

Chapter 19

Genetic studies in chronic pancreatitis in India

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Summary

Background Aims : Mutations in cationic trypsinogen (PRSS1) gene are causally associated with recurrent acute and chronic pancreatitis. We investigated whether mutations in PRSS1 gene are associated with hereditary and non-hereditary pancreatitis. Since a modifier role has been proposed for trypsin inhibitor (SPINK 1) mutations, the role of SPINK1 mutations in these patients was also analyzed.

Subjects and Methods: The coding regions of PRSS1 and SPINK1 genes were sequenced in 290 controls and 304 patients, of whom 106 were diagnosed as tropical calcific pancreatitis (TCP), 120 as idiopathic (ICP), 41 as alcoholic (ACP), and 37 as hereditary pancreatitis (HP). Twenty-four unaffected relatives of HP probands were also analyzed and genotype-phenotype correlations and statistical analyses were performed.

Results: No mutations in PRSS1 gene were detected in any of the patients including hereditary pancreatitis, while N34S mutation was observed in SPINK1 gene of majority of HP patients (73%). Similarly, 26.8% of ACP (11 of 41) and 32.8% (39 of 120) of ICP patients and 42.5% (45 of 106) of TCP patients had SPINK1 mutations. N34S mutation was observed in both homozygous as well as heterozygous condition. In comparison, only 2.76% of control population had N34S allele ($P < 0.001$). P55S mutation was observed in one patient each of ICP and ACP and 2 TCP patients and 3 normal individuals. Genotype-phenotype correlation did not suggest any significant difference in the age of onset, severity of disease or pancreatic endocrine insufficiency in patients with or without mutated SPINK1 and irrespective of the allelic status of N34S SPINK1. However, FCPD patients had an earlier age of onset but comparable prevalence of SPINK1 mutation as opposed to TCP patients without diabetes mellitus.

Conclusions: Irrespective of the etiology, mutations in PRSS1 gene are not associated with CP including hereditary pancreatitis. On the contrary, N34S mutation in SPINK1 gene shows significant correlation in these patients, albeit with variable prevalence in each etiological type. The results suggest a common genetic basis for TCP with additional factors responsible for the variability of phenotype in FCPD and TCP without diabetes mellitus. A comparable phenotype in terms of age of onset, diabetes mellitus and other phenotypic features in patients with or without SPINK1 mutations and N34S homozygotes and heterozygotes suggests that there may still be involvement of other genetic or environmental factors.

Introduction

Chronic pancreatitis (CP) is a global health care problem with varied etiologies. Alcohol is generally considered as an important risk factor for the development of chronic pancreatitis. However, additional factors like heredity, smoking, anatomical variations and metabolic disorders like hyperlipidemia and hypercalcemia have also been identified. About 20% to 30% of such cases fall in the category of idiopathic chronic pancreatitis (ICP) since the causal factor in them is yet to be known. Although the exact pathogenesis is not clear, autodigestion secondary to aberrant intraductal activation of zymogens by trypsin is a primary common event. Several genetic risk factors for CP have been identified recently.

The genetic basis of CP was first reported in 1996 by familial linkage analysis and confirmed by detection of missense mutations namely R122H and N29I in cationic trypsinogen gene (PRSS1) in hereditary pancreatitis (HP) patients. Subsequent efforts to investigate the presence of HP-associated PRSS1 mutations showed a very low incidence in ICP and complete absence in alcohol related pancreatitis (ACP). Overall, only about 60% cases of HP and less than 20% with a diagnosis of ICP have a mutated PRSS1 gene. The underlying causes of variability in penetrance are not clear, but the observations indicate the involvement of environmental as well as other genetic factors. More recently, N34S mutation of the serine protease inhibitor, Kazal type I (SPINK1), has been reported to be strongly associated with idiopathic and familial pancreatitis. Subsequent studies, however, have reported low prevalence of mutated SPINK1 gene in ICP patients. The role of SPINK1 mutations particularly N34S mutation is a matter of controversy with some suggesting a causal while others advocating a modifier role for this molecule.

Tropical calcific pancreatitis (TCP) is an idiopathic, juvenile, non-alcoholic form of CP widely prevalent in several tropical countries whereas Fibrocalculous pancreatic diabetes (FCPD) is a form of diabetes secondary to TCP. The disease differs from alcoholic pancreatitis by much younger age of onset, pancreatic calcification, a high incidence of insulin dependent but ketosis resistant diabetes mellitus and exceptionally high incidence of pancreatic cancer. We analyzed a large cohort of patients with hereditary and non-hereditary pancreatitis (ICP, ACP and TCP) to

determine if PRSS1 and SPINK1 mutations are associated with CP in India and also to understand their respective roles in the causation of disease. We found no mutations in PRSS1 gene but detected only SPINK1 mutations in all types of pancreatitis patients. We therefore propose genetic basis of CP (irrespective of its etiology) in India to be different to what has been observed in the Western countries. The observations made in this study may have implications in counseling and modification of the predisposition risk by avoiding exposure to possible precipitating factors such as alcohol, smoking and nutrition etc. in India.

Subjects and methods

Selection of patients

The diagnosis of CP was based on at least two separate episodes of abdominal pain and radiological findings of pancreatic calcifications by Computed tomography, Endoscopic ultrasonography and/or pathological findings like pancreatic ductal irregularities and dilatations on Endoscopic retrograde cholangiopancreatography. A detailed questionnaire including the clinical and family history and various investigations was collected from all the patients and their unaffected relatives willing to participate in the study. Clinical history included etiology, type and severity of pain, frequency of attacks, presence or absence of diabetes mellitus, age at onset of symptoms and of diabetes mellitus etc. Exclusion criteria for diagnosis of ICP included the absence of precipitating factors such as alcohol, gallstones, infection, trauma, medications and metabolic disorders, age over 65 years and a positive family history. Alcohol was considered causal in CP patients with a daily intake equivalent to more than 80 g of ethanol for at least two years. A diagnosis of HP was made on the basis of at least two affected first degree relatives or three or more second degree relatives in two or more generations. Patients were categorized as TCP based on the established WHO criteria.

Thus, in total 304 patients (120 ICP, 41 ACP, 106 TCP and 37 HP) and 24 unaffected relatives from HP families participated in the study. 290 healthy volunteers constituted the control population. Blood samples were drawn using EDTA as anticoagulant after collecting written informed consent.

DNA analysis

Genomic DNA was isolated from leucocytes following standard protocols. Since there is no report on genetics of CP in Indian population, PRSS1 and SPINK1 genes were sequenced to screen for the reported as well as for any novel mutations. The primer sequences were selected from the published sequences and nested PCR strategy was used to amplify PRSS1 gene since it is highly homologous with other trypsinogens. Sequencing was done on both the strands using Big dye terminator cycle sequencing ready kit on a DNA Sequencer. 580 control alleles were also sequenced to identify the prevalence of PRSS1 and SPINK1 variants in the general population.

Statistical analysis

All values are presented as median (range, 95% CI). Chi-Square test was used to analyze differences in prevalence of SPINK1 and N34S mutation among ICP, ACP, TCP and HP patients as well as controls. We categorized the study cohort based on presence or absence of N34S SPINK1 mutation and its zygosity. Phenotypic variability in features like age of onset and presence or absence of diabetes mellitus etc. among these groups was analyzed by applying Mann-Whitney U test using SPSS^R software. A p value less than 0.05 was considered statistically significant.

Results

Patient details

Our study cohort comprised 37 HP patients from 16 families and 267 patients with non-hereditary pancreatitis (120 ICP, 41 ACP and 106 TCP patients). There were 232 males and 72 females but all ACP patients were exclusively male. Majority of patients presented with pain in the abdomen (91%), while diabetes mellitus was the presenting symptom in the rest. The median age of onset for HP, ICP and TCP patients was comparable at 24.5, 23.5 and 25.0 years respectively, which was significantly lower than 36 yrs for ACP patients ($P < 0.001$). However, HP patients reported a longer duration of disease compared to other categories (Table 1).

DNA Analysis

On sequence analysis, none of the patients or controls carried either the common mutations or any novel variant in the coding region of PRSS1 gene. However, two commonly reported neutral polymorphisms 162Asp (GAC>GAT) and 246Asn (AAC>AAT) were observed in majority of patients (88%) as well as in the controls (90%, $P>0.05$). In comparison, 122 CP patients (40.1%) had at least one SPINK1 mutation. Majority of patients ($n=118$) carried N34S allele including 38 homozygotes and 80 heterozygotes. (Table 2) N34S mutation was found to be in complete linkage disequilibrium with IVS1-37T>C. P55S was observed in heterozygous state in only 4 patients (1.3%). The previously reported neutral polymorphism -253T>C was identified in heterozygous state in 3.5% of patients and 27.9% of controls. Eight out of 290 healthy controls also carried N34S mutation (2.76%, allele frequency= 0.014) while P55S was observed in only three individuals (1.03%, allele frequency=0.0017). Both mutations were present in heterozygous state and no other previously reported mutations like 2T>C, 41T>C etc. were detected in these patients.

Thirty-eight of 120 ICP patients (31.7%) carried N34S mutation ($P<0.0001$ vs. controls), of which 7 were homozygous. However, no significant difference in N34S SPINK1 mutation frequency was noted for early onset (35.7%) and late onset form (22.2%, $P=0.3872$) of ICP. Interestingly, we identified N34S mutation in 10 of 41 ACP patients (24.4%, $P<0.0001$ vs. controls), which is significantly higher compared to earlier studies reporting frequencies ranging between 5.6-6.0%. All of them were N34S carriers except one P55S heterozygote and one N34S homozygote. Till date, no N34S homozygote has been reported in this group of patients. This individual was a 31-year-old patient with persistent pain since the age of 20 yrs and diabetes for 2 years and very low alcohol intake for last 5 years. Although the diagnosis of ACP is based on a history of excessive alcohol intake in the background of recurrent attacks of AP, the amount of alcohol intake has been reported to vary from 25 g/day to more than 80 g/day for 5 years. Of 16 families matching the criteria of HP, 12 (75%) carried N34S SPINK1 mutation. 73% of HP patients ($P<0.0001$ vs. controls) were positive for N34S mutation and included 7 homozygotes. Interestingly, all N34S

homozygotes in this group were diabetic with the age of onset between 5 and 12 years. Of 24 unaffected relatives, 6 (25%) carried N34S SPINK1 mutation. The only homozygote was a 23-year-old individual without pancreatitis or diabetes mellitus, although his heterozygous parents had the disease.

SPINK1 mutations were also detected in 45 out of 106 (42.5%) TCP patients analyzed. Of 43 patients with N34S mutation, 10 were homozygous, 32 heterozygous and 1 compound heterozygote with P55S (Fig 1a). We detected SPINK1 mutations in both FCPD patients and TCP patients without diabetes mellitus in comparable frequency. A novel G to T transversion at 215 bp upstream in the SPINK1 promoter region (-215G>T) was also identified in 3 patients, who interestingly also carried an N34S allele, suggesting a compound heterozygote status (Fig 1b).

Genotype-phenotype correlation

We categorized the study cohort according to etiology and then compared the SPINK1 N34S positive and negative patients in each category as a function of various phenotypic markers (Table 3). The median age of onset and presentation for FCPD patients was 35.0 and 44.0 yrs respectively, which is significantly higher than that of TCP patients without diabetes mellitus 21.0 ($P<0.004$) and 26.0 yrs ($P<0.001$). The age of onset of symptoms was lower in the group with N34S SPINK1 compared to the group carrying wild type SPINK1 in each category but did not reach statistical significance except in HP patients ($P=0.045$). Analysis of N34S carrier frequency after categorizing our study cohort into groups by age showed interesting results. The <20 yrs group had a carrier frequency of 52.8% (28 of 53) which is significantly higher than that of 24.8% (36 of 145) in the 20-65 year-old group ($P<0.016$). Interestingly, majority of homozygotes (14 of 15) had CP before the age of 20 years. The only homozygote in the older group was a 54-year-old patient with mild disease and diabetes for 6 years. An increased contribution of environmental factors in the latter group may have contributed to this significant difference.

Diabetes mellitus as a feature of pancreatic endocrine insufficiency was equally prevalent in both groups and so were other parameters of disease severity, like pain, pseudocysts, pancreatic ductal abnormalities etc. (Table 3). A comparison of SPINK1 mutation frequency in FCPD patients and TCP patients without diabetes showed similar trends suggesting that neither these mutations nor their status is directly related to the presentation of diabetes mellitus (Table 4). Prevalence of N34S SPINK1 mutation (43.1%) in patients with diabetes was similar in patients without diabetes (37.9%) (Table 4). Relatively more N34S homozygotes were observed in patients with diabetes (38.7%) than the group without diabetes mellitus (14.7%). This may be due to additional genetic or environmental factors especially in the HP cohort since the prevalence of N34S mutation was comparable in both the groups. However, association between N34S and diabetes mellitus did not reach statistical significance in all categories of patients.

Data from HP families showed a variable genotype-phenotype association in individual families. In one of the families, the proband was a 40-year-old lady with pancreatitis at 21 and diabetes at 23 yrs (Fig 2). She was detected to be homozygous for N34S SPINK1, which was inherited from her obligate heterozygous healthy parents. Her father was diabetic for last 30 years but of her two obligate N34S heterozygote sons, the younger has both pancreatitis and diabetes for last 5 years, while the elder one is healthy. Three of her brothers were positive for N34S and had diabetes without evidence of pancreatitis. In another family, the proband was an 18-year-old N34S heterozygote, inherited from his heterozygous father who also had early onset of the disease. However, his elder brother and paternal grandmother are healthy despite being heterozygous for N34S SPINK1, whereas two aunts with N34S/WT have severe pancreatitis with diabetes and another heterozygote aunt is only diabetic.

Discussion

Chronic pancreatitis is a heterogeneous disease and its genetic basis in India has not been investigated. We analyzed 304 patients with hereditary and non-hereditary pancreatitis with the major objective of understanding the respective roles of PRSS1 and SPINK1 mutations in

its causation. In the present study, except two previously reported cSNPs, no PRSS1 mutation could be identified in any patient as well as in control individuals. Absence of PRSS1 mutations in HP and ICP patients is intriguing since such mutations have been reported in upto 60% of HP and about 20% of ICP patients. We describe absence of PRSS1 mutations in Indian patients with CP of different etiologies for the first time. This may most likely be related to their genetic makeup since no other study from abroad has reported absence of PRSS1 mutation in HP as well as non-hereditary CP patients. However, interaction with other factors like environmental, nutritional may also play an important role. These results strongly suggest that irrespective of its etiology, established mutations in PRSS1 are not a common cause of CP in the Indian population.

However, SPINK1 mutations were found to be strongly associated with all types of chronic pancreatitis. Most patients with SPINK1 variation had N34S mutation and the prevalence was significantly higher in HP compared to ICP, ACP and TCP ($P < 0.001$). The presence of N34S SPINK1 mutation in majority of HP patients is particularly interesting since PRSS1 mutations are lacking in these patients. N34S SPINK1 prevalence (2.76%) in controls is much higher than 1.5% in the French, 1.58% from USA and 0.36% from Germany but much lower than 4% in control population from Liverpool. Therefore, distribution of N34S allele among various populations might be more variable than originally assumed. The observed prevalence of mutated SPINK1 in ICP patients (32.5%) is significantly higher than earlier reports varying from 6.4-21.0% in other studies. A stronger genetic basis has recently been suggested for early-onset ICP than the late-onset ICP but we didn't find any significant association for N34S SPINK1 prevalence, although majority of patients in the older group (12 of 36) presented with diabetes as compared to only 7 of 84 early-onset patients ($P = 0.0103$). The higher age of onset for HP patients (24.5 yrs) in our study compared to majority of studies conducted abroad may be due to the presence of N34S SPINK1 mutation, which are hypothesized to perform a modifier role in comparison to the causal role played by PRSS1 mutations in the Western HP patients. A highly significant prevalence of SPINK1 mutations in our cohort of ACP patients suggests an important role for this genetic variant in our population. Decreased trypsin inhibitor to trypsinogen levels has been reported in the pancreatic juice of alcoholics compared with controls

without alcoholism. Alcohol might also affect SPINK1 regulation during the complex inflammatory processes in human alcoholic pancreatitis. Earlier studies have shown that in comparison with white patients, black patients are 2-3 times more likely to be hospitalized for CP than alcoholic cirrhosis. Thus, alcoholics in India may be more susceptible to CP due to a combination of factors like genetic makeup, racial difference in diet, type or quantity of alcohol or smoking etc. Although, the present knowledge suggests that ACP patients are likely to have higher interaction with the environmental factors in comparison to other types of chronic pancreatitis, there is a strong genetic basis for ACP patients in India. Since, SPINK1 mutations appear to predispose humans to an earlier age of onset, they may have an impact on the phenotypic presentation of ACP.

The exact relationship between the phenotype of FCPD and TCP is still not clear, although there is an overlap in their phenotypes. In our study cohort of 106 TCP patients, 30 were FCPD patients while remaining 76 were without diabetes at the time of presentation. Comparison of the age of onset shows that FCPD patients had a late age of onset with a difference of more than a decade (21 yrs vs. 35 yrs). It has also been shown that the patients with TCP are younger than the FCPD patients and majority of them have an abnormal Glucose tolerance test. This strongly suggests that FCPD may be gradually evolving diabetes in the background of TCP. Our study confirms the causal role of N34S mutation in SPINK1 gene in pancreatitis and simultaneously establishes the role of mutated inhibitor in the pathogenesis of TCP. These contradict the earlier observations by Rossi et al suggesting a genetic basis for FCPD only and proposing a clinical distinction within TCP with respect to presence or absence of diabetes mellitus. However, their limited sample size (8 FCPD and 4 TCP patients) raises serious concern about the validity of such a conclusion. Our results suggest a common genetic basis for TCP with additional genetic/environmental factors responsible for the variability of phenotype in TCP and FCPD.

The cohort in this study represents the conglomeration of CP patients usually seen in routine clinical practice in developing countries like India. Such high frequency of SPINK1 gene mutations in the background of complete absence of mutated PRSS1 gene is interesting. Till date, no

study has reported concurrence of mutated PRSS1 with SPINK1 suggesting that both the mutations work through different and independent mechanisms. However, this pancreatic protease-protease inhibitor system is very important, considering the physiological interaction between them in combating the prematurely activated trypsinogen inside the pancreas. Intrapancreatic levels of trypsin are expected to be elevated if mutations in the inhibitor molecule lead to loss of its inhibitory capacity. However, it is difficult to explain the phenotype of pancreatitis in the presence of a normal trypsin when the intact R122 autolysis "self-destruct" mechanism can take care of the prematurely activated trypsin molecule. This suggests impairment of other protective mechanisms involved in combating the prematurely activated trypsin molecule inside the pancreas. The exact mechanism is still not clear and it remains to be proven how, the prematurely activated trypsin is sustained inside the pancreatic acini and may cause a low-grade inflammation and disease. SPINK1 mutations have been proposed to significantly lower the threshold for pancreatitis from other factors. It is hypothesized that mutated inhibitor, with N34S mutation, may have functional consequences probably due to alteration of the protein structure.

The present state of research on the role of N34S SPINK1 is confusing with both causal as well as modifier role. A recent study supports the significance of SPINK1 mutations based on disappearance of pancreas in the homozygous knockout mice of SPINK1, although heterozygous mice showed no alteration in pancreatic tissue. We did not observe any significant difference in the phenotype between SPINK1 mutation positive and mutation negative groups as well as between SPINK1 N34S heterozygotes and homozygotes. Although, the association between N34S and diabetes mellitus was not statistically significant in all the categories of patients, N34S homozygosity was positively associated with diabetes mellitus. This suggests that N34S SPINK1 mutation may be involved in only modifying the phenotype. Several studies have argued about SPINK1 mutations as autosomal recessive or autosomal dominant. Our results may suggest autosomal dominant mode of inheritance with a low level of penetrance. At the same time, autosomal recessive model is also suggested by the high prevalence of N34S homozygotes (7.6%) in patients. Despite a significantly strong

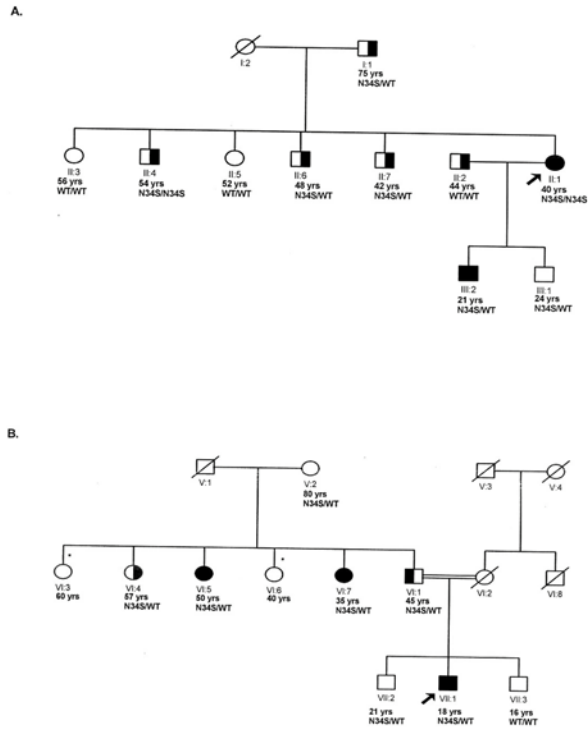
association of N34S with HP, analysis of HP families in our study shows variable inheritance pattern and association with the phenotype. This phenomenon of genetic heterogeneity is a characteristic of complex diseases with an important role of environment. Hence, it may be logical to suggest that the presence of a second mutation, either in the same gene or other genes in association with environmental factors is required to express the disease phenotype.

In conclusion, we demonstrated for the first time, the association of mutated pancreatic secretory trypsin inhibitor with tropical calcific pancreatitis, irrespective of the absence or presence of diabetes mellitus (FCPD). We also showed that N34S mutation in SPINK1 gene is strongly associated with all types of chronic pancreatitis, although the penetrance is quite variable. But mutations in cationic trypsinogen gene are not the important causes of chronic pancreatitis in Indian population. The presence of SPINK1 mutations in both FCPD and TCP patients without diabetes mellitus suggests a common genetic basis for tropical calcific pancreatitis. However, different genetic/environmental factors may be involved to account for phenotypic variability in TCP patients. Till date, N34S SPINK1 mutation is the only factor imparting a genetic basis to chronic pancreatitis in Indian patients. This may have implications in presymptomatic genetic testing, however, analysis of more such patients may validate such a conclusion.

Acknowledgements

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Figure 2



N34S/N34S, homozygotes; N34S/WT, heterozygotes; WT/WT, wild-type

◻ Chronic pancreatitis ◻ Diabetes mellitus

◼ Both chronic pancreatitis and diabetes mellitus ↗ Proband

* DNA samples not available

Table 1 Characteristics of the study population

	HP	ICP	ACP	TCP	Total
n	37	120	41	106	304
Sex (M/F)	28M/9F	87M/33F	41M/0F	76M/30F	232M/72F
Age of presentation (yrs)	39.5 (31.4-46.6)	27.5 (26.7-30.9)	40.0 (37.1-41.9)	32.0 (30.0-34.8)	31.0 (30.8-33.7)
Age of onset (yrs)	24.5 (18.1-34.5)	23.5 (22.8-27.3)	36.0 (32.9-37.9)	25.0 (25-29.8)	26.0 (26.3-29.3)
Duration of symptoms (yrs)	9.5 (6.7-15.9)	4.7 (3.9-5.4)	4.0 (3.7-5.7)	4.5 (4.2-6.6)	4.2 (4.0-5.3)

Values are median (range 95% confidence interval)

n, number of patients; HP, hereditary pancreatitis; ICP, idiopathic chronic pancreatitis; ACP, alcohol related pancreatitis; TCP, tropical calcific pancreatitis

Table 2 Distribution and status of the PRSS1 and SPINK1 mutations in chronic pancreatitis patients

	HP	ICP	ACP	TCP	Total
n	37	120	41	106	304
PRSS1 mutation*	-	-	-	-	-
SPINK1 mutation	27 (73%)	39 (32.5%)	11 (26%)	45 (42.5%)	122 (40.1%)
N34S	27	38	10	43	118
Homozygote	20	7	1	10	38
Heterozygote	7	31	9	33	80
P55S	-	1	1 (2.4%)	2 (1.9%)	4 (1.3%)
Homozygote	-	-	-	-	-
Heterozygote	-	1	1	2	4

n, number of patients; HP, hereditary pancreatitis; ICP, idiopathic chronic pancreatitis; ACP, alcohol related pancreatitis; TCP, tropical calcific pancreatitis

* Neither the common nor any novel mutation were detected in the PRSS1 gene;

Figures in parentheses indicate percentage

Chapter 20
**The pathology of fibrocalculous
pancreatopathy (CCP)**

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