mutations in SPINK1 are the L14P mutation in the signal peptide of the protein, and mutations R65Q, R67C, D50E and Y54H in the mature inhibitor. The four latter mutations were found in very few patients which makes their disease association uncertain. A polymorphic variant, P55S, has been found in patient with pancreatitis as well as healthy individuals.

Of the five missense mutations, only N34S has been subjected to functional analysis with a negative result. The other five mutations have not been analyzed with respect to their functional effects.

## II. AIMS

Elucidating the functional effects of mutations implicated in hereditary pancreatitis has been the common goal of research projects in our laboratory. In recent years, many studies have looked for mutations in pancreatic secretory trypsin inhibitor (SPINK1) in pancreatitis patients. As a result, several genetic factors have been identified in SPINK1 that increase susceptibility to pancreatitis. However, the functional effects of these mutations are unknown.

**The aims of this thesis project were as follows:**

- 1. Searching for new pancreatitis-associated mutations in the *SPINK1* gene.** Our aim was to find new mutations in SPINK1 which show a stronger association with pancreatitis than previously described variants. Determining the functional effects of such mutations may lead to a better insight into the pathomechanism of pancreatitis.
- 2. Testing the functional effects of SPINK1 mutations.**
  - 2.1.** Determining the effects of the newly identified **L14R mutation and L12F polymorphism and the previously known L14P** mutation in the signal peptide on transcription of the gene and secretion of the inhibitor.
  - 2.2.** Determining the effects of missense mutations in the mature protein (**N34S, R65Q, R67C, P55S, D50E and Y54H**) on transcription, trypsin inhibitory activity and secretion.
  - 2.3.** Verifying the causative relationship between rare mutations **L14R, L14P, R65Q, R67C, D50E and Y54H** and pancreatitis.

### **III. MATERIALS AND METHODS**

#### **Genetic studies**

Analysis of the *SPINK1* gene in pancreatitis patients and healthy controls was performed by the laboratories of Dr Claude Férec (Université de Bretagne Occidentale, Brest, France) and Dr Heiko Witt (Department of Hepatology and Gastroenterology, Charité, Berlin, Germany) using denaturing HPLC and direct sequencing.

#### **Plasmid construction**

SPINK1 cDNA was cloned into a eukaryotic expression vector. Sequence variants were generated by site-directed PCR mutagenesis. Epitope tag for labeling proteins was inserted by PCR. For bacterial expression, the cDNA region coding for the mature inhibitor was cloned into a SUMO fusion vector.

#### **Testing secretion of the inhibitor**

HEK 293T cells were transiently transfected with plasmids encoding SPINK1 variants in native and epitope tagged form. SPINK1 is secreted into the cell media from transfected cells. To compare secretion rates, quantity of secreted SPINK1 was assayed using two independent methods.

#### **A) Assaying secretion through trypsin inhibitory activity**

Secretion of SPINK1 variants was followed for 48 hours after transfection through trypsin inhibitory activity of conditioned media. Samples of media were mixed with human cationic trypsin and residual trypsin activities were determined by photometry using a chromogenic substrate. Residual activities were converted to SPINK1 concentrations using a calibration curve obtained with purified recombinant SPINK1. SPINK1 concentrations were normalized by specific activities of cotransfected  $\beta$ -galactosidase to control for transfection efficiency.

## **B) Assaying secretion with Western blots**

48 hours after transfection, quantity of secreted SPINK1 was assayed in cell media and extracts of transfected cells using Western blots. Samples were subjected to SDS-PAGE and blotted onto a PVDF membrane. Media were assayed using an antibody raised against the native SPINK1 protein as well as a commercial antibody raised against the affinity tag. After the antibody against the native protein raised in rabbit, a secondary antibody raised against rabbit IgG was used. Cell extracts were assayed with the antibody against the affinity tag.

## **Determining trypsin inhibitory activity ( $K_i$ )**

SPINK1 variants were expressed in *Escherichia coli* as SUMO fusion proteins. Trypsin inhibitory activity of SPINK1 variants was determined by *progress curve* analysis against human cationic trypsin. For mutants secreted from HEK 293T cells, SPINK1 was isolated from cell media and inhibitory activities against all human trypsin isoforms were assayed with *progress curve* analysis.

## **Assaying transcription of the SPINK1 gene**

Cells transfected with SPINK1 variants were collected 48 hours after transfection as cell extracts. Total RNA from cell extracts was prepared and reverse transcribed into cDNA. SPINK1 mRNA levels were assayed with semi-quantitative PCR using gene-specific primers on cDNS templates. Control reactions were performed using primers specific for the GAPDH gene.

## IV. RESULTS

- 1. New mutation in the signal peptide of SPINK1 in families with autosomal dominant hereditary pancreatitis.** The L14R mutation was found in a Bulgarian family living in France and a German family. The mutation segregates with pancreatitis through several generations with almost complete penetrance. We also found a polymorphic variant, L12F, which is not associated with pancreatitis and was only found in African populations.
- 2. The new L14R mutation and the previously described L14P mutation abolish SPINK1 secretion, while the L12F variant has no effect.** Secretion of SPINK1 variants was assayed by measuring residual trypsin activities and with Western blots. SPINK1 mRNA levels were the same for all three variants indicating that they do not influence transcription of the gene. SPINK1 mRNA levels were measured by semi-quantitative RT-PCR. Mutants L14P and L14R were not detected in extracts of transfected cells indicating that these mutants are degraded within the cells.
- 3. Missense mutations affecting the mature inhibitor have no major effect on trypsin inhibitory activity.** Inhibitory activity against human cationic trypsin was determined for all variants after expression in *E. coli*. For mutants secreted from HEK 293T cells (N34S and R65Q), inhibitory activities against all three trypsin isoforms was determined.  $K_i$  values were in the subnanomolar range and did not show significant differences.

**4. Rare missense mutations impair secretion of SPINK1. Mutation R65Q significantly reduces while mutations R67C, D50E and Y54H practically abolish secretion of the inhibitor** as assayed by measuring residual trypsin activities and with Western blots.

SPINK1 mRNA levels were the same in all variants except R65Q indicating that most of the mutations do not affect transcription of the gene. Mutation R65Q may reduce transcription efficiency. SPINK1 mRNA levels were measured by semi-quantitative PCR.

Western blots on extracts of transfected cells showed that mutants with impaired secretion can be detected in cells in varying amounts, indicating that mutant proteins are retained and degraded by the cells at different rates.

The polymorphic variant P55S, which is not associated with pancreatitis, has no effect on secretion of SPINK1. Mutation N34S, which is a risk factor for chronic pancreatitis, also has no effect on secretion. Thus, the N34S mutation does not affect either trypsin inhibitory activity or secretion of SPINK1.

## V. CONCLUSIONS

The aim of this thesis research was to determine the functional effects of pancreatitis-associated mutations in pancreatic secretory trypsin inhibitor (SPINK1). From the model of the disease pathomechanism it is generally assumed that these mutations cause a loss of SPINK1 function in the pancreas. However, no experimental evidence has been available for this hypothesis.

Previously identified SPINK1 mutations do not show a strong association with pancreatitis. Thus, these mutations are referred to as disease modifiers instead of disease-causing mutations.

To learn more about the pathomechanism of pancreatitis, we searched for new mutations in SPINK1 which have a strong association with the disease. In two families with hereditary pancreatitis, we identified a new mutation affecting the signal peptide of the inhibitor and causing the amino acid substitution L14R. The new mutation segregates with autosomal dominant hereditary pancreatitis with a very high penetrance. We also found a polymorphic variant, L12F, which is not associated with pancreatitis and is found in the Central African population.

Mutations L14P and L14R abolish secretion of SPINK1 while the L12F polymorphism has no effect on secretion. Transcription efficiencies from the wild-type and mutant genes are identical and an effect on translation is very unlikely given the distance of the mutations from the translation start point. Our results show that mutations L14P and L14R cause **autosomal dominant hereditary pancreatitis** by **abolishing secretion** of the pancreatic secretory trypsin inhibitor. Thus, these mutations should be regarded as **disease-causing mutations** as opposed to disease modifiers.

Next, we investigated the functional effects of missense mutations affecting the mature SPINK1 protein. The most common of these mutations is N34S which is an established risk factor for chronic pancreatitis. The polymorphic variant P55S is found in pancreatitis

patients as well as healthy individuals. The rare mutations R65Q, R67C, D50E and Y54H have been found in very few patients which makes their disease association uncertain.

As these mutations affect the mature inhibitor, they might influence its trypsin inhibitory activity. In the first step of the functional analysis, we determined trypsin inhibitory activities ( $K_i$ ) against human cationic trypsin for all six variants and the wild type inhibitor.  $K_i$  values showed no significant differences indicating that these mutations have no major effect on trypsin inhibitory activity.

Transcription of missense mutants was found to be identical with the exception of the R65Q mutation which may decrease transcription efficiency. Secretion of wild-type, N34S and P55S SPINK1 was the same while the R65Q mutant significantly decreased, and mutations R67C, D50E and Y54H practically abolished secretion of the inhibitor. These mutations probably result in misfolding of the protein and mutant SPINK1 is retained and degraded by cells at varying rates. The strong functional effect of rare mutations suggests that there is a **causative relationship** between these mutations and the development of pancreatitis.

The dramatic effect of mutations D50E and Y54H can be explained by the fact that these two side chains form a hydrogen bond in the crystal structure of recombinant human SPINK1. Our data indicate that this hydrogen bond is essential for proper folding of the inhibitor. The functional effect of mutation N34S is still unknown and might be caused by the intronic mutations reported to be in linkage disequilibrium with N34S.

Our results show that disease-associated mutations in SPINK1 are indeed **loss-of-function mutations** impairing SPINK1 function in the pancreas. Missense mutations in the signal peptide and in the mature protein both result in decreased SPINK1 secretion and thus share a **common pathomechanism** in generating a protease-antiprotease imbalance that results in susceptibility to pancreatitis.

## VI. PUBLICATIONS

### Publications from the material of the dissertation:

- I. **Király O**, Wartmann T, Sahin-Tóth M. Missense mutations in pancreatic secretory trypsin inhibitor (SPINK1) cause intracellular retention and degradation. *Gut* 2007 May 24 (IF.: 7,692)
- II. **Király O**, Boulling A, Witt H, Le Maréchal C, Chen J-M, Rosendahl J, Battaglia C, Wartmann T, Sahin-Tóth M, Férec C. Signal peptide mutations that impair secretion of pancreatic secretory trypsin inhibitor (SPINK1) cause autosomal dominant hereditary pancreatitis. *Human Mutation* 2007 **28**(5):469-76. (IF.: 7,923)

### Other publications:

- III. **Király O**, Guan L, Szepessy E, Tóth M, Kukor Z, Sahin-Tóth M. Expression of human cationic trypsinogen with an authentic N terminus using intein-mediated splicing in aminopeptidase P deficient *Escherichia coli*. *Protein Expr Purif.* 2006 **48**(1):104-11. (IF.: 1,553)
- IV. Kereszturi E, **Király O**, Csapó Z, Tárnok Z, Gádoros J, Sasvári-Székely M, Nemoda Z. Association between the 120-bp duplication of the dopamine D4 receptor gene and Attention Deficit Hyperactivity Disorder: Genetic and molecular analyses. *Am J Med Genet B Neuropsychiatr Genet.* 2007 **144**(2):231-6. (IF.: 3,521)
- V. Kereszturi E, **Király O**, Barta C, Molnár N, Sasvári-Székely M, Csapó Z. No direct effect of the -521 C/T polymorphism in the human dopamine D4 receptor gene promoter on transcriptional activity. *BMC Mol Biol.* 2006 **7**:18. (IF.: 4,485)
- VI. Szántai E, **Király O**, Nemoda Z, Kereszturi E, Csapó Z, Sasvári-Székely M, Gervai J, Rónai Z. Linkage analysis and molecular haplotyping of the dopamine D4 receptor gene promoter region. *Psychiatr Genet.* 2005 **15**(4):259-70. (IF.: 2,366)

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