High Prevalence of Serine Protease Inhibitor Kazal Type 1 Gene Variations Detected by Whole Gene Sequencing in Patients with Fibrocalculous Pancreatic Diabetes

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Abstract

Aim of Study: The aim is to study the prevalence and pattern of serine protease inhibitor Kazal type 1 (SPINK1) gene variations in patients with fibrocalculous pancreatic diabetes (FCPD) using whole gene sequencing. Materials and Methods: A total of 56 consecutive patients of FCPD were recruited for the study. Diagnosis of FCPD was based on the presence of diabetes mellitus in patients having chronic pancreatitis with radiological evidence of ductal calcifications, in the absence of other known causes for pancreatitis. Ethylenediaminetetraacetic acid samples were collected from all patients, and complete gene sequencing was performed for SPINK1 gene using Sanger technique. Results: Overall 35 patients (62.5%) were detected to have genetic alterations in SPINK1 gene. N34S polymorphism was seen in 23 participants (41.07%) out of which 3 were homozygous. N34S was seen to be in linkage disequilibrium with IVS1 − 37T>C (18/23) and IVS3-69insAAAA (19/23) polymorphisms. Seven patients (12.5%) had a 272 C>T 3'UTR polymorphism while one patient (1.8%) had a P55S polymorphism. Two patients (3.5%) had an IVS3 + 2T>C mutation which has been shown to be associated with loss of function of SPINK protein. Overall 48.2% of FCPD patients had genetic variations that were significant compared to the control population. There was no difference in anthropometric and biochemical parameters between those with or without SPINK1 gene variations. Conclusions: Variations in SPINK1 gene are frequently observed in FCPD. N34S polymorphism was the most common variation followed by intronic variations. Two patients had the pathogenic intronic IVS3 + 2T>C mutation. Whole gene sequencing of the SPINK1 gene enabled detection of an additional 7.1% of patients with significant SPINK1 gene variations as compared to targeted screening for the N34S variation.

Keywords: Calcific, fibrocalculous pancreatic diabetes, genetics, pancreatitis, tropical

INTRODUCTION

Fibrocalculous pancreatopathy (FCP) is a rare secondary cause of diabetes mellitus seen almost exclusively in the tropical parts of the world. It is also known as tropical calcific pancreatitis. It is characterized by pancreatic intraductal calcifications along with progressive and irreversible destruction of the pancreatic parenchyma.[1] Genetic as well as environmental factors have been implicated in the pathogenesis of FCP. Chronic malnutrition, cassava intake, and micronutrient deficiencies are some of the environmental factors proposed to play a role in pathogenesis.[2-4] Genetic alterations in the serine protease inhibitor Kazal type 1 (SPINK1) gene have been shown to be consistently associated with FCP.

The reported prevalence of SPINK1 gene variations in patients with diabetes secondary to FCP, i.e., fibrocalculous pancreatic diabetes (FCPD) ranges from 33% to 46%.[5-6] Most studies have evaluated isolated specific genetic alterations in SPINK1 gene. The objective of the present study was to investigate the prevalence and pattern of SPINK1 gene variations by whole gene sequencing in patients with FCPD.

MATERIALS AND METHODS

A total of 56 patients of FCPD were recruited for the study from the Outpatient Department of Vydehi Institute of Medical Sciences and Research Centre, Bengaluru, Karnataka, India. All patients were diagnosed with chronic pancreatitis with radiological evidence of ductal calcifications, in the absence of other known causes for pancreatitis. Genomic DNA was extracted from ethylenediaminetetraacetic acid (EDTA) blood samples using a DNA isolation kit. The complete SPINK1 gene was sequenced using Sanger technique. The study was approved by the Institutional Ethics Committee. Informed consent was obtained from all patients after explaining the study protocol.

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Sciences and Research Centre. Diagnosis of FCPD was made based on fulfillment of the following criteria:

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.

Anthropometric and relevant biochemical assessment was performed for all the patients as per the routine standard of care. Informed written consent was obtained from all the participants. The study was approved by the Institutional Ethics Committee.

Ethylendiaminetetraacetic acid samples were collected from all patients and stored in a −80°C laboratory freezer. Gene sequencing was performed for SPINK1 gene for all the samples at Sandor Life Sciences, India. The samples were processed for DNA isolation from the peripheral blood leukocytes using conventional methods. The coding exons, intronic regions, and the untranslated and flanking regions of SPINK1 gene were polymerase chain reaction (PCR) amplified using primers designed in-house. The amplification was confirmed by agarose gel electrophoresis, which was followed by post-PCR purification. The purified PCR products were sequenced by an ABI 3700 capillary sequencer (Thermo Fisher Scientific, USA) using BigDye termination sequencing.

Insilico analysis was performed for the observed variations using the software MutationTaster (Schwarz JM. Charité – Universitätsmedizin Berlin). Control data of healthy population for the SPINK1 gene variations were obtained from Sandor Life Sciences laboratory database. Statistical analysis was carried out using SPSS version 18 (IBM). Categorical data were analyzed by Fisher’s exact test and continuous data were analyzed by Student’s t-test and Mann–Whitney U tests. P <0.05 was considered statistically significant.

RESULTS

The study was carried out from January 2014 to April 2016 and included a total of 56 patients of FCPD. The male to female ratio was 1.43 (33 males and 23 females). The mean duration of diabetes was 4.49 years (range: 0–20 years). None of the patients had a family history suggestive of FCP/FCPD. The mean duration of diabetes was 4.49 years (range: 0–20 years). None of the patients with homozygous N34S variations were in linkage with these two intronic variations. There was no difference in clinical and biochemical parameters between those with or without SPINK1 gene alteration [Table 2].

The observed frequency of SPINK1 gene variations noted in our study was compared to healthy control population derived from the Sandor Life Sciences laboratory database. The results

### Table 1: Baseline parameters of the study group

| Parameter                | Mean±SD  |
|--------------------------|----------|
| Age (years)              | 34.9±8.61|
| Age at diagnosis (years) | 30.4±8.97|
| Duration of diabetes (years) | 4.49±5.3 |
| BMI (kg/m²)              | 18.05±2.16|
| HbA1c (%)                | 9.8±3    |
| Hemoglobin (g/dl)        | 12.8±1.45|
| Serum albumin (g/dl)     | 3.86±0.52|
| Serum calcium (mg/dl)    | 9.15±0.48|

BMI: Body mass index, HbA1c: Glycated hemoglobin, SD: Standard deviation

### Table 2: Comparison of parameters between patients with or without serine protease inhibitor Kazal type 1 gene variation

| Parameter                | Variation present | Variation absent | P*  |
|--------------------------|-------------------|-----------------|-----|
| Age at diagnosis (years) | 31.12±7.99        | 30±10.38        | 0.66|
| BMI (kg/m²)              | 17.70±2.12        | 18.82±2.08      | 0.06|
| HbA1c (%)                | 10.01±3.28        | 9.55±2.59       | 0.61|
| Hemoglobin (g/dl)        | 12.80±1.40        | 12.77±1.61      | 0.95|
| Serum calcium (mg/dl)    | 9.06±0.47         | 9.32±0.49       | 0.07|

*P<0.05 considered statistically significant. BMI: Body mass index, HbA1c: Glycated hemoglobin

### Table 3: The prevalence of serine protease inhibitor Kazal type 1 gene variations in fibrocalculous pancreatic diabetes patients and healthy controls

| Variant               | FCPD (n=56), % | Controls (n=200), % | P*  |
|-----------------------|----------------|---------------------|-----|
| c.101A>G (N34S)       | 41.1           | 1                   | <0.01|
| IVS1-37T>C            | 35.7           | 1                   | <0.01|
| IVS3-69insAAA         | 35.7           | 1                   | <0.01|
| c.481 C>T (3’UTR)     | 12.5           | 16                  | 0.67|
| 0.67c.163C>T (P55S)   | 1.7            | 0                   | 0.22|
| IVS3+2T>C             | 3.5            | 0                   | 0.04|

*P<0.05 considered statistically significant. FCPD: Fibrocalculous pancreatic diabetes
The present study reports a high prevalence of SPINK1 gene variations in FCPD patients. Although N34S is the most frequent genetic alteration in the SPINK1 gene in patients with FCPD, additional variations can be identified by whole gene sequencing. Two patients had a unique pathogenic IVS3 + 2T>C mutation.

The SPINK1 gene codes for the protein SPINK1 also known as pancreatic secretory trypsin inhibitors. The SPINK protein is secreted by the pancreatic acinar cells and provides the primary defense against intra-acinar activation of pancreatic enzymes by prematurely activated trypsinogen. SPINK binds to the active site of trypsin and is proposed to block 20% of the trypsin activity in the pancreatic acinar cell. SPINK1 gene is currently given the status of a modifier gene by virtue of conferring increased susceptibility to developing pancreatitis in the event of mutations.

The association of SPINK1 gene variations with pancreatitis was first reported in the year 2000 by three independent groups of investigators in patients with idiopathic and/or hereditary chronic pancreatitis. Subsequent studies reported a similar high prevalence of SPINK1 gene variations in patients with FCP. Most of these reports on SPINK1 gene variations in FCP were from the Indian subcontinent. This suggests a common genetic predisposing factor between idiopathic chronic pancreatitis of the West and FCP of the East. There have been mixed reports with regard to the presence of SPINK1 gene variation in patients with alcoholic pancreatitis. The role of SPINK1 gene in pancreatitis has also been confirmed by animal studies. Animal studies on the knockout of SPINK gene have demonstrated a high predisposition to the development of pancreatitis. Similarly, animal models with increased expression of SPINK were seen to be resistant to the development of pancreatitis.

More than forty variants of SPINK1 gene have been reported in literature till date. Previous studies in FCPD patients have reported a high frequency of the N34S variation in SPINK1 gene, ranging from 33% to 45.6%. In contrast, the prevalence of the same variation in controls was much lower and ranged from 1.3% to 4.7%. In a study by Hassan et al., which included participants from India and Bangladesh, the N34S variation was present in 33% of patients with FCPD. In comparison, only 4.4% of nondiabetic participants and 7.7% of patients with Type 2 diabetes carried the N34S variation in that study. Bhataia et al. in a study from northern India reported the N34S SPINK1 variation in 43% of FCPD patients and 47% of FCP patients. In contrast, only 2.2% of their controls had the N34S variation. Nearly 41.07% of the FCPD patients in the present study were seen to carry the N34S SPINK1 variation which is comparable to the previous studies. In comparison, only 1% of the controls were seen to harbor the variation.

Previous studies have reported cosegregation of intronic variations IVS1-37T>C, IVS2 + 268A>G, IVS3-66-65insTTTT, and IVS3-604G1A with N34S. The role of these intronic variations in FCPD is yet to be elucidated. In the present study, IVS1-37T>C and IVS3-66-65insTTTT variations were seen in twenty patients each, and both these variations were in strong genetic disequilibrium with the N34S variation. Only two patients with these variations did not carry the N34S variation. The IVS3 + 2T>C mutation affects the consensus splicing site in intron 3 which leads to skipping of the entire exon 3 where the trypsin binding site is located. Previous studies from Japan have reported a high prevalence of IVS3 + 2T>C SPINK1 variation in patients with idiopathic and hereditary chronic pancreatitis. Two patients were seen to carry the IVS3 + 2T>C variation in the present study. In comparison, none of the controls had this variation. On the whole, an additional 7.1% of the patients had significant variations of the SPINK1 gene after excluding the N34S variation.

The exact mechanism as to how N34S variation affects the function of SPINK1 protein is not yet clear. While some studies showed no change in the function of the N34S variant of SPINK1 protein, in a more recent study by Halangk et al., N34S alteration was seen to increase the susceptibility of SPINK1 for inactivation by cathepsin L. Another factor that may play a role is the cosegregation of N34S with the intronic variants, and these intronic variations may be the actual pathologic factors.

There was no difference in the clinical features between the patients with or without SPINK1 gene variations. Similar observations have been noticed in previous studies, wherein there was no genotype–phenotype correlation in patients with SPINK1 gene variations. Few studies have, however, noted an association between the presence of SPINK1 gene variation and pancreatic ductal adenocarcinoma. None of the patients in our cohort had pancreatic carcinoma.

Other genes found to be associated with FCPD are cathepsin B, chymotrypsin C, carboxypeptidase A1, glycoprotein 2, cladin 2, cystic fibrosis transmembrane regulator, and PRSS1 (cationic trypsinogen). However,
these findings have not been replicated in subsequent studies. Further studies with the aid of next-generation sequencing may help in assessing the relevance of these genes in the pathogenesis of FCPD.

**Conclusions**

A high prevalence of SPINK1 gene variations has been observed in this study. The most frequent variation observed was the N34S variation followed by intronic variations. Two patients had a pathogenic intronic variant IVS3 + 2T>C. Whole gene sequencing of the SPINK1 gene enables detection of additional patients with SPINK1 gene variations as compared to targeted screening for the N34S variation. The present study reinforces the role of SPINK1 gene variations in the pathogenesis of FCPD.

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**Conflicts of interest**

There are no conflicts of interest.

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