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Original Article

Genetic study of *SPINK1* and *PRSS1* in idiopathic chronic pancreatitis and allele frequencies of *SPINK1* and *PRSS1* in the southern Thai population♣

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Abstract

Certain cases of chronic pancreatitis (CP) occur without an apparent causative factor, diagnosed as idiopathic CP (ICP). Several studies have reported that ICP was associated with mutations in the *SPINK1* and *PRSS1* genes, but the studies were not conclusive as these polymorphisms can also occur in healthy individuals. In the present study, we aimed to study the genotypes of *SPINK1* and *PRSS1*. Our study recruited nine ICP patients having no known risk factors. Symptoms relating to ICP were recorded. Genetic studies were performed in all ICP patients and 230 healthy controls. *SPINK1* mutations in cases and controls were genotyped by genotyping assay. The entire *PRSS1* gene was sequenced in all cases and controls to look for any mutations. Statistical analysis of genotypes and minor allele frequencies (MAF) was performed. All of the 9 ICP patients had *SPINK1* mutations. The *SPINK1* N34S heterozygous mutation (MAF 2.17%) was detected in 10 out of 230 controls, while the IVS3+2T>C mutation was not detected in any. *PRSS1* mutations were detected in no cases or controls. In summary, *SPINK1* mutations were associated with ICP. *SPINK1* N34S carrier status was found in the general population with low prevalence, while IVS3+2T>C was rarely found in the controls.

Keywords: idiopathic chronic pancreatitis, single nucleotide polymorphisms, *SPINK1*, *PRSS1*

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1. Introduction

Chronic pancreatitis (CP) is a detrimental disease characterized by irreversible fibrosis and progressive inflammation of the pancreatic parenchyma. The condition can be a primary stage of either or both of emasculated pancreatic exocrine and endocrine functions. CP usually presents with classical features such as abdominal pain, steatorrhea and diabetes (Kleeff *et al.*, 2017) Alcohol consumption is the major cause of CP in adult patients, while other factors such as anatomical anomalies or metabolic diseases can worsen pancreatic inflammation (Kleeff *et al.*, 2017; Lerch & Gorelick, 2013). However, 10-25% of individuals with CP have no apparent risk factors, and these cases are classified as idiopathic chronic pancreatitis (ICP) (Wang *et al.*, 2013).

Many studies have reported that genetic factors are potential causes of ICP. (Hasan, Moscoso, & Kastrinos, 2018; Tandon, 2007; Zou *et al.*, 2018). There are several genes reported to be related to the pathogenesis of ICP such as protease serine 1 (*PRSS1*), serine inhibitor Kazal type 1 (*SPINK1*), the cystic fibrosis transmembrane conductance regulator and chymotrypsin C. (Hasan *et al.*, 2018; Wang *et al.*, 2013; Zou *et al.*, 2018). Patients with *SPINK1* and *PRSS1* mutations are at a higher risk of pancreatic dysfunction (Wang *et al.*, 2013; Zou *et al.*, 2018). The majority of genetic susceptibilities in both of these genes are in the form of single nucleotide polymorphisms (SNPs). According to previous studies, there are two common *SPINK1* mutations usually found in ICP, c.101A>G (p.N34S) and IVS3+2T>C (Kalinin *et al.*, 2006; Muller *et al.*, 2019). The *SPINK1* gene is located on chromosome 5q32, and encodes for a serine protease inhibitor to prevent intrapancreatic activation of trypsin or autodigestion. (Hirota, Ohmuraya, & Baba, 2006; Pfützer *et al.*, 2000; Threadgold *et al.*, 2002) (Threadgold *et al.*, 2002).

PRSS1 gene is positioned on chromosome 7q35. This gene provides two major distinct variations of SNPs, c.86A>T (p.N29I) and c.365G>A (p.R122H), both of which are related to ICP (Hirota *et al.*, 2006; Threadgold *et al.*, 2002). Healthy populations possibly have these genetic disorders as well as confirmed patients. The frequencies of these SNPs vary geographically (Hasan *et al.*, 2018; Hirota *et al.*, 2006). Therefore, the frequencies of *SPINK1* and *PRSS1* mutations in healthy population should be considered. To our knowledge, the allele frequencies of *SPINK1* (c.101A>G, p.N34S and IVS3+2T>C) and *PRSS1* (c.365G>A, p.R122H and c.86A>T, p.N29I) mutations in southern Thailand have not been previously reported.

This study aimed to evaluate mutations of *SPINK1* at rs17107315 (c.101A>G, p.N34S), rs148954387 (IVS3+2T>C) together with mutations of *PRSS1* at rs111033566 (c.86A>T, p.N29I), rs111033565 (c.365G>A, p.R122H) in ICP patients. The study aimed to report genetic information of each ICP patient, also the variant frequency of these markers in healthy controls in the southern part of Thailand. Identification of these genetic factors would improve the options for treatment and prevention of this disease. In addition, both genetic information and variant frequency provide important reference data for further studies.

2. Materials and Methods

2.1 Study population and diagnostic criteria

Nine patients (seven males and two females) with ICP, under 50 years, were recruited from Songklanagarind Hospital during 2012-2019. The affected individuals and healthy volunteers provided informed consent to participate in this study. Blood samples were collected from healthy volunteers at the age of 35 to 60 years old. The study was approved by the Human Research Ethics Committee of the Faculty of Medicine, Prince of Songkla University (REC.62-122-10-1). All of the patients resided in southern Thailand, and none had a history of alcohol consumption or precipitating medication. All patients suffered from chronic upper abdominal pain and had clinical features and blood chemistries consistent with pancreatitis, and were diagnosed with acute pancreatitis at first presentation. Their symptoms tended to exacerbate more frequently than patients with ordinary acute pancreatitis. Together with the endoscopic retrograde cholangiography results, the diagnosis of the individuals was classified as ICP.

The electronic medical records of each patient were evaluated based on demographic and clinical features including gender, age at disease onset, fever, jaundice, cholangitis, diabetes mellitus and magnetic resonance cholangiopancreatography (MRCP) features including size of pancreatic duct, form of pancreatic parenchyma, pancreatic calcification and pseudocyst, and location of any pancreatic duct stones. The controls were adult subjects residing in southern Thailand who had no history of alcohol consumption or pancreatic diseases. Genetic information of these healthy controls was used for calculation of MAF for each SNP.

2.2 Molecular analysis

2.2.1 DNA isolation

DNA was extracted from peripheral blood samples taken from each subject by a DNA extraction kit (QIAAMP DNA Mini kit, QIAGEN, Germany). For the healthy controls, leftover DNA from a previous study (Sangkhathat, Maneechay, Chaiyapan, Kanngern, & Boonpipattanapong, 2015) stored at the biological banking system of the Translational Medical Research Center, Faculty of Medicine, Prince of Songkla University was used. Evaluation of DNA quality and quantity was performed using a NanoDrop (Thermo Fisher Scientific, USA) device, which confirmed that the DNA concentration of every sample was at least 50 ng/ µl, which was the minimum required concentration for the current study.

2.2.2 Genotyping methods

To detect *SPINK1* mutations, each DNA sample was genotyped by a TaqMan SNPs Genotyping Assay on the 7500 Fast Real-Time PCR system (Applied Biosystems, USA). The TaqMan probes used for the *SPINK1* variants (rs17107315, rs148954387) were VIC-and FAM-dye-labeled. Each PCR

reaction in a 96-well plate was carried out in a 20 µL sample including 2 µL of DNA template, 10 µL of TaqMan universal master mix (Applied Biosystems, USA), and 0.5 µL of 40X primer probe mixture. The real-time thermal cycler condition was used a 2-step PCR: denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95 °C for 15 seconds and annealing-extension at 60 °C for 60 seconds. Genotypes were classified by an allelic discrimination plot compared with the homozygous and heterozygous positive controls of these SNPs.

As we had no samples with known genotypes of *PRSS1* SNPs (rs111033566, rs111033565) to be used as positive controls, the TaqMan SNPs Genotyping Assay was not suitable for *PRSS1* genotyping. PCR and direct nucleotide sequencing were performed for both *PRSS1* SNPs.

2.2.3 Genotyping quality control

A sample was considered acceptable for analysis based on genotype call rates and concordance, according to the following criteria. The genotype call rate for each run had to be more than 95%. Genotypes of both groups were randomly revalidated in at least 20% of the population; in addition, genotype concordance of the duplicates had to be higher than 99%. Experiments which did not meet these criteria were repeated, and if they failed any one of these requirements again, they were excluded from the final analysis.

2.2.4 Sequencing analysis

Samples with *SPINK1* variants were amplified by a PCR C1000 Thermal Cycler (Bio-RAD, USA). Due to the absence of positive controls with known *PRSS1* SNPs, genotyping experiments were performed with a direct sequencing method using the same protocol as for *SPINK1*. The primers targeting to regions of *SPINK1*, which of both SNPs (rs148954387, rs17107315) used single oligonucleotide.

Primers for *PRSS1* (rs111033566, rs111033565) were designed and synthesized by Thermo Fisher Scientific, as presented in Table 1. The PCR reactions followed the manufacturer's guidelines. Annealing temperatures were individually optimized for all primers (as shown in Table 1). The products were purified by a laboratory-grade purification kit (QIAAMP DNA Mini kit, QIAGEN, Germany), following the manufacturer's protocol. Finally, the products were sequenced by Sanger's sequencing method (ATCG, Thailand). The sequence information was presented as electrophero grams, which could be compared with references from the NCBI genomic database.

2.2.5 Statistical analysis

Simple statistical analysis was used to assess genotype and allele frequencies in the patients and healthy controls. The frequencies of genotypes were also reported as percentages. The minor allele frequencies (MAF) of the SNPs were calculated and information of allele frequency was reported.

3. Results and Discussion

3.1 Baseline patient characteristics

Seven males and 2 females were included in this study (Table 2). Every patient presented with severe abdominal pain and was diagnosed with acute pancreatitis. Three patients had related complications caused by exocrine insufficiency, two with cholestatic jaundice and one with cholangitis. One patient had diabetes mellitus, a long-term complication originating from endocrine pancreatic insufficiency (Table 2). MRCP studies reported atrophic pancreatic parenchyma in four patients (44.44%), pancreatic pseudocyst in two patients (22.22%), intrapancreatic duct stone in seven patients (77.77%) and pancreatic calcification in one patient (11.11%). The position of the intrapancreatic duct stones, sizes of pancreatic ducts and associated anomalies were also noted on the MRCP reports (Table 3).

3.2 Analysis of *SPINK1* and *PRSS1* mutations in the study ICP patients

The genotype data of the nine cases were analyzed for rs17107315 (c.101A>G, p.N34S), rs148954387 (IVS3+2T>C), rs111033565 (c.365G>A, p.R122H) and rs111033566 (c.86A>T, p.N29I). The study results including the mutated genes and genotypes are presented in Table 4. *SPINK1* mutations were found in all patients. There were 2 types of *SPINK1* mutation found in this study, N34S (c.101A>G) in exon 3 and IVS3+2T>C in the consensus splicing donor site on intron 3. Six participants had N34S (c.101A>G) mutation, two of whom harbored a heterozygous mutation of this polymorphism (n=2, 22.22%) while the other participants (n=4, 44.44%) had a homozygous mutation. A heterozygous mutation of IVS3+2T>C was identified in 3 patients (n=3, 33.33%). In addition, one of the patients having the IVS3+2T>C heterozygous mutation also had a heterozygous mutation of N34S (c.101A>G), while a homozygous mutation of IVS3+2T>C was identified in 1 patient (n=1, 11.11%). However, no *PRSS1* mutations (R122H or N29I) were detected in any of the patients. The genotype

Table 1. Primers used for DNA amplification

SNPs	Primer sequence		Product size (bp)	Tm (°C)
	Forward	Reverse		
rs17107315	5' TTG CTT TTC TCG GGG TGA GA 3'	5' CCC AAT CAC AGT TAT TCC CCA GA 3'	303	60
rs148954387	5' TTG CTT TTC TCG GGG TGA GA 3'	5' CCC AAT CAC AGT TAT TCC CCA GA 3'	303	60
rs111033566	5' TAA TTA GCA GAA AGC AAT CAC AGG CT 3'	5' GAC AGG AAG ACT CTG AAC AAT GAC AT 3'	226	59
rs111033565	5' CTG ATA CCA CCC ACT GTT CGT T 3'	5' AGG TGA GAG GAT TCA AGT TAA GGG 3'	244	57

SNPs: single nucleotide polymorphisms, Tm: melting temperature, bp: base pairs

Table 2. Baseline characteristics and presenting symptoms of study patients

Patient (n=9)	Gender	Age of onset	Symptoms					
			Abdominal pain	Fever	Jaundice	AP	DM	Cholangitis
A	Male	41	+	-	+	+	-	-
B	Male	14	+	-	-	+	-	-
C	Male	12	+	-	-	+	-	-
D	Female	27	+	-	+	+	-	-
E	Male	30	+	-	-	+	+	-
F	Male	10	+	-	-	+	-	-
G	Female	13	+	-	-	+	-	-
H	Male	10	+	-	-	+	-	+
I	Male	13	+	-	-	+	-	-

(-) stands for normal condition or nonappearance of symptom (+) stands for the appearance of a symptom, AP : acute pancreatitis, DM : diabetes mellitus

Table 3. Characteristics of symptoms as evaluated by MRCP in each ICP patient

Patients	Pancreatic parenchyma	Pancreatic duct size/mm	Pancreatic calcification	Pancreatic pseudocyst	Intrapancreatic duct stone	Location of duct stone(s)	Associated anomalies
A	Atrophic	15.7	-	-	+	Distal CBD	Pancreatic duct stricture
B	-	11.0	-	-	+	Head	-
C	-	27.0	-	-	+	Head	Double principle duct
D	Atrophic	27.0	-	-	+	Head	-
E	Atrophic	18.2	-	-	+	CBD, Head, Body, Tail	-
F	-	3.8	-	+	-	-	-
G	Atrophic	6.0	-	+	-	-	-
H	-	4.0	+	-	+	Distal CBD	-
I	-	7.0	-	-	+	Head	-

[as above](-) stands for normal condition or nonappearance of each diagnosis, (+) stands for the appearance of each symptom, CBD : common bile duct

Table 4. Genotype distribution of rs17107315, rs148954387, rs111033566 and rs111033565 polymorphisms in patients suffering with both types of pancreatitis and healthy controls

SNP ID	Gene	Chr: position	Allele	Genotype					
				Cases N= 9 (%)			Controls N= 230 (%)		
			(A/B)	AA	AB	BB	AA	AB	BB
rs17107315	*SPINK1*	5: 147828115	A/G	3 (33.33)	2 (22.22)	4 (44.44)	220 (95.65)	10 4.35)	0 (0.00)
rs148954387	*SPINK1*	5: 147828020	T/C	5 (55.55)	3 (33.33)	1 (11.11)	230 (100.00)	0 (0.00)	0 (0.00)
rs111033566	*PRSS1*	7: 142750600	A/T	0 (0.00)	0 (0.00)	0 (0.00)	230 (100.00)	0 (0.00)	0 (0.00)
rs111033565	*PRSS1*	7: 142751938	G/A	0 (0.00)	0 (0.00)	0 (0.00)	230 (100.00)	0 (0.00)	0 (0.00)

[as above](-) stands for normal condition or nonappearance of each diagnosis, (+) stands for the appearance of each symptom, CBD : common bile duct

frequencies of these polymorphisms are shown in Table 4. According to the appearance of these polymorphisms, the MAF of the N34S variant in these subjects was 55.55%, while the IVS3+2T>C variant had 27.77% MAF.

3.3 Analyses of *SPINK1* and *PRSS1* mutations in the healthy controls

The genotype data of the 230 healthy controls were analyzed for the same SNPs of the two genes as in the patients. (rs17107315, rs148954387, rs111033565 and rs111033566). While 2 *SPINK1* variants were found in the patients, only the N34S (c.101A>G) variant was identified in the controls. Moreover, the N34S (c.101A>G) variant found in the healthy controls was heterozygous in all individuals, (n=10, 4.35%). As a result, the MAF of this polymorphism in the healthy controls was 2.17%, while IVS3+2T>C in the healthy controls was 0%. Additionally, the *PRSS1* mutations R122H and N29I were not found in this group of healthy Thai controls. Thus, genotype frequency and MAF of both SNPs were 0%. The details of the genotypes of the *SPINK1* and *PRSS1* polymorphisms in the control group are shown in Table 4.

3.4 Discussion

Even though most CP patients (40-70% of cases) have a known causative risk of the disease, e.g., alcohol consumption or drug use, up to 25% of cases have no identifiable etiology for the CP and are defined as idiopathic CP (ICP) (Kleeff *et al.*, 2017; Lerch & Gorelick, 2013; Wang *et al.*, 2013). Genetic factors may explain the pathogenesis in these cases. Therefore, identification of genetic susceptibility may indicate options of treatment and disease prevention. Various studies have suggested that genetic factors may explain the pathogenesis of at least some of these idiopathic cases (Muller, Sarantitis *et al.* 2019; Pfützer, Barmada *et al.* 2000; Raphael & Willingham 2016; Zou, Tang *et al.* 2018), and especially polymorphisms of *SPINK1* and *PRSS1* (Cho, Shin, & Lee, 2016; Joergensen, Brusgaard, Crüger, Gerdes, & de Muckadell, 2010; Nishimori *et al.*, 1999; Raphael & Willingham, 2016; Schneider *et al.*, 2002; Sobczynska-Tomaszewska *et al.*, 2006). Therefore, identification of genetic susceptibility may indicate options of treatment and disease prevention. In the current study, we evaluated *SPINK1* (N34S and IVS3+2T>C) and *PRSS1* (R122H and N29I) mutations in ICP patients in Southern Thailand. All 9 cases diagnosed as ICP by the classical criteria were found to have mutations on *SPINK1* affecting both alleles, leading to loss-of-function status.

SPINK1, located on 7q32, encodes the serine protease inhibitor Kazal type 1 (Hirota *et al.*, 2006; Schneider *et al.*, 2002; Threadgold *et al.*, 2002). *SPINK1* plays an important role as the first defender against prematurely activated trypsinogen (Pfützer *et al.*, 2000; Schneider *et al.*, 2002). *SPINK1* binds directly to the catalytic site of trypsin to prevent over activation of trypsin. Autodigestion and inflammation ultimately occur following both insufficient trypsin inhibitor and irregular protein structure of the inhibitor (Hirota *et al.*, 2006; Pfützer *et al.*, 2000; Tandon, 2007). Moreover, *SPINK1* mutation has two major variants relating to ICP (Tandon, 2007). A missense mutation of codon 34 (c.101A>G) is the most common variant reported globally (Bhatia *et al.*, 2002; Tandon, 2007). The substitution of asparagine by serine causes a side chain orientation rearrangement of the protein fold in the C-terminus direction and significantly decreases the inhibitory capacity of trypsin inhibitor (Bhatia *et al.*, 2002; Pfützer *et al.*, 2000). The prevalence of N34S varies geographically among different populations. South India has reported the highest prevalence at 42-46% in ICP patients (Bhatia *et al.*, 2002; Midha, Khajuria, Shastri, Kabra, & Garg, 2010; Tandon, 2007; Wang *et al.*, 2013) , while other countries such as Bangladesh and China are around 20% (Schneider *et al.*, 2002) and 0% (Wang *et al.*, 2013). The prevalences of N34S in early onset ICP in some industrialized countries such as the USA and Germany have been reported at 40.4% and 43%, respectively (Wang *et al.*, 2013). In our study, 6 of 9 patients had N34S mutations, in which four were homozygous mutations and the other two were heterozygous mutations. In cases with a N34S heterozygous mutation, another mutation on IVS3+2T>C may explain the loss of both alleles. In cases with heterozygous mutation also corresponded to the increase of risk factor by genetic susceptibility, which could increase the risk of pancreatitis from environmental factors (Threadgold *et al.*, 2002). *SPINK1* N34S was reported as autosomal recessive by most studies, however autosomal pattern was also found in some report (Barman, Premalatha, & Mohan, 2003; Hirota *et al.*, 2006; Tandon, 2007; Threadgold *et al.*, 2002). The N34S genotype frequency in the controls of the current study was 4.35% and the minor allele frequency (MAF) in these healthy controls was 2.17% which was similar to a study from India (Bhatia *et al.*, 2002). The MAF of the N34S mutations has also been found to vary in studies from different regions, such as Germany and Liverpool, UK which reported a 2.5% variation of prevalences among the controls and 1.25% MAF (Threadgold *et al.*, 2002). In our study, the N34S mutation was identified in all 10 healthy controls as heterozygous mutations. All the mutation carriers did not have any sign of disease, consistent with the autosomal recessive trait of this mutation (Pfützer *et al.*, 2000).

IVS3+2T>C is another common *SPINK1* pathogenic mutation caused by substitution of an intervention sequence at intron 3. The IVS3+2T>C mutation causes skipping of the whole of exon 3, where the trypsin binding site is located, leading to the loss of trypsin inhibitor (Wang *et al.*, 2013). In this study, IVS3+2T>C was identified in four out of nine patients, one with a homozygote and three with heterozygotes. There have been several previous reports indicating that this mutation was often found in Asian populations, especially Japan, Korea and China (Hasan *et al.*, 2018; Kume *et al.*, 2005; Nishimori *et al.*, 1999). According to a study done on Chinese and Japanese patients, ICP patients carrying *SPINK1* IVS3+2T>C mutation frequently had early onset of pancreatitis (Wang *et al.*, 2013). However, the *SPINK1* IVS3+2T>C mutation have been found in only low prevalences in western countries. A cohort study from Germany reported that the IVS3+2T>C mutation prevalence was 1% in ICP patients .(Witt *et al.*, 2000) Another study from England reported that only 0.03% of ICP patients had this variant.(Pfützer *et al.*, 2000) In both our study and a study from Japan, no IVS3+2T>C variants were detected in healthy subjects. IVS3+2T>C–related ICP has also been identified as an autosomal recessive trait (Hasan *et al.*, 2018; Hirota *et al.*, 2006). In one study, an ICP patient having the heterozygous mutation of this variant also had a compound heterozygous mutation of *SPINK1*. This patient had the baseline characteristics of ICP such as early onset, atrophic pancreatic parenchyma and chronic abdominal pain, and the study concluded that the compound heterozygous mutation may have contributed to the development of ICP in this patient. Some reports have suggested that the heterozygous variant of IVS3+2T>C could be a disease risk factor or disease modifier, while some articles have reported an autosomal dominant pattern of SPINK1 inheritance, which may explain the other heterozygous cases found in our study (Király *et al.*, 2007; Pfützer *et al.*, 2000).

PRSS1, located on 7q35, is another gene whose mutations cause more severe damage than *SPINK1* (Cho *et al.*, 2016; Hirota *et al.*, 2006). Cationic trypsinogen, encoded by *PRSS1* (protease serine 1), is a zymogen precursor of trypsin (Hasan *et al.*, 2018; Shelton, Umapathy, Stello, Yadav, & Whitcomb, 2018; Threadgold *et al.*, 2002). Cationic trypsinogen converts inactive pancreatic zymogen into an active digestive enzyme leading to premature activation before excretion from the pancreas (Hasan *et al.*, 2018; Raphael & Willingham, 2016). *PRSS1* mutations normally occur at exons 2 and 3 (Tandon, 2007). There are two

commonly identified pathogenic SNPs of *PRSS1*. A missense variant (c.365G>A) in exon 3 results in amino acid replacement from arginine to histidine substitution (p.R122H) leading to elimination of a trypsin cleavage site and loss of trypsin inactivation (Cho *et al.*, 2016; Hasan *et al.*, 2018; Raphael & Willingham, 2016). Another substitution from A to G in codon 29 of *PRSS1* (c.86A>T, p.N29I) has an effect on the regulatory site (Cho *et al.*, 2016). Neither of the R122H or N29I mutations were found in any cases or healthy controls in our study, similar to other Asian studies in which *PRSS1* mutations were reported at low prevalences or even absent in ICP patients and healthy controls (Chang *et al.*, 2009; Cho *et al.*, 2016; Nishimori *et al.*, 1999; Oh *et al.*, 2009). These mutations have been reported to have an inheritance pattern of autosomal dominant and are considered to be a pathogenic variant (Hasan *et al.*, 2018; Joergensen *et al.*, 2010; Nishimori *et al.*, 1999; Tandon, 2007). The prevalence of *PRSS1* mutations among pancreatitis patients is different among geographically diverse populations, although most have been reported to be heterozygous mutations (Cho *et al.*, 2016; Hasan *et al.*, 2018; Hirota *et al.*, 2006; Wang *et al.*, 2013; Zou *et al.*, 2018).

A limitation of our study was the low number of ICP cases. The allele frequencies of SNPs in both genes in ICP patients need further study before conclusive prevalences can be drawn. Therefore, further studies should be based on larger numbers of both ICP cases and controls for better power of analysis, and also both ICP patients and mutated controls should be followed up for longer periods of time, due to the possibility of long term complications and disease onset.

4. Conclusions

In conclusion, *SPINK1* mutations could be identified in all our ICP patients. The *SPINK1* N34S mutation was also found in healthy individuals from southern Thailand, but with low prevalence and exclusively heterozygous, indicating carrier status. Genotyping of *SPINK1* is advisable in ICP patients and the genotyping should begin with N34 genotyping. In cases with a homozygous N34S variant, the lesion is likely a causative mutation. If there are no variants or only a heterozygous one on N34, IVS3+2T>C should be looked for. *PRSS1* genotyping can be reserved for cases without *SPINK1* pathology.

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