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Study of a panel of genetic mutations in fibrocalcific pancreatic diabetes (FCPD): SPINK1 (N34S) mutation unlikely to be relevant

Vijay Budhwar, Susmita Dutta, Kaushik Pandit, Pradip Mukhopadhyay, Nitai P. Bhattacharyya & Sujoy Ghosh✉

Panel of known genetic mutations (SPINK1, PRSS1, PRSS2, CTRC, and CFTR) in patients with Fibrocalcific pancreatic diabetes (FCPD)compared to Type 2 Diabetes (T2DM) and healthy controls with emphasis on SPINK1 (N34S) mutations. Whole blood samples were used to detect mutations by PCR followed by Sanger sequencing. In-silico analysis of N34S performed, to explore role in pathogenesis. Isolated SPINK1 N34S mutations found in 5.88%, 6% and 2% in FCPD, T2DM, controls respectively (p = ns). In-silico analysis of N34S variant: conflicting role. 2/51 (3.92%) SPINK1 (IVS1-37 T > C) positive, 2/51 (3.92%) SPINK1 P55S positive, 1/51 (2%) SPINK 1 (IVS3 + 2 T > C) positive and none of them SPINK1 (IV3-69insTTT) positive and none of these variants found in T2DM & healthy individuals. PRSS1, CTRC exon 2–3 mutation was found 4/51 (7.8%) and 1/51 (2%) patients of FCPD respectively. None of the patient had mutations in PRSS2, CTRC Promoter region & exon 1, CTRC exon 4–5, CTRC exon 6, CTRC exon 7–8, CFTR ΔF508, CFTR G551D, CFTR G542X, CFTR R117H and CFTR W1282X. Different variants of SPINK1, PRRS1 and CTRC were found in FCPD. Isolated SPINK1 N34S unlikely to cause disease by itself.

Keywords FCPD, Genetic mutations and SPINK1 (N34S)

Disorders of pancreas causing diabetes is classified as pancreatic diabetes[1,2], also referred as type 3c diabetes as per the (American Diabetes Association classification) and it accounts for less than 1% of all cases of diabetes across the world[3].

While alcohol induced chronic pancreatitis is the commonest cause in this group, in tropical countries like India, the etiology frequently includes, and 'tropical chronic pancreatitis' (TCP) which is characterized by calculi formation in the pancreatic duct in addition to the chronic inflammation of the pancreas[4,5].The diabetes resulting from TCP is called Fibrocalcific pancreatic diabetes (FCPD).

The exact etiology of FCPD remains unclear. Studies have suggested association of certain gene variants with exocrine pancreatic function, and the risk of developing TCP and FCPD. Most common genetic mutation observed to be associated with FCPD is Serine protease inhibitor Kazal type 1(SPINK1)[6]. Association of Cationic trypsinogen (PRSS1), Anionic trypsinogen (PRSS2), Chymotrypsinogen C (CTRC), Cystic fibrosis transmembrane conductance regulator (CFTR) gene mutations too have been observed, though the data is limited& conflicting[7,8].

SPINK1, also known as pancreatic secretory trypsin inhibitor (PSTI), plays a major role in protecting pancreatic damage from unregulated trypsinogen activation. The human PSTI gene on chromosome 5 is approximately 7.2 kb long and consists of four exons[9]. The SPINK1 gene codes for a 56–amino acid mature peptide that is synthesized in pancreatic acinar cells. It is estimated that SPINK1 has the ability to inhibit about 20% of total potential trypsin activity within the pancreas thus providing the first line of defense against pancreatitis from aberrant intra-pancreatic activation of trypsinogen and subsequent pancreatitis[10].

Previous studies in FCPD patients have reported a high frequency of the N34S variants in SPINK1 gene, ranging from 33% to 45.6%[6,11,12]as compared to controls which ranged from 1.3% to 4.7%. In a study by Hassan et al., which included participants from Southern Indiaand Bangladesh, the N34S variation was present in 33% of patients with FCPD[6]. In comparison, only 4.4% of Non Diabetes participants and 3.7% of patients with Type 2 diabetes carried the N34S variation in that study. Several other intronic variants have been

Department of Endocrinology and Metabolism, Institute of Post Graduate Medical Education & Research, 244 AJC Bose Road, Kolkata 700020, India. ✉email: drsujoyghosh2000@gmail.com

reported to be associated with FCPD. The variants observed include variations observed in the SPINK1 gene such as – IVS1 − 37 T > C (c.56-37T > C), IVS3-69insTTTT (c.195-66_195-65insTTTT), and IVS3 + 2 T > C (c.194 + 2 T > C) and SPINK1 P55S mutations[6].

The presence of certain mutations does not imply causation of disease and is neither taken to be significantly associated with a disease condition, without comparing its association with other similar disease states and healthy individuals.

The objective of the present study was to check a panel of known genetic mutations (SPINK1, PRSS1, PRSS2, CTRC and CFTR) in patients with Fibrocalcific pancreatic diabetes (FCPD)and compare the occurrence of such mutation in FCPD as compared to subjects with Type 2 Diabetes Mellitus (T2DM) and healthy controls.

We especially wanted to determine whether isolated SPINK1 (N34S) mutations were more commonly found in FCPD, as compared to those with T2DM and healthy controls.Finally we wanted to explore the pathogenic nature of these SPINK1 (N34S) mutations in an in-silico analysis.

Materials and methods

This was a single-center prospective study, approved by Institutional Ethics Committee, Institute of Post Graduate Medical Education and Research, Kolkata, West Bengal, India. The Declaration of Helsinki was followed when conducting the study. Every patient was given a thorough explanation of the purpose and nature of every procedure before providing their written consent. We recruited patients of FCPD, age > 18 years were recruited from the Endocrinology Outpatient clinic for the study. Furthermore, controls were recruited as healthy individuals without diabetes and T2DM patients who were recruited based on similar ethnic backgrounds. Appropriate radiological investigations were conducted to exclude any abnormal pancreatic morphology. Diagnosis of FCPD was made based on fulfillment of the following criteria described[13]:

1. Diagnosis of diabetes mellitus as per the American Diabetes Association criteria.
2. Evidence of chronic pancreatitis based on radiological evidence of ductal calcifications.
3. Absence of other known causes of pancreatitis such as alcoholism, hypertriglyceridemia, hypercalcemia, biliary duct stones and anatomical abnormalities of the pancreas.

Each patient completed a proforma that included information about their medical history, current medications, age at diabetes onset, and duration of diabetes, steatorrhea, and family history of diabetes. They then had a thorough clinical examination, which included a determination of any micro- and macrovascular problems. To perform retinal screening, digital fundus imaging was used. Using a hand-held biothesiometer, the vibration perception threshold (VPT) for neuropathy was determined (Vibrometer, Diabetic Foot Care, Madras Engineering Service, India). Average of three measurements was taken.

The VPT was measured at six positions and the highest score was taken. Neuropathy was defined by the VPT score, which was categorised as follows: normal (< 15 V), mild (15–20 V), moderate (20–25 V), and severe (> 25 V)[14]. Blood samples were taken for the following tests: HbA1c (average glycosylated haemoglobin), lipid profile, liver function test, renal function test, complete blood count (CBC), and plasma glucose fasting. Using a Bio-Rad D10, high performance liquid chromatography was used to measure HbA1c. An automated haematology analyzer measured the CBC. This autoanalyzer (Cobas, Integra 400 Plus; Roche) assessed LFT, RFT, plasma glucose, and lipid profile.

Genomic DNA isolation

Venous blood samples were collected in ethylenediamine tetra acetic acid (EDTA) vials from FCPD patients, T2DM and healthy control. Blood samples were collected from family members (1st degree relatives) of mutation positive FCPD patients. Genomic DNA was isolated from 200 µl of blood samples by using NucleoSpin® Blood kit (NucleoSpin® Blood 250 preps; Cat No: 740951.250; MACHEREY–NAGEL).

Quantification and purity

Quantification and purity of isolated DNA from blood were determined by measuring absorbance at 260 and 280 nm using a Bio Spectrometer (Eppendorf Bio Spectrometer® basic) in duplicate. To check the purity of DNA, a ratio of absorbance at 260 and 280 nm ranging ~1.8 was used and also check the DNA contamination by measuring absorbance at 260/230 nm. While the 260/230 ratio is more informative, good quality DNA. To check the purity of DNA and for high-quality DNA, the 260/230 ratio was 2.0–2.2 was used. Concentration of DNA was recorded in ng/mL. All samples in our study satisfying this criterion were included.

Mutation screening for SPINK1 gene

Using the polymerase chain reaction (PCR), each of the SPINK1 gene's four exons and surrounding intronic regions was amplified independently. Supplementary Table 1 provides a summary of the primer nucleotide sequences for the PCR amplifications. The primer sequences were selected based on prior research[9]. The reaction programme consisted of a hot start (5 min at 94 °C), 35 more cycles (1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C), and 7 min of incubation at 72 °C. Agarose gel electrophoresis verified the amplification, and post-PCR purification followed next. Using the BigDye® Terminator v3.1Cycle Sequencing Kit and the ABI 3730 XL DNA Analyzer, the purified PCR products were sequenced.

Mutation screening for PRSS1 gene

PCR was performed to detect the mutations of the PRSS1 gene (R122H & N29I) in exons 3 and 2. Supplementary Table 2 provides a summary of the primer nucleotide sequences for the PCR amplifications. The primer sequences were selected based on prior research[15]. The VeritiPro Thermal Cycler (Applied Bio system) was used to perform

the amplification. The parameters were as follows: 35 cycles of denaturation at 94° C for 30 s, primer annealing at a specified temperature for 30 s, extension at 72° C for 30 s, and final extension at 72° C for 7 min. The initial denaturation took place at 95° C for 2 min. After staining with ethidium bromide, the PCR products were separated on a 2% agarose gel using a DNA size marker of 50–1000 bp. The gel documentation system (Biorad) was then used to visualise and semi-automatically evaluate the results. Using the BigDye® Terminator v3.1Cycle Sequencing Kit and the ABI 3730 XL DNA Analyzer, the purified PCR products were sequenced.

Mutation screening for PRSS2 gene

The PRSS2 gene underwent mutational analysis by the use of PCR restriction fragment length polymorphism analysis. Because of the significant sequence homology between PRSS1 and PRSS2, and since we had occasionally encountered difficulties with direct sequencing using the original PCR product as a template, we perform nested PCR.

Using the 1:200 dilutions of the original PCR results, we carried out the nested amplification for exon 4 following the first amplification. Supplementary Table 3 provides a summary of the primer nucleotide sequences for the PCR amplifications. The primer sequences were selected based on prior research[16]. 30 cycles of 94 °C for one minute, 58 °C (for the first amplification/first PCR) or 64 °C (for the nested amplification/second PCR) for 1 min, and 72 °C for 1 min were the cycle conditions. The nested PCR products underwent semi-automated analysis by the gel documentation system (Biorad) after being digested with Fast Digest Hpy8I (Thermo Scientific Cat. No. ER1571) and electrophoresed on a 2% agarose gel with a DNA size marker of 50–1000 bp. The results were then stained with ethidium bromide. In each case, the BigDye® Terminator v3.1Cycle Sequencing Kit and the ABI 3730 XL DNA Analyzer were used to do Sanger sequencing.

Mutation screening for CTRC gene

Based on the published nucleotide sequence (GenBank # NT_004873), we designed primers complementary to intronic regions flanking exons 1–8 of CTRC. Supplementary Table 4 provides a summary of the primer nucleotide sequences for the PCR amplifications. The primer sequences were selected based on prior research[17]. In the VeritiPro Thermal Cycler (Applied Bio system), amplification was performed as follows: five minutes of initial denaturation at 94 °C; thirty-five cycles of denaturation at 94 °C for two minutes, primer annealing at 60° for two minutes, two minutes of extension at 72 °C, and ten minutes of final extension at 72 °C.

After staining with ethidium bromide, the PCR products were separated on a 2% agarose gel using a DNA size marker of 50–1000 bp. The gel documentation system (Biorad) was then used to visualise and semi-automatically evaluate the results. In each case, the BigDye® Terminator v3.1Cycle Sequencing Kit and the ABI 3730 XL DNA Analyzer were used to do Sanger sequencing.

Mutation screening for CFTR gene

Using PCR, we identified mutations in the CFTR gene. Supplementary Table 5 provides a summary of the primer nucleotide sequences for the PCR amplifications. The primers were selected based on prior research[18]. In the VeritiPro Thermal Cycler (Applied Bio system), amplification was performed as follows: five minutes of initial denaturation at 94 °C; thirty-five cycles of denaturation at 94 °C for two minutes, primer annealing at 60° for two minutes, two minutes of extension at 72 °C, and ten minutes of final extension at 72 °C. After staining with ethidium bromide, the PCR products were separated on a 2% agarose gel using a DNA size marker of 50–1000 bp. The gel documentation system (Biorad) was then used to visualise and semi-automatically evaluate the results. Sanger sequencing was performed using ABI 3730 XL DNA Analyzer and BigDye® Terminator v3.1Cycle Sequencing Kit in all cases.

We checked nature of these particular variants (pathogenic or not) (SPINK1 N34S) from the Genome Aggregation Database (gnomAD) (https://gnomad.broadinstitute.org/gnomAD v2.1.1).

Sample size

For sample size calculation, we considered approximate prevalence of DM to be 10%[19]and prevalence of FCPD amongst DM to be 0.3%[20]. We recruited 51 cases of FCPD by non-probability purposive sampling in our study considering fitted mean sample size for rare diseases with prevalence 1–5/10,000 to be 35.6 (CI: 23.3–54.3)[21]. Double the number of cases amongstT2DM and healthy individuals were taken as controls with aforementioned inclusion criteria.

Statistical analysis

Statistical analyses were performed using SPSS software (version 21; SPSS, Inc. Chicago, IL, USA). Categorical variables were expressed as frequencies and percentages. The data was tested for normality using Kolmogorov–Smirnov test. Parametric data are presented as mean ± SD and non-parametric continuous data expressed as median with inter quartile range. The analysis of numerical variables was performed using the independent sample t test. The comparison of categorical data parameters was performed by using the Chi-square test / Fisher's exact test. P value < 0.05 was considered statistically significant.

Results

A total of 51 subjects with FCPD, 100 subjects with T2DM and 100 healthy controls were recruited. Clinical and demographical profile of FCPD patients is shown in Table 1.

Variable		FCPD (n = 51; male = 21, female = 31)	T2 DM (n = 100; male = 40, female = 60)
Age in years	Male	37.42 ± 10.33	43.42 ± 10.22
	Female	32.66 ± 8.17	39.34 ± 9.67
	Overall	34.62 ± 9.33	40.64 ± 10.32
Age at detection of diabetes	Male	32.66 ± 10.57	37.36 ± 10.62
	Female	25.89 ± 8.15	33.8 ± 9.84
	Overall	28.74 ± 9.74	34.64 ± 10.43
Duration of Diabetes (years)		5.9 ± 6.42	6.1 ± 4.73
BMI (kg/m^2)	Male	20.34 ± 3.0	25.72 ± 2.96
	Female	19.9 ± 2.88	26.3 ± 3.15
	Overall	20.99 ± 2.91	25.8 ± 3.04
Waist Hip Ratio	Male	0.89 ± 0.05	0.94 ± 0.04
	Female	0.86 ± 0.04	0.91 ± 0.05
Pain abdomen		88%	Nil
	Before detection of DM	58%	
	After detection of DM	24%	
	Simultaneous	6%	
Steatorrhea		61%	Nil
	Before detection of DM	25%	
	After detection of DM	32%	
	Simultaneous	4%	
Family history of FCPD		Nil	Nil
Family history of pancreatic cancer		Nil	Nil
Family history of DM		35%	46%
Diabetic Keto acidosis		Nil	Nil
HbA1C (%)		7.98 ± 1.28	7.4 ± 1.31
Insulin (U/kg)		0.55 ± 0.27	0.26 ± 0.18
VPT (> 25 V)		14%	20%
Retinopathy		2%	18%
Albuminuria (> 30mcg/gm)		8%	14%
Cerebrovascular accident		2%	3.3%
Myocardial infarction		None	3.3%
Ankle Brachial Index (< 0.9)		None	6.6%

Table 1. Clinical and demographical profile of FCPD & T2DM Patients.

Genetic analysis

The overall study results, mutation analysis and number of mutations observed in FCPD patients are illustrated in Fig. 1. Over All 23.5% (12/51) patients were positive for known genetic mutations. Total of 18 mutations were detected in 51 patients with 4 patients having 2 mutations and one patient had 3 known mutations.

SPINK1 mutation

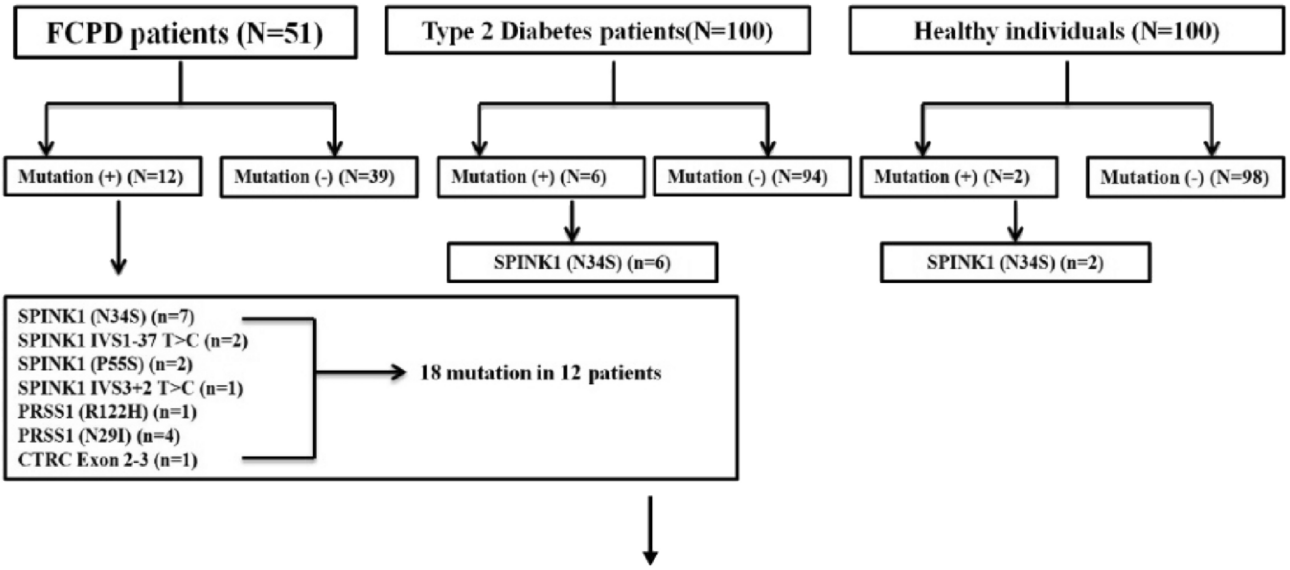
In SPINK1, we have looked for SPINK1 (N34S), SPINK 1 (IVS1-37 T > C), SPINK1 P55S, SPINK 1 (IVS3 + 2 T > C), SPINK1 (IV3-69insTTT). These variants are present in overall 8/51 (15.7%) FCPD patients. SPINK1 N34S (homozygous mutations) along with other mutations was present in 7/51 (13.7%) of FCPD (Table 2) cases.

Isolated SPINK1 (N34S) mutation

Isolated SPINK1 N34S was present in only 3/51 (5.88%) FCPDcases. In addition 6/100 (6%) of T2DM, 2/100 (2%) of healthy controls (who radiologically had normal pancreas) were also SPINK1 N34S positive and hence isolated SPINK1 (N34S) association with FCPD was found to be non significant (Table 2).

4 FCPD cases, in addition to SPINK1 N34S also had other mutations. 2 Patients (case no 1 and 21) were both SPINK1 (N34S) & SPINK1 (P55S) positive, case no 2 had both SPINK1 (N34S) & SPINK 1 (IVS1-37 T > C) and case no 16 had SPINK1 (N34S), SPINK 1 (IVS3 + 2 T > C) & PRSS1 (N29I) positivity. None of the T2DM and healthy controls had these additional variants positivity.

2/51 (3.92%) were SPINK 1 (IVS1-37 T > C) positive, 2/51 (3.92%) were SPINK1 P55S positive, 1/51 (2%) were SPINK 1 (IVS3 + 2 T > C) positive and none of them were SPINK1 (IV3-69insTTT) positive and none of these variants were found in T2DM & healthy individuals.



FCPD Case ID	Mutations in FCPD
01	SPINK1N34S, SPINK1 P55S
02	SPINK1N34S, SPINK1 IVS1-37T>C
05	SPINK1N34S
06	PRSS1N29I
07	SPINK1 IVS1-37T>C
10	PRSS1N29I
13	CTRC EXON 2-3
16	SPINKN1N34S, SPINK 1 IVS3+2T>C, PRSS1N29I
21	SPINKN1N34S, SPINK1 P55S
22	PRSS1 R122H, PRSS1N29I
25	SPINK1N34S
48	SPINK1N34S

Fig. 1. Mutation data of FCPD, Type 2 DM & healthy individuals.

Variant	No. of FCPD (n = 51), %	No. of T2DM (n = 100), %	No. of Healthy (n = 100), %	P value
Isolated SPINK1 (N34S) mutation	(3/51); 5.88%	(6/100); 6%	(2/100); 2%	ns
SPINK1 (N34S) along with other mutations	(4/51); 7.84%	(0/100); 0%	(0/100); 0%	0.0003
Overall SPINK1 mutation	(8/51); 15.68%	(6/100); 6%	(2/100); 2%	0.004

Table 2. Comparison of SPINK1 mutations in FCPD, T2DM and healthy Controls.

Mutation analysis of family members of FCPD patient with SPINK1 positivity

The first degree relatives of these mutation positive cases were screened for mutations. 19 family members of 7 mutation positive patients gave their consent. All of them were asked for history of pain abdomen and steatorrhea; underwent USG (to look for any pancreatic abnormality) and HbA1C testing. For case no 25 (who was herself SPINK1N34S positive), patient's mother, father and brother gave samples for mutation analysis and

her father was also positive for SPINK1 (N34S). He was asymptomatic, euglycemic and had normal pancreas on USG. Pedigree chart is shown in supplementary Fig. 1.

In-silico analysis to look the pathogenic nature of SPINK1 (N34S) mutations

We checked nature of these particular variants (pathogenic or not) (SPINK1 N34S) from the Genome Aggregation Database (gnomAD) (https://gnomad.broadinstitute.org/gnomAD v2.1.1) and there was conflicting interpretations of pathogenicity of SPINK1 (N34S) shown in Fig. 2. The SPINK1 N34S variant has been identified in FCPD, T2DM & also in healthy individuals. When these variant was cross checked in the Genome Aggregation Database (gnomAD) (https://gnomad.broadinstitute.org/gnomAD v2.1.1) it was found to have conflicting role in pathogenesity raising suspicion about its association with FCPD.

PRSS mutation

Overall 5 PRSS mutations were detected in 4/51 (7.84%) patients. All of them had PRSS1 (N29I) mutation positive and 1 (2%) patient was PRSS1 (R122H) positive in addition to PRSS1 (N29I) mutation. None of them were PRSS2 (G191R) positive. Case no 16 was SPINK1 (N34S) & SPINK 1 (IVS3 + 2 T > C) & PRSS1 (N29I) positive. PRSS1 mutations data shown in FCPD, T2DM and healthy controls were shown in supplementary table 6.

Mutation analysis of family members of FCPD patient with PRSS1 positivity

For case no 10, (who was himself positive for PRSS1N29I) patient's parents and all siblings (2 brothers & 2 sisters) gave sample for mutation analysis. Only one among them (younger sister) gave history of intermittent pain abdomen and steatorrhea. She also had thinned out pancreas on USG, however MPD was not dilated and there was no calcification. Like the index patient she was also positive for PRSS1N29I. Younger brother of same patient though asymptomatic, had thinned out pancreas along with dilated MPD and parenchymal calcification on USG. He however had h/o alcohol intake (~ 14 units/week). He was negative for known mutations. Pedigree chart was shown in supplementary Fig. 2.

CTRC & CFTR gene mutation

Only 1 patient (case no 13) among 51 FCPD patients (2%) was CTRC Exon 2–3 positive. None of the patient had mutations in CTRC Promoter region & EXON 1, CTRC EXON 4–5, CTRC EXON 6, CTRC EXON 7–8, CFTR ΔF508, CFTR G551D, CFTR G542X, CFTR R117H and CFTR W1282X.

Subgroup analysis of mutation positive and mutation negative patients

Among 51 FCPD patients, 12 (23.52%) were detected to have known genetic mutations and 39 (76.47%) didn't harbor the same in our study. A subgroup analysis showed that mutation positive patients were no different to

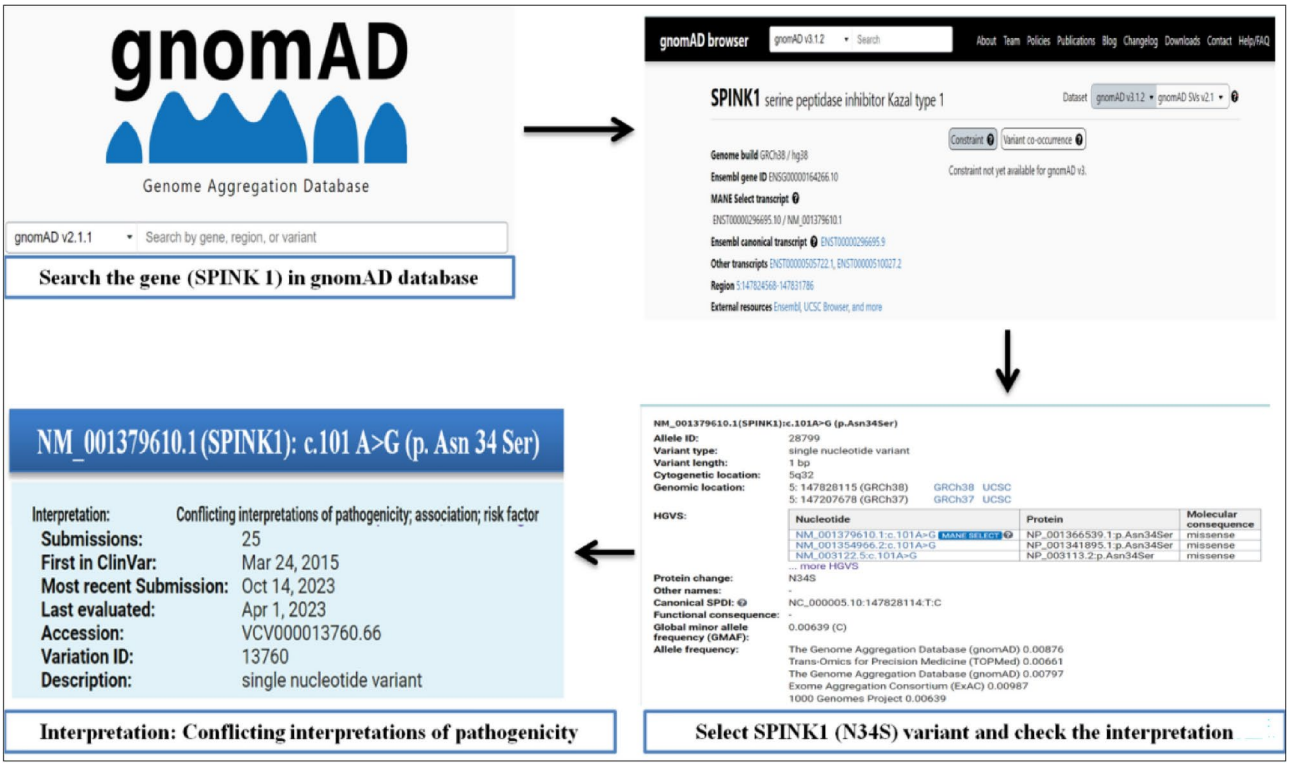


Fig. 2. SPINK1 (N34S) interpretation from Genome Aggregation Database.

mutation negative patients in terms of age of detection of DM, symptomatology, insulin dose and microvascular complications as shown in Table 3.

Discussion

FCPD is a form of type 3c (pancreatogenic) diabetes mellitus, predominantly limited to the tropics and characterized by pancreatic intra-ductal calcifications with typically ketosis-resistant diabetes; associated with progressive and irreversible destruction of the pancreatic parenchyma. SPINK1 is a pancreatic secretory trypsin inhibitor, secreted from the pancreatic acinar cells, which prevents premature activation of zymogens within the pancreas and pancreatic duct. There is overwhelming evidence that the SPINK1 (N34S) variant associated to chronic pancreatitis, since it is present in 13%–40% of patients with idiopathic chronic pancreatitis[22,23]. However the presence of mutations does not imply causation of disease and is neither taken to be significantly associated with a disease condition without comparing its association with other similar disease states and healthy individuals. An in-silico analysis and functional study is important to establish possible causation of disease by these variants.

In the current study, we attempted to look into known genetic mutations (SPINK1, PRSS1, PRSS2, CTRC and CFTR) from previous studies associated with FCPD with PCR method followed by Sanger sequencing along with T2DM and healthy controls. In-silico analysis was done to look the pathogenic nature of SPINK1 (N34S) mutations.

Previous studies in FCPD patients have reported a high frequency of the N34S variation in SPINK1 gene, ranging from 33% to 45.6%[24] as compared to controls which ranged from 1.3% to 4.7%. In a study by Hassan et al.[6]which included participants from Southern India and Bangladesh, the N34S variation was present in 33% of patients with FCPD. In comparison, only 4.4% of non-diabetic participants and 3.7% of patients with Type 2 diabetes carried the N34S variation in that study. Bhatia et al. in a study from northern India reported the N34S SPINK1 variation in 43% of FCPD patients and 47% of FCP patients[25]. In contrast, only 2.2% of their controls had the N34S variation. In a study from southern India by Kolly et al.[12]observed 41.07% of the FCPD patients with N34S SPINK1 variation. In comparison, only 1% of the controls were seen to harbor the variation. Very limited data from previous studies showed association of PRSS, CTRC and CFTR gene with TCP[7,8,26].

In contrast to previous studies, overall SPINK1 mutation positivity was present in 15.7% (8/51) FCPD patients while 13.72% (7/51) cases were SPINK1 (N34S) positive along with other variants.

Isolated SPINK1 N34S mutations were present 5.88% in FCPD, 6% in T2 DM and 2% in healthy individuals and the association was statistically not significant (0.32).Nonetheless, SPINK1 N34S has been proven to be considerably associated with FCPD when clubbed with other SPINK1 mutations. These results suggest that SPINK1 (other than N34S variant) is a susceptibility gene for FCPD. Our in-silico analysis also suggests that N34S variant is unlikely to cause disease on its own.

Mahurkar S et al.[27] proposed a two hit model to explain the sequence of events in the pathogenesis of tropical calcific pancreatitis (TCP). Functional studies employing the human recombinant N34S SPINK1 did not reveal any changes to trypsin inhibitor secretion or capability. Acinar cells gradually vanished in an animal model lacking in SPINK3, the murine orthologue of human SPINK1, as a result of poor regeneration and autophagic cell death.

Therefore, it is possible to infer that SPINK1 is crucial for the integrity and regeneration of acinar cells. Nonetheless, four intronic variations of SPINK1 have been found to be in perfect linkage disequilibrium, with one of them possibly pathogenic[27].

Mutations contribute to protein diversity. (a) Protein sequence heterogeneity is caused by translational errors such as amino acid mis-incorporation, ribosomal frame-shift, stop codon read through, and premature termination, as well as transcriptional errors such as nucleotide mis-incorporation, RNA polymerase slippage, and error in promoter regionand post translational modification (b) Multiple transcripts and proteins can be produced from a single gene sequence by phenotypic alterations[28].

SPINK1 N34S mutation is frequently observed in patients with chronic pancreatitis (CP), recent functional studies have demonstrated that this mutation does not significantly alter the trypsin inhibitory activity of the SPINK1 protein[29]. This finding challenges the traditional understanding of the mutation's role in pancreatic pathology.

SPINK1 (Serine Peptidase Inhibitor, Kazal Type 1) functions as a protective protein by inhibiting trypsin, an enzyme involved in pancreatic digestion. Mutations in SPINK1, including N34S, have been associated with an increased risk of chronic pancreatitis due to impaired inhibition of trypsin. However, the study cited indicates

	Mutation positive (n = 12)	Mutation negative (n = 39)	P value
Age at detection (years)	28.42 ± 10.9	28.84 ± 9.4	0.90
Pain Abdomen (%, n)	83.3% (10)	87% (34)	0.62
Steatorrhea (%, n)	58.3% (7)	59% (23)	1.0
Insuline dose (U/kg)	0.61 ± 0.31	0.54 ± 0.27	0.59
Retinopathy (%, n)	8% (1)	Nil	0.235
Nephropathy (%, n)	25% (3)	12.8 (5)	0.372
Neuropathy	20% (2)	12.8 (5)	1

Table 3. Analysis of mutation positive and mutation negative patients.

that despite the presence of the N34S mutation, the overall inhibitory function of SPINK1 remains intact. This suggests that the pathogenesis of chronic pancreatitis associated with the SPINK1 N34S mutation might not be solely attributed to a loss of inhibitory activity. Other factors, such as interactions with additional genetic or environmental triggers, could contribute to disease development. The presence of the mutation might influence disease progression or severity through mechanisms beyond direct alterations in trypsin inhibition.

For Fibrocalcific pancreatic diabetes (FCPD), which is often linked to chronic pancreatic damage and fibrosis, the role of the SPINK1 N34S mutation might be less straightforward. Given that the mutation does not impair trypsin inhibition, its role in FCPD could be mediated through other pathways or genetic interactions. In summary, while the SPINK1 N34S mutation is a known risk factor for chronic pancreatitis, its lack of effect on trypsin inhibition underscores the complexity of its role in pancreatic diseases and highlights the need for further research to elucidate its precise contributions to FCPD[29].

The SPINK1 c.194 + 2 T > C mutation, which is more prevalent in the Chinese population, results in a significant reduction in SPINK1 protein levels. This mutation disrupts the normal splicing of the SPINK1 gene, leading to lower levels of functional SPINK1 protein. The reduction in SPINK1 levels impairs its ability to inhibit trypsin effectively, thereby contributing to the development and progression of pancreatitis.

In contrast, the SPINK1 N34S mutation, although associated with chronic pancreatitis, does not significantly affect the trypsin inhibitory activity of the SPINK1 protein[29]. This suggests that the pathophysiological mechanisms of the N34S mutation might involve factors other than a direct loss of inhibitory function.

The protective role of SPINK1 against pancreatitis is well-documented, particularly in animal models[30]. SPINK1 functions as a crucial trypsin inhibitor, preventing premature activation of digestive enzymes within the pancreas and thereby protecting pancreatic tissue from autodigestion and inflammation. The effectiveness of SPINK1 in safeguarding against pancreatitis underscores the importance of maintaining adequate levels and functional activity of this protein. In summary, while the SPINK1 N34S mutation does not affect the protein's inhibitory function, the c.194 + 2 T > C mutation leads to decreased SPINK1 protein levels and contributes more directly to pancreatitis. These findings highlight the critical role of SPINK1 in protecting against pancreatic damage and illustrate how different mutations can impact disease outcomes through varying mechanisms[30].

PRSS1 mutation positivity is only seen in overall around 4/51(7.8%) of FCPD patients. All patients in this group are positive for PRSS1 (N29I) mutation. 1/51 (2%) patients have PRSS1 (R122H) mutation. None of the T2DM and Healthy control is positive for these mutations. PRSS1 mutations in respective groups showed significant difference among three groups of FCPD, T2DM and healthy controls. Only 1/51 (2%) patient among 51 FCPD patients is positive for CTRC exon 2–3 mutation. None of the patient has mutations in CTRC Promoter region & exon 1, CTRC exon 4–5, CTRC exon 6, CTRC exon 7–8, CFTR ΔF508, CFTR G551D, CFTR G542X, CFTR R117H and CFTR W1282X.

Subgroup analysis demonstrated that mutation positive patients were no different to mutation negative patients in terms of age of detection of DM, symptomatology, insulin dose and microvascular complications. Similar observations have been noted in previous studies, wherein there no genotype–phenotype correlation was found in patients with SPINK1 gene variations.

Conclusion

Isolated SPINK1 N34S in FCPD is as commonly found as in individuals with T2DM & healthy controls. However SPINK1 N34S when associated with other mutations of SPINK1 is found to be significantly associated with FCPD. In-silico analysis also revealed that SPINK1 N34S has conflicting role in pathogenecity in FCPD. The presence of certain mutations does not imply causation of disease and is neither taken to be significantly associated with a disease condition, without comparing its association with other similar disease states and healthy individuals.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Author contributions

VB: data collection, first draft SD: lab work KP: funding, review of draft PM: data analysis, review of draft NPB: scientific inputs, supervision of lab work SG: Conceptualisation, review of Draft.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to S.G.

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