Original Article

SPINK1 and CFTR Mutation Patterns and Their Correlation with Clinical Features in Iranian Patients with Chronic Pancreatitis

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ABSTRACT

Background:
In cases of chronic pancreatitis, a set of behavioral, genetic, and environmental factors are involved. The SPINK1 and CTFR genes are important genetic factors, but they might have different patterns in different regions. There are only few studies conducted to investigate the prevalence of these mutations, especially in Iran. In this study, we determined the prevalence of mutations in SPINK1 and CFTR in patients with chronic pancreatitis in an Iranian referral hospital.

Materials and Methods:
A total of 56 patients diagnosed with chronic pancreatitis (based on the criteria of the American Pancreatic Association) were included in this cross-sectional study. Using specific primers for the CFTR exon 11 and SPINK1 exon 3, two conventioan PCRs were performed, and products were examined by nucleotide sequencing. The raw data were edited with bioinformatics software against the reference sequence. Mutated samples were added to the patient data file and analyzed with the rest of the variables in SPSS software.

Results:
Among the 56 patients, the average age±SD of the patients was 48.1±14.9 years, 20 were women (35.7%), 44 (78.6%) were ever-smokers and 25 people (44.6%) had a history of long-term alcohol consumption. According to the results of mutation analysis, seven people (12.5%) had CFTR gene mutation (F508del and Gly469Asp), three people (5.4%) had SPINK1 gene mutation (S34N), and one person (1.8%) had both CFTR and SPINK1 gene mutation. The average age and other demographical data of mutated people were not statistically different from other patients. CFTR mutation was observed more in men (85.7%), smokers (85.7%), with gallstones (57.1%), and in people with SPINK1 mutation, it was seen more in ever-smoking patients, diabetics, and those with gallstones, and family history of acute pancreatitis (2/3; 66.7%).

Conclusion:
We observed S34N and F508del mutations in the SPINK1 and CFTR genes are associated with chronic pancreatitis, which is in agreement with previous studies. The younger age, smoking, gallstone, and diabetes are probable risk factors for chronic pancreatitis. The limited sample size, lack of a control group, and small number of examined exons may affect our findings and significant results.

Keywords: Chronic pancreatitis, Cystic fibrosis, Mutation analysis

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INTRODUCTION

Chronic pancreatitis (CP) is a long-lasting inflammation of the pancreas that causes permanent structural damage and gradual loss of both digestive and hormonal functions. It is estimated to affect 30-50 out of 100,000 people, but some studies suggest it could be as high as 120-143 out of 100,000(1-3). The condition reduces the quality of life, increases the chance of pancreatic cancer, and is a significant source of illness. The main cause of CP is drinking too much alcohol (40-70% of cases), followed by idiopathic CP (ICP), which is when no clear cause can be identified before genetic testing (up to 25% of patients)(3-5).

CP is believed to be irreversible once it develops. Therefore, finding inherited risk factors that have a strong impact could lead to better prevention and treatment options(2). In the last 20 years, it has become clearer that ICP is largely genetic and that uncommon harmful variants (here meaning those with a frequency of less than 1%) in the CFTR gene (which makes a protein that regulates fluid transport) and the three genes that control trypsin activity, namely PRSS1 (which makes a type of trypsin), SPINK1 (which makes a trypsin inhibitor), and CTRC (which makes another enzyme that breaks down proteins) play a key role(5). Some of these rare harmful variants in these four genes are also more common in patients with CP caused by alcohol(2,6).

Most earlier studies had many limitations, such as screening few patients, testing only one or two genes, not having proper control data from the normal population, choosing to genotype only known harmful variants or a few selected exons, and not assessing other causes, especially smoking, which has been proven to be more important (3,7). These drawbacks have prevented us from obtaining a more precise estimate of how much the rare harmful variants in the four genes contribute to CP worldwide. They have also made it more difficult to understand the complex interactions between genes and the environment for this disease. Furthermore, the effect of rare harmful variants on when and how severely CP starts is still unknown (8). This issue was previously looked at in the case of hereditary pancreatitis, which is passed down in a dominant way, a rare reason for CP that makes up less than 1% of cases; however, there were conflicting findings in the main studies (3,9). There have not been many studies on recognizing rare harmful variants in ICP, and the available studies always have small numbers of ICP patients because of the nature of the disease (for example, 35 in Sandhu and colleagues, 45 in Xiao and colleagues, and 61 in Cho and colleagues); this makes it hard to reach solid conclusions (7). In this study, we share results from a thorough analysis of rare harmful variants in the SPINK1, and CFTR genes in a group of patients diagnosed with CP.

MATERIALS AND METHODS:

Study Setting and Patients

In this cross-sectional study, all cases with CP who were referred to the Firoozgar Hospital affiliated with the Iran University of Medical Sciences, Tehran, Iran, from Jan 2021 to Dec 2021 and met the American Pancreatic Association (APA) criteria were enrolled. The Ethics Committee of the Iran University of Medical Sciences approved the study protocol (IR.IUMS.REC.1400.1016). The inclusion criteria were diagnosis of CP based on APA criteria, signing informed consent, and age of more than 18 years. Patients who did not meet the inclusion criteria or did not fill in the checklist data, patients who had any benign or malignant pancreatic disorder simultaneously, and patients who had other disorders such as anemia with limitations for taking blood samples were excluded. We collected data on demographic characteristics of patients, history of comorbidities, history of alcohol consumption and smoking, CP clinical signs and symptoms, abdominal computed tomography (CT) result, endoscopic ultrasound (EUS), and endoscopic retrograde cholangiopancreatography (ERCP) result, and surgical history.

Sampling and DNA Extraction

A total of 2cc blood samples were obtained from each participant. Sera were separated by centrifugation at 3000 rpm for 10 min and kept at -20 C° until further use. Isolation of genomic DNA was done by the DNA Extraction Mini Kit (Favorgen, Taiwan) according to the manufacturer's instructions. The NanoDrop-1000 spectrophotometer was used for isolated DNA quality assessment.

PCR amplification

A conventional PCR was used for amplification of CFTR exon 11 and SPINK1 exon 3. The specific primer sequences were obtained from the previous studies (8,10). The amplicon size for amplification of CFTR exon 11 was 491 bp, and for SPINK1 exon 3, it was 308 bp. The heating protocol was set at the ABI-Veriti thermal cycler (Applied Biosystems, Foster City, CA, USA) as follows: 1 step at 95°c for 5 min; 35 cycles at 95°c for 30s, 55°c (for the SPINK1) and 52°c (for the CFTR) for 30s, 72°c for 30s; and a final step at 72°c for 5 min. The visualization was done on the 1.5% agarose gel electrophoresis against UV radiation, and a Gel Doc 2000 gel documentation system (BIO-RAD, Hercules, CA) was used for imaging.

Nucleotide sequencing and Mutation analysis

After purification of specific bands on the gel via a FavorPrep™ GEL/PCR Purification kit (Favorgen, Taiwan), samples were sent for nucleotide sequencing, which was done by an ABI 3700™ DNA analyzer (Applied Biosystems, USA). The raw data were edited and trimmed by the CLC Workbench 5 software using the reference sequences NG_008356 and NG_016465. The BLAST online software (http://www.ncbi.nlm.nih.gov/blast) was used to identify and confirm the sequence's similarity. The consensus sequences were aligned by the CLC Workbench 5 software and compared with the reference sequences. For the mismatched bases, the amino acid product was screened according to the reading frames of the reference sequences.

Statistical analysis

The SPSS software version 25 was used for quantitative and qualitative data analysis. The values less than 0.05 were considered statistically significant.

RESULTS

Demographics

Of a total of 56 patients diagnosed with CP, the mean age±standard deviation was 48.09 ± 14.87 (y), 36 (64.3%) were male, and 20 (35.7%) were female. History of long-term alcohol consumption was reported in 44.6% (25/56) of cases, and 78.6% (44/56) of the cases were ever-smokers. The most common cofounding reported was gallstone (73.2% [41/56]), and 41.1% (23/56) had diabetes. The mostcommon symptoms were stomachache and nausea (both 91.1% (51/56)). The parenchymal calcification and ductal beading were identified by CT scan in 98.3% and 71.4%, respectively. According to the EUS findings, the majority had irregular MPD contour, MPD dilation, or MPD calculi (96.4% (54/56)). The main duct abnormality was seen in 98.2% (54/56) via ERCP. One patient died during the study period (Table 1).

Table 1. Demographics of mutations detected in our studied patients with chronic pancreatitis

No	Case ID	Sex	Age	BMI	CFTR		SPINK1
					F508del	Gly469Asp	S34N
1.	46	Male	29	30	+	-	+
2.	1	Male	27	26	+	+	-
3.	4	Male	52	24	+	-	-
4.	13	Male	53	31	+	-	-
5.	21	Male	49	21	+	-	-
6.	32	Male	39	28	+	-	-
7.	39	Female	35	21	+	+	-
8.	7	Female	71	31	-	-	+
9.	55	Female	43	27	-	-	+

Mutation analysis

Mutation analysis revealed that a total of seven cases (12.5%) had CFTR mutations, three cases (5.4%) had SPINK1 mutations, and one (1.8%) had both mutations. CFTR exon 11 mutations included F508del in 12.5% (7/56) of the cases (1521 to 1523 deletion of CTT), 1408G>A (Gly469Asp) in 3.5% (2/56) of the cases and both Gly469Asp and F508del in 3.5% (2/56) of the cases. The SPINK1 exon 3 mutations included S34N (101A>G) in all three mutant cases. One case had both S34N and F508del (Table 1).

Mutant cases were younger and had higher BMI than wild-type (WT) cases in both CFTR and SPINK1 genes. However, the differences were not statistically significant. The majority of CFTR and SPINK1 mutant cases were ever-smokers (6/7), but neither this nor other variables had significant differences.

CP symptoms, including nausea and vomiting, were more common in mutant patients than in WT cases. According to the EUS and ERCP results, all mutant cases of both genes

were identified by irregular MPD contour, MPD dilation, main duct abnormality, and hyperechoic MPD margin. Especially for the SPINK1 mutant cases, there were branch duct abnormalities and dilated side branches in all mutant cases. However, they were not statistically significant (Table 2).

DISCUSSION

Several studies have reported that mutations in various genes associated with CP, such as PRSS1, PRSS2, SPINK1, CTRC, CFTR, CPA1, CEL-HYPB1, CASR, CLDN2, and TRPV6, contribute to the disease (1,2,4,5,11-13). However, their association is not completely clear. These studies represent a list of genetic markers for screening the mostcommon genetic risk factors in CP development (1,2,5).

The present study was carried out on 56 patients with CP diagnosed based on APA criteria by experienced clinicians who performed extra sampling for the identification of CFTR and SPINK1 mutations. The results showed that the

Table 2. Comparison of different variables in CFTR exon 11 and SPINK1 exon three genes mutants (n=56).

	Scale	Mutant	Wild type	Total	P value
CFTR exon 11		**(n=7)**	**(n=49)**	**(n=56)**	
Demographics					
Age (year)	Mean ± SD	40.1 ± 57.8	49.1±16.8	48.1±9.6	0.148
BMI (kg/m^2)	Mean ± SD	25.4±86.0	28.4±86.3	28.4±48.4	0.094
Male	N (%)	6 (16.7)	30 (83.3)	36 (64.2)	0.252
Current smoking	N (%)	6 (13.6)	38 (86.4)	44	1.00
History of alcohol consumption	N (%)	3 (12)	22 (88)	25	1.00
Diabetes	N (%)	3 (13)	20 (87)	23	1.00
Gallstone	N (%)	4 (9.8)	37 (90.2)	41	0.37
Family history of CP	N (%)	3 (23.1)	10 (76.9)	13	0.335
Family history of acute pancreatitis	N (%)	3 (20)	12 (80)	15	0.37
CP symptoms					
Nausea/Vomiting	N (%)	6 (11.8)	45 (88.2)	51	0.370
Abdominal pain	N (%)	5 (9.8)	46 (90.2)	51	0.113
Loss of appetite	N (%)	4 (9.5)	38 (90.5)	42	0.35
Steatorrhea	N (%)	2 (22.2)	7 (77.8)	9	0.312
CT scan, EUS, ERCP					
Irregular MPD contour	N (%)	7 (13)	47 (87)	54	1.00
MPD dilation	N (%)	7 (13)	47 (87)	54	1.00
Hyperechoic MPD margin	N (%)	7 (15.9)	37 (84.1)	44	0.326
Main duct abnormality	N (%)	7 (12.7)	48 (87.3)	55	1.00
MPD calculi	N (%)	6 (11.1)	48 (88.9)	54	0.236
Dilated side branches	N (%)	6 (11.5)	46 (88.5)	52	0.423

Table 2. Comparison of different variables in CFTR exon 11 and SPINK1 exon three genes mutants (n=56).

SPINK1 exon 3		**(n=3)**	**(n=53)**	**(n=56)**	
Demographics					
Age (year)	Mean ± SD	47.2±67.3	48.1±11.4	48.1±9.6	0.960
BMI (kg/m^2)	Mean ± SD	29.2±33.0	28.4±43.5	28.4±48.4	0.734
Male	N (%)	1 (2.8)	35 (97.2)	36 (64.2)	0.288
Current smoking	N (%)	2 (4.5)	42 (95.5)	44	0.522
Alcohol	N (%)	1 (4)	24 (96)	25	1.00
Diabetes	N (%)	2 (8.7)	21 (91.3)	23	0.562
Gallstone	N (%)	2 (4.9)	39 (95.1)	41	1.00
Family history of CP	N (%)	1 (7.7)	12 (92.3)	13	0.555
Family history of acute pancreatitis	N (%)	2 (13.3)	13 (86.7)	15	0.172
CP symptoms					
Nausea/Vomiting	N (%)	2 (3.9)	49 (96.1)	51	0.249
Abdominal pain	N (%)	2 (3.9)	49 (96.1)	51	0.249
Loss of appetite	N (%)	2 (4.8)	40 (95.2)	42	1.00
Steatorrhea	N (%)	0 (0)	9 (100)	9	1.00
CT scan, EUS, ERCP					
Irregular MPD contour	N (%)	3 (5.6)	51 (94.4)	54	1.00
MPD dilation	N (%)	3 (5.6)	51 (94.4)	54	1.00
Hyperechoic MPD margin	N (%)	3 (6.8)	41 (93.2)	44	1.00
Main duct abnormality	N (%)	3 (5.5)	52 (94.5)	55	1.00
MPD calculi	N (%)	2 (3.7)	52 (96.3)	54	0.105
Dilated side branches	N (%)	3 (5.8)	49 (94.2)	52	1.00

mutations in CFTR exon 11 included F508del (12.5%), Gly469Asp (3.5%), Gly469Asp/ F508del (3.5%), and that the mutation in the SPINK1 exon 3 included S34N (5.3%). Additionally, one case was identified by S34N/F508del (1.7%). The age of both mutated groups was lower than that of the wild types, and the BMI was lower only in the mutated CFTR group.

The majority of the mutated CFTR cases were male (85.7%), smokers (85.7%), had symptoms of nausea/ vomiting (85.7%), abdominal pain (71.4%), irregular MPD contour (100%), MPD dilation (100%), hyperechoic MPD margin (100%), and main duct abnormality (100%). The majority of the SPINK1 mutant cases were female (66.7%), smokers (66.7%), had diabetes (66.7%), gallstones (66.7%), and a family history of acute pancreatitis (66.7%). Their symptoms included nausea/vomiting, abdominal pain, loss of appetite in 66.7% and irregular MPD contour, MPD dilation, hyperechoic MPD margin, main duct abnormality, and dilated side branches in 100% of the mutated cases.

The study of Jena and colleagues (7) showed that the ratio of male/female in total 200 CP cases was 2.12:1, which is somewhat similar to our study (1.7:1).

Also, they (7) found that severe abdominal pain, exocrine and endocrine insufficiency, and parenchymal atrophy were

significantly more common in the patients with mutation, which is similar to our findings in abdominal pain and diabetes. Abdominal pain is also common in other studies by Balakrishnan and others (14), Midha and colleagues (15), and Layer and co-workers (16).

Jena and others (7) reported that 20.5% of the patients with CP had steatorrhea, which is consistent with previous studies by Midha (15), Shetty (17), and Garg (18) and their colleagues who reported 5-15 % of steatorrhea.

Compared with our study, we found a similar percentage of steatorrhea in 16% of the cases. Type 2 diabetes mellitus was found in 41% of our cases, which is nearly two-fold higher than in previous studies (7,15,18).

Our cases showed that 96.4% of them had dilated pancreatic ducts, which is higher than the studies by Jena and colleagues (7) (65.5%) and Shetty (17) (75%). The age of the mutated cases of SPINK1 and CFTR genes in several studies was reported to be lower than that of the non-mutated cases, which is similar to our study (1,5,7,19).

Also, we found that abdominal pain was common in the mutated cases, similar to Jena and colleagues (7).

Another study documented that the CFTR mutant cases did not have a history of alcohol consumption or smoking (5). This contrasts with our findings, which showed that 85.7% of the mutated CFTR and 66.7% of the SPINK1 cases were smokers, while alcohol abuse was less frequent (42% and 33%, respectively).

The SPINK1 mutation causes its protein to dysfunction as a trypsin inhibitor and leads to the induction of idiopathic CP (5). Its N34S mutation is accompanied by severe CP. This mutation is found in 1% of the general population (5). The Jena et al. (7) study showed that 50% of patients did not have the SPINK1 mutation, that 10% were homozygous, and that 40% were heterozygous for N34S. Other studies reported 18% to 40% mutation in the SPINK1 gene (9,12,17,18,20-22).

However, Chen and colleagues (23) study found 6.3%, and Litvinova and others (1) found 8.6% SPINK1 mutants, which are rather similar to our findings (5.3%) and other European reports (11).

Muller and co-workers (4) documented that acute pancreatic history, abdominal pain, and higher pancreatic morphological abnormalities were more common in cases with the SPINK1 mutation, which is similar to our findings. Masson and others (11) demonstrated that even heterozygous mutations of the SPINK1 gene were sufficient to cause the disease and that N34S could increase the risk of CP by about 10-15 fold. N34S is found in ~0.75% of the French population (23).

Threadgold and colleagues (21) demonstrated that the SPINK1 N34S mutation rate in CP was 18% and in the general population was 2.5%, compared with our findings, where we found it in 5.3% of cases. A study showed that the SPINK1 and PRSS1 mutants could have a family history of CP in several generations (1), which is also found in our studied patients, where 33.4% of the SPINK1 and nearly half of the CFTR mutants (42.8%) had a family history of CP. The CFTR protein is a cAMP-dependent ion channel found in epithelial cells of secretory organs such as the lung, the pancreas, and the digestive system. The mutation of this gene leads to cystic fibrosis (CF)(5). Nearly 2000 CFTR mutations have been identified with various clinical significance in pancreatitis development (5). CFTR variants were reported in 18.1% of a study in Russia (1).

They found 6.7% F508del, which was nearly half of our studied samples, although they examined 105 CP cases and reported variants in the other exons except exon 11. Our study showed a similar finding to the Sharer and colleagues' (24) study, in which they reported 13% of CFTR mutation in 134 patients with CP. Interestingly, we found Gly469Asp (1408G>A) for the first time in two cases (3.5%). However, the new variant (2619+86delT) was found in the study of Litvinova and others (1), which needs more studies to clarify the prevalence and functional significance of these new mutations.

The CP development risk by CFTR mutation was calculated by cumulative OR as 2.432 (1) and 3.71 (19). The clinical significance of genetic variation for screening of CP was reported with SPINK1, PRSS1, and CPA1 genes, while CTRC and CFTR mutations could be found in people without CP (1,5,25). Our study could not prove this for our population due to the lack of a control group.

The limitations of our study should not be neglected. Firstly, the sample size, the limited duration of the study, and the budget restricted us from obtaining more comprehensive results. Secondly, because of logistic issues, using a high-throughput method for all critical exons of target genes and using more genes in the analysis were avoided. Also, we did not have a control group to compare the percentage of mutations in patients with CP and control groups.

Failing to calculate the survival rate due to not having a follow-up study setting and lacking a group of other pancreatic diseases rather than CP to compare were another limitation.

In conclusion, patients with CP should be characterized by genetic markers to help them with the disease prognosis, hereditary risk awareness, and personalized medicine facilitation.

The SPINK1 N34S and CFTR F508del mutations are common in CP cases and are accompanied by severe types of pancreatitis, clinical severity, and structural abnormality. These mutations may be related to many factors such as

environment, unknown genetic mutations, and patient physiopathological status. Although we could not describe significant results by our variables analysis and outcomes, major limitations of our study restricted us from obtaining more comprehensive results. Further studies in a prospective and follow-up setting are recommended to clarify the exact role of altered genes in CP outcomes.

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