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PREVALENCE OF SPINK 1 AND CASR GENE MUTATIONS IN ACUTE AND RECURRENT ACUTE PANCREATITIS : A STUDY FROM CENTRAL INDIA

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ABSTRACT

Background: Genetic factors may play an important role in the pathogenesis of acute pancreatitis. It has been observed in various studies that the presence of risk factors alone like alcohol abuse or gall bladder stones does not lead to attacks of pancreatitis in all the patients. This leads to assumption that genetic factors may decrease the threshold for the development of pancreatitis in presence of one or more risk factors. We observed that there is a paucity of data regarding the role of genetics in acute pancreatitis (AP) and recurrent acute pancreatitis (RAP) in our part of the world and we aimed at studying the prevalence of genetic mutations in such patients.

Methods: Our study intended to find the prevalence of SPINK1 N34S (Serine protease inhibitor kazal type 1) and CaSR (Calcium sensing receptor) gene mutations in patients of AP and RAP. A total of 50 patients and 25 age and gender matched controls entered our study. Blood samples were obtained from all the cases and controls for routine investigations and genetic analysis. SPINK 1 N34S and CaSR gene mutation studies were done in all the patients and controls.

Results: Alcohol (64%) followed by gallbladder stone disease (20%) was the most common aetiology of pancreatitis. SPINK 1 N34S mutation was present in 21 patients and 2 controls whereas CaSR gene mutation was present in 13 patients and 2 controls. Patients with SPINK 1 N34S and CaSR gene mutations were younger than the patients without these mutations. Prevalence of both SPINK1 N34S and CaSR gene mutations was higher in patients of RAP than AP. These mutations were not associated with aetiology or severity of pancreatitis.

Conclusion: The prevalence SPINK 1 N34S and CaSR gene mutations was higher in patients of AP and RAP. Identification of these mutations in patients of AP can help in the identification of patients who are at increased risk of recurrent attacks of AP.

KEYWORDS : Genetics, Environment, Aetiology

INTRODUCTION

Acute Pancreatitis (AP) is an inflammatory condition of the pancreas with or without the involvement of peri-pancreatic tissues or distant sites. Some patients of AP have increased susceptibility to develop recurrent attacks of AP, referred to as Recurrent Acute Pancreatitis (RAP) which itself is an established risk factor for the development of Chronic pancreatitis (CP)[1-3].

The most common aetiology of pancreatitis is gallstones and alcohol abuse but not all the patients with gallstones or alcohol abuse develop AP.[4] Moreover it is not feasible to find out the aetiology of pancreatitis in about one-third of the cases .It is becoming clear that environmental and anatomical factors such as alcoholism, tobacco, gallstones, pancreatic divisum, etc. trigger pancreatitis only in susceptible individuals and the spectrum of presentations is based on host susceptibility, controlled largely by genetic factors.

In the pathogenesis of pancreatitis, various groups of genetic mutations may play an important role. Mutations in the cationic trypsinogen (PRSS1), pancreatic secretory trypsin inhibitor (SPINK1), cystic fibrosis transmembrane conductance regulator (CFTR) and calcium-sensing receptor (CASR) have been found in different types of pancreatitis.[5,6]

SPINK1 gene is located on chromosome 5q32 and encodes serine protease inhibitor Kazal type 1 (SPINK1) protein. This protects the pancreas against premature trypsinogen activation and inhibits up to 20% of intrapancreatic trypsin.[7] SPINK1 variants are common among the general population with a prevalence of nearly 2%.[8] N34S is the most common missense mutation occurring in SPINK1 gene. Pathogenic SPINK1 mutations lower levels of trypsin inhibitor and thereby increase susceptibility to pancreatitis.

Pancreatitis may be initiated in the homozygous N34S state, however the heterozygous genotype may only cause a lowering of the enzyme level and it requires other additional factors (genetic and environmental) to initiate the disease.[9] Therefore in general SPINK1 polymorphism is hypothesized to be a susceptibility or disease modifying factor.

(CaSR) is a member of the G-protein-coupled receptor (GPCR) superfamily and is located on chromosome 3q 13.3-21.[10] CASR plays an important role in calcium homeostasis.

Recent studies have reported that (CaSR) gene mutations in combination with the presence of serine protease inhibitor Kazal type 1 (SPINK1) N34S mutation increase the risk of CP.[11,12]

Since the distribution of these mutations varies among countries [13] and a limited data on the role of genetics in AP and RAP is available in our part of the world ,we intended to study the prevalence of these mutations in such patients.

AIMS AND OBJECTIVES

1. To study the prevalence of SPINK1 N34S and CASR gene mutations in patients with AP and RAP
2. To study the relationship of these mutations with the severity of the pancreatitis.

MATERIALS AND METHODS

Patients And Controls

This prospective, observational case control study was conducted on 50 patients of AP and RAP along with 25 age and gender matched healthy controls from 1 February 2017 to 31 July 2018 in the department of Gastroenterology and Hepatobiliary sciences at SAMC, PGI, Indore, India. The diagnosis of AP was based on two of the following three criteria: (1) abdominal pain characteristic of AP, (2) serum amylase and / or lipase ≥ 3 times the upper limit of normal, and (3) characteristic radiological findings of AP. Recurrent acute pancreatitis was defined as more than one documented prior episode of acute pancreatitis. Pancreatitis was considered to be alcohol related when patient had history of alcohol use during the week before admission and gall stones related when radiology was suggestive of gall bladder stones. Voluntary healthy controls included the hospital staff and relatives of other patients attending the outpatient department. The controls had no personal or family history of any pancreatic disease and were not related to the patients. Patients were divided in to mild, moderate and severe as per the revised Atlanta guidelines. Patients with known chronic pancreatitis were excluded from the study Blood samples were obtained at admission for genetic study and other routine investigations. The SPINK1 N34S mutation and CaSR gene polymorphisms, were assessed in all the patients and controls. Institutional review board approval was taken prior to this study. This study was conducted in compliance with the ethical standards of the responsible institution on human subjects as well as with the Helsinki Declaration.

DNA Preparation

Peripheral blood samples were drawn in to EDTA containing tubes. DNA was extracted from EDTA blood using QIAGEN kit (QIAamp DNA Blood Mini Kit). Quality and concentration of isolated DNA was measured by Qubit 3.0 fluorometer (Invitrogen, life technologies). To avoid repeated freezing and thawing of DNA, we stored the purified DNA at 4°C for immediate use or aliquoted the DNA and stored at –20°C for long-term storage.

Identification Of Genotypes Of *SPINK1* Gene

Exon 3 of the SPINK 1 gene polymorphism was identified for polymerase chain reaction (PCR-RFLP) amplification of the HPYCH4-III restriction (Invitrogen) site located between exon, using the specific forwards (5'-CCA TCT TAC CCA ACC TCA GTA G-3') and reverse (5'-TGA TGA CAG ATC GTT GGG GGC TAG A-3') primer. PCR cycling conditions were initial denaturation at 95°C for 1 min, followed by 35 cycles of denaturing at 95°C for 60 s, annealing at 50°C for 60 s and extension at 72°C for 60 s and a final incubation at 72°C for 5 min. The resulting amplified PCR product was conformed run on 2% agarose gel. After conforming, amplified PCR product (595bp) was digested with 10 unit of HPYCH4-III (Invitrogen, life technologies) restriction enzyme and incubated at 37°C for 12 hours. After digestion the products were run on 3% agarose gel and after staining with ethidium bromide; visualized on transilluminator. Identification of genotypes were determined based on the expected product size. (Table 1)**.**

Identification Of Genotypes Of CaSR gene

Part of exon 7 of the CaSR gene comprising the 3 single-nucleotide polymorphisms (G/T at codon 986, A/G at codon 990, and C/G at codon 1011) were amplified by PCR-RFPL amplification of the Hin1I, BsaHI, PSTI restriction site located between exon, using the specific forwards (5'-CAG AAG GTC ATC TTT GGC AGC GGC A-3') and reverse (5'-TGC AGA CCT GTT TCC TGG ACG GT C-3') primer. PCR cycling conditions of CaSR gene were run at 93°C for 10 minutes followed by 35 cycles of 93°C for 45s, 63°C for 30s, 72°C for 45 seconds, and final extension at 72°C for 10 minutes. The resulting amplified PCR product was conformed run on 2% agarose gel. After conforming, amplified PCR product (269bp) was digested with 10 unit of Hin1I, BsaHI, PST I (Invitrogen, life technologies) restriction enzyme and incubated at 37°C for 12 hours. After digestion the product were run on 2% agarose gel and after staining with ethidium bromide; visualized on transilluminator. Identification of genotypes were determined based on the expected product size. (Table 1)

Table 1. Gene Identification On Basis Of Product Size

Genotypes	Product size (bp)
***SPINK1* gene**	
N/N (NORMAL)	299bp,139bp,137bp,20bp
S/S (MUTANT)	190bp,139bp,137bp,109bp,20bp
N/S (HETEROZYGUS)	299bp,190bp,139bp,137bp,109bp,20bp
***CaSR* gene**	
T/T (NORMAL)	269 bp
G/G (MUTANT)	241 bp and 28 bp
T/G (HETEROZYGUS)	269bp, 241 bp and 28 bp

Statistical Analysis

Data was presented as mean and standard deviation for continuous variables and number and percentages for discrete variables. Normality of data was checked by Kolmogorov smirnov test. Student t test and Mann Whitney U test was applied to see the significant difference in mean and median of continuous data for parametric and non-parametric data respectively. Chi Square test was used to see the significant difference in frequency of genotypes and other discrete variables between two groups. Multinomial logistic regression was applied to see the independent associated factors for acute pancreatitis. p=<0.05 was considered significant.

RESULTS

The mean age at presentation of the patients with pancreatitis did not differ significantly from control group*(p=0.96)*. A total of 31 patients (62%) were diagnosed with AP and rest 19 patients (38%) with RAP. The mean age of patients with AP (36.64±9.06 years) was similar to RAP patients (33±8.96 years) *p=0.17.* Age and sex characteristics of patients and controls has been shown in table 2.

Table 2. Age And Sex Characteristics Of Patients And Controls

	CASES (n=50)	CONTROLS (n=25)
Age (years, mean± sd)	35.26±9.11	34.8±7.86
Male	43(86%)	21(84%)
Female	7(14%)	4(16%)

sd : standard deviation, n:number, %: percentage

As per revised Atlanta guidelines,18 patients (36%) were having moderately severe disease followed by mild (17 patients, 34%) and severe disease (15 patients, 30 %). Alcohol (32 patients, 64%) followed by gallbladder stones (10 patients,20%) was the most common aetiology of pancreatitis (Table 6).

SPINK 1 N34S Mutation

SPINK 1 N34S mutation was present in twenty one (42%) patients and only two (8%) controls. The prevalence of SPINK1 N34S mutation was statistically higher in patients as compared to control group (*p=0.01*). The mean age (34.52 ± 9.56 years) of patients having SPINK 1 N34S mutation was statistically lower than patients without it (37.79 ± 8.91 years) *p=0.032.* Twelve (63%) out 19 patients having RAP had SPINK1 N34S mutation while only 9 (29%) out of 31patients having AP had SPINK1 N34S mutation The prevalence of SPINK1 N34S mutation was statistically higher in RAP than AP patients *(p=0.01)* (Table 3)

Table 3. Distribution Of SPINK 1 N34S Mutation Among Various Groups

SPINK 1 GENE MUTATION	CASES		CONTROL		ACUTE PANCREATITIS		RECURRENT ACUTE PANCREATITIS	
	n	%age	N	%age	n	%age	N	%age
NORMAL	29	58	23	92	22	71	7	36.8
HETEROZYGOUS	19	38	2	8	9	29	10	52.6
HOMOZYGOUS	2	4	0	0	0	0	2	10.5
Total	50	100	25	100	31	100	19	100
	p=0.01				p=0.01			

n:number, %age: percentage, p: p value

CaSR GENE MUTATION

CaSR gene mutation was present in thirteen patients (26%) and only two (8%) controls. The prevalence of CaSR gene mutation was statistically higher in patients as compared to control group *(p=0.02).* The mean age (34±11.3 years) of patients having CaSR gene mutation was less as compared to patients without CaSR gene mutation (36.7±8.33 years) *p=0.046.* Seven (36.84%) out 19 patients having

RAP had CaSR gene mutation while only 6(19.3 %) out of 31 patients having AP had CaSR gene mutation. The prevalence of CaSR gene mutation was statistically higher in RAP than AP patients (*p=0.04*). (Table 4)

Out of three CaSR gene polymorphisms studied, only polymorphism G/T at 986 (*p=0.034*) was found to be significantly higher in patients than controls whereas in case of polymorphism A/G at 990 *(p=0.32)* and polymorphism C/G at 1101 (*p=0.07)* , no significant difference between the cases and control was observed.

Table 4. Distribution Of CaSR Gene Mutation Among Various Groups

CaSR GENE MUTATION	SUBJECTS		CONTROL		ACUTE PANCREATITIS		RECURRENT ACUTE PANCREATITIS	
	n	%age	N	%age	n	%age	n	%age
NORMAL	37	74	23	92	25	80.6	12	63.2
HETEROZYGOUS	11	22	2	8	5	16.12	6	31.57
HOMOZYGOUS	2	4	0	0	1	3.22	1	5.26
Total	50	100	25	100	31	100	19	100
	p=0.02				p=0.042			

n:number,%age: percentage, p: p value

It was observed that neither the presence of SPINK 1 N34S (*p=0.205*) nor CaSR gene (*p=0.24*) mutation correlated with the severity of pancreatitis (Table 5)

Table 5. Distribution Of Gene Mutations In Relation To Severity

REVISED ATLANTA CLASSIFICATION	SPINK1 N34S MUTATION		CASR GENE MUTATION	
	Present	Absent	Present	Absent
MILD	5	12	1	16
MODERATELY SEVERE	7	11	7	11
SEVERE	9	6	5	10
p value	0.20		0.24	

Presence of SPINK1 N34S mutation was not significantly associated with any specific aetiology; alcohol(*p=0.93*), gallstone disease (*p=0.87*),hypertriglyceridemia *(p=0.08)*.Similarly the presence of CaSR gene mutation was not significantly associated with any aetiology :alcohol (*p=0.06*) ,gallstone aetiology (*p=0.7*) (Table 6)

Table 6. Frequency Of Genetic Mutations With Different Aetiologies

ETIOLOGY OF PANCREATITIS	PATIENTS		SPINK1 N34S MUTATION PRESENT				CaSR GENE MUTATION PRESENT			
			WITH AETIOLOGY UNDER CONSIDERATION		WITH OTHER AETIOLOGIES		WITH AETIOLOGY UNDER CONSIDERATION		WITH OTHER AETIOLOGIES	
	n	%age	n	%age	N	%age	n	%age	N	%age
GALLSTONES	10	20	3/10	30	18/40	45	3/10	30	10/40	25
ALCOHOL	32	64	13/32	40.6	8/18	44.4	10/32	31.25	3/18	16.7
HYPERTRIGYCERIDEMIA	3	6	3/3	100	18/47	38.2	0	0	13/47	27.6
POST-ERCP	1	2	0	0	21/49	42.85	0	0	13/49	26.5
HYPERCALCEMIA	1	2	0	0	21/49	42.85	0	0	13/49	26.5
IDIOPATHIC	3	6	2/3	66.6	19/47	40.4	0	0	13/47	27.6

n: number, %age: percentage

DISCUSSION

The prevalence of SPINK1 N34S mutation in patients was statistically higher than in control group (*p=0.01*). Similar results were obtained by Tukiainen et al.[14] who observed that the N34S mutation occurred in 7.8 % of cases and 2.6 % of controls although the frequency of mutation was relatively low as compared to our study. This difference in the prevalence of mutations may be due to different study population.

If a genetic factor is supposed to lower the threshold of a disease, the presence of disease with that particular genetic factor in younger population may be a pointer towards the role of genetic factor in that disease. In our study, patients with SPINK1 N34S mutation had significantly younger age*(p=0.032)* than patients without them which is consistent with study conducted O'Reilly et al. [15] which showed similar results. The prevalence of SPINK1 N34S mutation was statistically higher in RAP patients as compared to AP patients (*p*=0.01), a finding supported by Aoun et al. [16] who observed similar results. This can be explained by the fact that the presence of a genetic mutation predisposes to repeated attacks of RAP after a sentinel attack in the presence of continuous insult to pancreas.

The exact mechanism by which SPINK 1 N34S predisposes to pancreatitis is not clear. It is a protease inhibitor and protects the pancreas against premature trypsinogen activation. SPINK 1 mutations lower levels of trypsin inhibitor and thereby increase susceptibility to pancreatitis.[17,18] The presence of SPINK 1 N34S mutation seems to be disease modifying rather than causing the disease itself because same mutation was present with high frequency in the control group in our study. We can safely assume that the presence of SPINK 1 N34S mutation in presence of other pancreatic insults like alcohol makes one more susceptible not only to a sentinel attack of pancreatitis but also to recurrent attacks.

In our study, it was observed that the SPINK1 N34S mutation was not significantly different in mild and severe disease (*p=0.205)* which is consistent with a study done by Rai et al [19]. The presence of SPINK1 N34S mutation had no relation to severity of pancreatitis. Once the process of acute inflammation begins in a susceptible patient, SPINK1 N34S mutation may have no role in the progression of inflammatory cascade which eventually gets impacted by multiple factors including the cytokine polymorphism among others. The presence of SPINK 1 N34S mutation was not significantly related to any of the aetiologies of acute pancreatitis which is consistent with the study done by Koziel et al.[20]

The prevalence of CaSR gene mutation was statistically higher in patients as compared to controls (*p=0.02*). CaSR gene polymorphism at 990 has been seen in CP patients in previous studies.[21] However in our study polymorphism G/T at 986 (*p=0.034*) was found to be significantly higher in patients than controls but polymorphism A/G at 990 *(p=0.32)* and polymorphism C/G at 1101 (*p=0.07)* was not significantly higher in cases than controls. CaSR gene mutations were not significantly related to any of the aetiologies or severity of disease. Individuals carrying the CaSR gene mutations may experience mild decrease in serum ionized calcium levels which alters the cytosolic calcium ion concentrations in acinar cells in a concentration-dependent manner, and may alter the risk of acute pancreatitis.[22]We assume that CaSR gene mutations predispose individuals to development of AP and RAP rather than having a casual role, as the frequency of CaSR gene mutation was high in the control group too. While CaSR gene mutations have established association with chronic pancreatitis ,we could not find any study on CaSR gene mutation in AP and RAP. Thus our results need to be reproduced and validated in further studies.

Thus we conclude that SPINK1 N34S and CaSR gene mutations are associated with AP and RAP.

The association of SPINK 1 N34S and CaSR mutations was stronger for RAP than AP suggesting role of SPINK 1 N34S and CaSR mutations in predisposition to recurrent attacks of pancreatitis in these patients.

Although at this stage there seems to be limited therapeutic implication of our study but we can identify the group of AP patients who are predisposed to have repeated attacks of pancreatitis. Thus we can counsel them that any further pancreatic insult can be much more dangerous than patients of pancreatitis without such mutations. Patients can thus be encouraged to have strict abstinence from alcohol, to undergo cholecystectomy on priority basis and to tightly control their triglyceride levels among other corrective measures.

There were few limitations in our study:
1.There were less number of patients in our study so the results need to be reproduced on a larger scale.
2.There can be other genetic factors apart from SPINK 1 N34 S and CaSR which may play a role in the pathogenesis of AP and RAP.

Abbreviations:
AP: Acute Pancreatitis
CP : Chronic Pancreatitis
RAP : Recurrent Acute Pancreatitis
CaSR: Calcium Sensing Receptor
SPINK 1 N34S: Serine Protease Inhibitor Kazal Type 1

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