Trypsinogen Copy Number Mutations in Patients With Idiopathic Chronic Pancreatitis

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Background & Aims: **We have recently reported that the triplication of a ~605 kilobase segment containing the *PRSS1* (encoding cationic trypsinogen) and *PRSS2* (encoding anionic trypsinogen) genes causes hereditary pancreatitis. Here we went further to investigate whether this copy number mutation could account for some unidentified French white patients with idiopathic chronic pancreatitis (ICP) or familial chronic pancreatitis (FCP) as well as Indian patients with tropical calcific pancreatitis (TCP).** ***Methods:*** **Patients and controls were screened by means of previously described quantitative fluorescent multiplex polymerase chain reaction and/or genotyping of the microsatellite marker rs3222967.** ***Results:*** **The ~605 kilobase triplication and a novel duplication (confirmed by fluorescence in situ hybridization) of the trypsinogen locus were detected in 10 and 2 of 202 ICP patients, respectively (age of disease onset, ≤20 years) but were absent in 282 French controls. In addition, the duplication mutation was found in 2 of 1044 ICP patients whose age of disease onset was >20 years. However, the 2 trypsinogen copy number mutations were observed in neither 103 FCP patients nor 268 Indian TCP patients.** ***Conclusions:*** **Our findings revealed the molecular basis of 6% of the young ICP patients and further demonstrated that chronic pancreatitis is a genomic disorder. Our findings also add to the mounting evidence showing that trypsinogen gene mutations do not appear to play an important role in the pathogenesis of TCP in the Indian population. Finally, a dividend of this study is that we have provided convincing evidence to show that all 5 previously described copy number variations involving *PRSS1* or/and *PRSS2* are artifacts.**

Chronic pancreatitis is a persistent inflammation of the pancreas characterized by attacks of abdominal pain, irreversible morphologic changes, and deficiency of pancreatic enzymes.[1] Hereditary pancreatitis (MIM #167800)—a rare form of chronic pancreatitis—is inherited as an autosomal dominant disease.[2] Nevertheless, genetic studies of hereditary pancreatitis have provided the strongest support for the century-old theory that chronic pancreatitis is an autodigestive disease,[3] which was thought to be initiated by prematurely activated trypsin within the pancreas. Since the mapping and cloning of one gene for hereditary pancreatitis in 1996,[4–7] complementary observations have pointed to the pivotal role of a gain of trypsin in the etiology of chronic pancreatitis. First, several gain of function missense mutations (eg, p.D19A,[8] p.D22G,[8,9] p.K23R,[8,9] p.N29I/T,[10] and p.R122H[11,12]) in the *PRSS1* gene (encoding cationic trypsinogen, the most abundant isoform of trypsinogen[13]; MIM #276000) have been reported to be disease-causing. Second, loss of function variations in *PRSS1* or *PRSS2* (encoding anionic trypsinogen, the second major isoform of trypsinogen[13]; MIM #601564) have been reported to be disease-protecting.[14,15] Finally, a diverse range of variations including missense, splicing, frame-shifting, and nonsense mutations in the *SPINK1* gene (encoding trypsin's physiologic inhibitor; MIM #167790) have also been reported to be associated with idiopathic chronic pancreatitis (ICP), familial chronic pancreatitis (FCP), tropical calcific pancreatitis (TCP; MIM #608189), or even to cause autosomal dominant hereditary pancreatitis.[16–26]

The above findings were invariably made from studies of point mutations or microinsertions/deletions. Recently, copy number mutations (CNMs) including 2 large genomic deletions of the *SPINK1* gene[27,28] and a ~605 kilobase (kb) triplication containing both *PRSS1* and *PRSS2* on chromosome 7[29] (Figure 1*A*) have also been identified in patients with chronic pancreatitis. (The term *CNM* is used here to distinguish from the neutral term *copy number variation* [CNV]. CNV refers to a DNA segment of ≥1 kb that is present in different copy numbers with respect to a reference genome sequence.[30]) The 2 large deletions in *SPINK1* are clearly disease-causing; whereas the first one results in gene disruption,[27] the second one removes the entire gene.[28] The ~605 kb trypsinogen triplication was also considered to be disease-causing because (1) it segregated with the disease in 5 French white families with hereditary pancreatitis, (2) it is absent in 200 healthy controls from the same population, and (3) an increased expression of trypsinogen resulting from an increased gene dosage of *PRSS1*/*PRSS2* is

Abbreviations used in this paper: CNM, copy number mutation; CNV, copy number variation; FCP, familial chronic pancreatitis; FISH, fluorescence in situ hybridization; ICP, idiopathic chronic pancreatitis; kb, kilobase; QFM-PCR, quantitative fluorescent multiplex polymerase chain reaction; TCP, tropical calcific pancreatitis.

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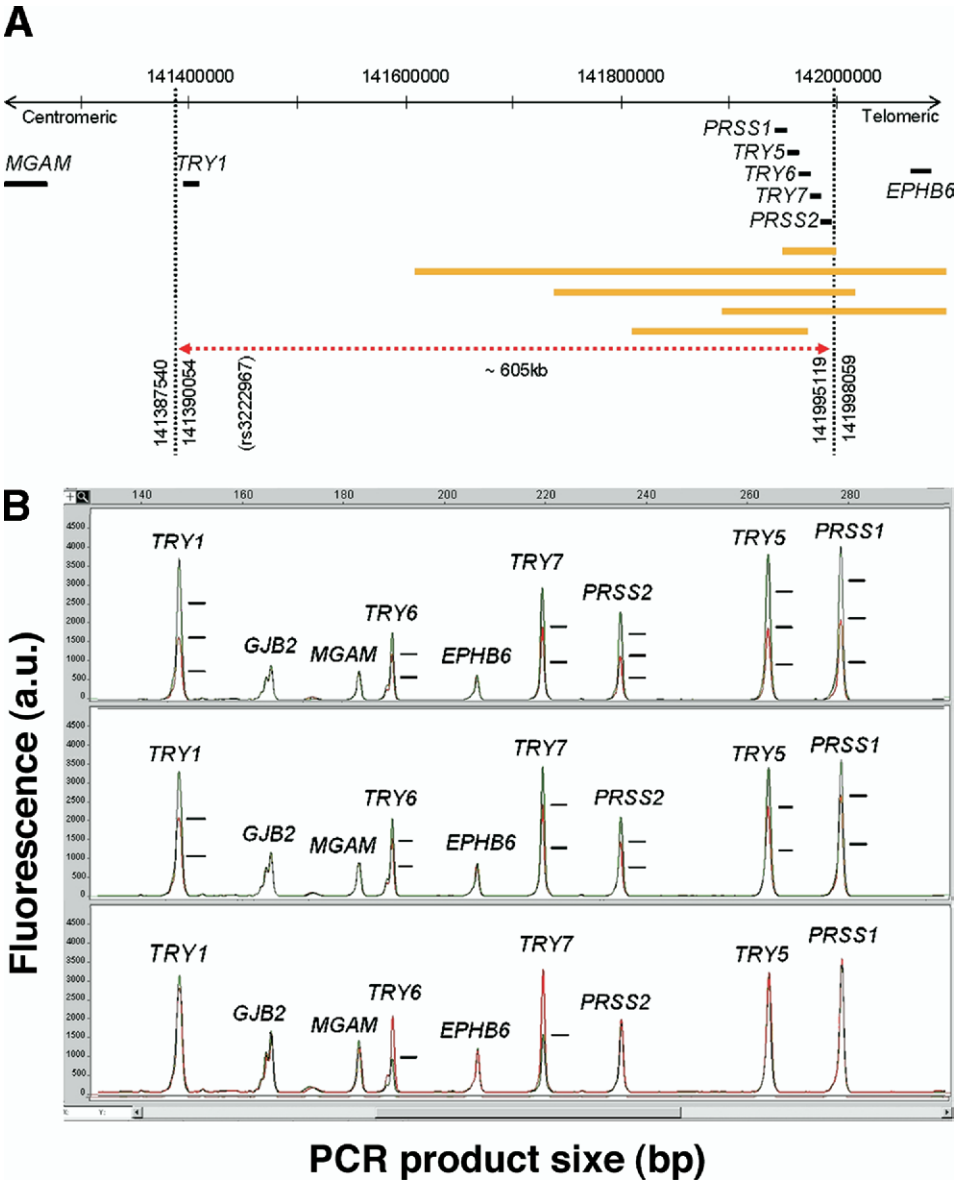

Figure 1. Detection of trypsinogen CNMs in patients with ICP. (*A*) Schematic illustration of the ~605 kb triplication of the trypsinogen locus and its flanking regions on chromosome 7q34. (Adapted from reference 29). *Vertical dotted lines* indicate uncharacterized centromeric and telomeric regions flanking the triplicated ~605 kb segment. Five *horizontal yellow bars* indicate "CNVs involving *PRSS1* and/or *PRSS2*" logged in the Database of Genomic Variants (Table 1). (*B*) Detection of triplication (*top panel*) and duplication (*middle panel*) of trypsinogen locus in ICP patients by QFM-PCR. Note that illustrated triplication carrier possesses a total of 4 copies of *TRY1*, *PRSS1*, *TRY5,* and *PRSS2* but only 3 copies of *TRY6* and *TRY7*. *Lower panel* illustrates our QFM-PCR analysis of sample NA11919 that was previously reported to carry a trypsinogen CNV gain (Table 3); no CNV was found in *TRY1*, *PRSS1*, *TRY5,* and *PRSS2,* whereas only 1 copy of *TRY6* and *TRY7* was identified. Electropherogram of the analyzed subjects (*in green*) was superimposed on that of a normal control who was known to carry 2 copies of *TRY6* and *TRY7* (*in red*) after normalization against the control amplicon *GJB2* located on chromosome 13. Increased or decreased fold in peak height of the *TRY1*, *PRSS1*, *TRY5*, *TRY6*, *TRY7,* and *PRSS2* amplicons in the analyzed samples is indicated by *horizontal bars*.

consistent with our current understanding of the role of trypsin in the etiology of chronic pancreatitis.[29]

The initial aim of this study was to investigate whether the ~605 kb trypsinogen triplication could account for some unidentified French white patients with ICP or FCP. Because previously reported mutations in *PRSS1* are absent in TCP patients, we also investigated whether increased copy number of the trypsinogen locus could be the primary mechanism responsible for TCP. During this process, we had become aware of 5 "gain" or "loss" CNVs involving *PRSS1* and/or *PRSS2* detailed in the Database of Genomic Variants (http://projects.tcag.ca/variation/; Figure 1*A* and Table 1). That all of them, particularly the trypsinogen gain CNVs, were identified in subjects without any known phenotypes[31–33] poses a serious challenge to our previous finding.[29] To obtain further insights into this issue, we decided to screen a further set of control samples from different ethnic origins and, most importantly, to investigate whether we can replicate the presence of these CNVs in the original samples by using our own method, quantitative fluorescent multiplex polymerase chain reaction (QFM-PCR).[29]

Materials and Methods

Patients

Clinical diagnosis of chronic pancreatitis was based on 2 or more of the following criteria: presence of a typical history of recurrent pancreatitis, radiologic findings such as pancreatic calcifications and/or pancreatic irregularities revealed by endoscopic retrograde pancreatography or by magnetic resonance imaging of the pancreas and/or pathologic sonographic findings, as previously described.[15] Patients were classified as having ICP when both precipitating factors (eg, alcohol abuse, trauma, medication, infection, metabolic disorders) and a positive family history were not reported. The only difference between ICP and FCP is that a positive family history was reported in the latter, but it did not satisfy the criteria we previously used to

Table 1. Five CNVs Involving *PRSS1* and/or *PRSS2* Reported in Three Genome-Wide Studies[a]

Variation	Genomic position	Known gene(s)	Frequency of gain or loss	Total no. of control samples analyzed	Reference
0623	chr7:141,948,871–142,002,444	*PRSS2*	Loss: 1	46 lymphoblastoid cell lines (Coriell Cell Repository) of diverse ethnic origin	31
3707	chr7:141,610,138–142,240,984	*C7orf34, PRSS1, PRSS2, KEL, EPHB6, TRPV6, TRPV5, OR9A2*	Loss: 28	270 HapMap individuals	32
2715	chr7:141,739,700–142,018,646	*PRSS2, PRSS1*	Loss: 26	270 HapMap individuals	32
4567	chr7:141,887,558–142,030,287	*PRSS1, PRSS2*	Gain: 3, loss: 2	95 individuals	33
4566	chr7:141,813,914–141,973,680	*PRSS1*	Gain: 1, loss: 2	95 individuals	33

[a]From the Database of Genomic Variants (http://projects.tcag.ca/variation/; as of July 31, 2007). Variations were arranged in the same order (from top to the bottom) as they appeared in Figure 1*A*.

define hereditary pancreatitis (ie, 3 or more affected members involving at least 2 generations[29]). TCP was diagnosed in accordance with the established World Health Organization criteria as described earlier.[20]

Four groups of patients participated in this study: all patients had not been found to carry any known point mutations or microinsertions/deletions in the *PRSS1* and *SPINK1* genes after mutational screening by our previously established methods;[20–23,34] some of them (ie, groups Ia and II) had also not been found to carry any known mutations in the *CFTR* gene, by means of denaturing high-performance liquid chromatography[35] (Table 2). Eighty-two unrelated healthy white-French subjects (newly recruited after the completion of our previous study[29]) and 63 healthy Dravidians (ethnically matched to the Indian TCP patients) served as controls. The ethical review committee of respective institutions approved this study, and all patients gave informed consent for genetic analysis.

In addition, DNA samples from 25 Moroccans, 25 Ivorians, and 25 Vietnamese were further recruited; and several DNA samples reported to carry trypsinogen CNVs were purchased from the Coriell Cell Repositories (Camden, NJ) (Table 3).

Mutational Screening

Except for group Ib, all the patients and controls were screened by means of QFM-PCR as previously described.[29] It is important to point out that because of the presence of a common deletion polymorphism involving *TRY6* and *TRY7*,[36] all the QFM-PCR analyzed samples in this study were normalized against a same control sample that was known to possess 2 non–*TRY6/TRY7*-deletion alleles. This served to ensure an accurate estimation of the *TRY6/TRY7* copy number in the analyzed subjects. The group Ib patients were initially genotyped for the microsatellite marker rs3222967, which is located ~55 kb telomeric to *TRY1* (Figure 1*A*), as previously described.[29] Samples found to carry at least one 163 allele were then subjected to the abovementioned QFM-PCR analysis; the frequency of the 163 allele in the French white control population was determined to be only 1.2%; and the trypsinogen triplication and duplication mutations identified by QFM-PCR were found to carry at least one 163 allele (Table 4). For cross-platform comparison, the genotype of CEPH-134702 is 169-169.

Results and Discussion

Trypsinogen Triplication and Duplication Mutations in Patients With Idiopathic Chronic Pancreatitis

Previous studies have shown that pancreatitis-predisposing genetic factors are more easily found in children and adolescents with ICP than in adults.[17,37–39] Thus, we initially analyzed group Ia of ICP patients (age of disease onset, <20 years) for both theoretical and practical considerations. Triplication of the entire trypsinogen gene family (top panel, Figure 1*B*) was found in 10 of the 202 ICP individuals (5%). (Because both the nonfunctional *TRY6* and *TRY7* genes are flanked by other trypsinogen members (Figure 1*A*) and the common *TRY6* and *TRY7* deletion polymorphism[36] appears not to be associated with the disease [unpublished preliminary data], we consider the trypsinogen locus on chromo-

Table 2. Classification of Participating Patients

Group	No.	Ethnicity	Diagnosis	Previous mutational screening
Ia	202	French white	ICP (age of disease onset was either known to be ≤20 y or the diagnosis was made at the age of ≤20 y)	None of them carried any of the known point mutations or microinsertions/deletions in the *PRSS1*, *SPINK1* and *CFTR* genes after mutational screening by our previously established methods[23,34,35]
Ib	1044	French white	ICP (the remaining subjects)	Same as above except that *CFTR* gene was not screened
II	103	French white	FCP	Same as Ia
III	268	Dravidian (India)	TCP	None of them carried any of the known point mutations or microinsertions/deletions in the *PRSS1* and *SPINK1* genes after mutational analysis as described before[20]

Table 3. Trypsinogen CNVs Containing DNA Samples

Reference no.[a]	Description	Reported CNV[b]	Copy number of *TRY6 and TRY7*[c]
NA06985	CEPH/UTAH pedigree 1341 (father)	Unknown	1
NA06991	CEPH/UTAH pedigree 1341 (child)	Loss: variation 3707	0
NA06993	CEPH/UTAH pedigree 1341 (mother)	Unknown	1
NA10469	Pygmy population	Gain: variation 4567	0
NA10843	CEPH/UTAH pedigree 1423 (mother)	Unknown	2
NA11919	CEPH/UTAH pedigree 1423 (child)	Gain: variation 4567	1
NA17076	Human variation panel: Puerto Rican	Gain: variations 4566 and 4567	2
NA18524	International HapMap project: Han Chinese in Beijing, China	Loss: variation 3707	0
NA18537	International HapMap project: Han Chinese in Beijing, China	Loss: variation 3707	0
NA18855	International HapMap project: Yoruba in Ibadan, Nigeria (mother)[d]	Loss: variation 3707	0
NA18857	International HapMap project: Yoruba in Ibadan, Nigeria (child)[d]	Unknown	1

[a]Coriell Cell Repositories.
[b]See Table 1 for indicated CNV.
[c]Determined in this study.
[d]From same family.

some 7q34 to be intact if CNMs or CNVs were not found in the other gene members.) More importantly, we identified a novel CNM, duplication of the trypsinogen locus (middle panel, Figure 1*B*), in 2 of these ICP subjects. Altogether, trypsinogen triplication and duplication account for 6% (12/202) of group Ia of ICP patients (Table 4). Encouraged by this finding, we further screened group Ib of ICP patients (age of disease onset, >20 years) but found only the duplication of the trypsinogen locus in 2 subjects (Table 4).

Neither the triplication nor the duplication was found in the 103 FCP subjects as well as in a total of 282 healthy French controls (the previous 200[29] plus the newly recruited 82). In addition, both the above CNMs at the trypsinogen locus were not detected in the 268 Indian TCP patients (as well as the 63 Indian controls); this adds to the growing evidence showing that trypsinogen gene mutations might not play a role in the pathogenesis of TCP as well as other types of chronic pancreatitis from India.[40–42]

Trypsinogen Triplication Mutation Identified in Idiopathic Chronic Pancreatitis Probably Arose From a Common Founder With That Found in Hereditary Pancreatitis

The size of the triplicated segment identified in the 10 ICP patients was found to be identical to that of our previously found ~605 kb triplication, as analyzed by walking QFM-PCR.[29] Moreover, genotyping of the microsatellite marker rs3222967 suggested that the triplication in the 10 ICP patients probably occurred on the same haplotype that harbored the known ~605 kb triplication; the triplication in all informative hereditary pancreatitis patients from our previous study always had the genotype of 163-163-169,[29] and all 10 ICP triplication carriers were found to have at least two 163 and one 169 alleles (Table 4). (Note that the frequency of the 163 allele in the French population is only 1.2%.) Finally, it is hard to envisage how this kind of triplication

Table 4. ICP Patients With Triplication or Duplication of Trypsinogen Locus

Patient	Sex	CNM	Age of disease onset (y)	Age at diagnosis (y)	Genotype of rs3222967
Group Ia (n = 202; see Table 2)					
523	M	Triplication	11	11	163-163-169-169
637	F	Triplication	15	15	163-163-169-169
663	M	Triplication	18	18	163-163-169-169
802	M	Triplication	Unknown	13	163-163-169-167
1016	M	Triplication	Unknown	19	163-163-169-169
1521	M	Triplication	10	10	163-163-169-169
1926	M	Triplication	11	12	163-163-169-169
2129	M	Triplication	13	15	163-163-169-169
2246	F	Triplication	20	47	163-163-169-169
2326	M	Triplication	10	24	163-163-169-169
2193	M	Duplication	6	15	163-169-169
1620	F	Duplication	4	19	163-169-169
Group Ib (n = 1044; see Table 2)					
344	F	Duplication	Unknown	39	149-163-169
1215	M	Duplication	38	40	163-169-169

could occur recurrently from the standpoint of mutational mechanism.

The above observations, when considered together, strongly suggest that the triplication identified in the 10 ICP subjects and that previously found in the 5 hereditary pancreatitis families most probably arose through a common founder. If this were the case, one might argue that the ICP subjects carrying the triplication might belong to families with hereditary pancreatitis that escaped clinical attention. Although the ICP patients were recruited through different clinics, this seems very unlikely because of the following reasons. First, our other patients, diagnosed with either hereditary pancreatitis[29] or FCP, were also referred to us by these clinics. Second, we have revisited some of the ICP patients carrying the triplication mutation, and in all revisited cases, we found no evidence to contradict the original diagnosis. Third, if one assumes that the triplication mutation should always cause hereditary pancreatitis, this mutation should more likely be found in the subjects with FCP rather than in those with ICP. Contrary to this expectation, none of the FCP patients was found to carry the triplication mutation. Last, variable penetrance of some human genetic diseases caused by CNMs has also been previously demonstrated; for example, in both Charcot-Marie-Tooth disease type 1A[43] and hereditary neuropathy with liability to pressure palsies,[44] the disease-causing CNMs in some probands were found to be inherited from healthy parents. Cascade mutation screening in the triplication carriers should help to clarify this issue.

Trypsinogen Duplication Mutation Was Independently Confirmed by Fluorescence in Situ Hybridization

The size of the newly identified duplicated segment was also shown to be identical to that of the known ~605 kb triplication by walking QFM-PCR, and genotyping of rs3222967 suggested that either the duplication arose from the loss of 1 of the 2 allele-163–containing copies from the triplication, or the triplication arose from gain of an additional allele-163–containing copy from the duplication (Table 4). We are currently working on the mechanisms underlying the triplication/duplication mutations. Irrespective of its exact origin, the duplication was independently confirmed by fluorescence in situ hybridization (FISH) performed on cultured peripheral blood lymphocytes from one of the patients carrying this mutation (Figure 2).

Because the duplication was only detected in 4 ICP subjects, it was impossible to perform a genotype-phenotype correlation. However, on the basis of the observations that triplication of the *SNCA* gene and the *PLP1* gene appeared to cause more severe disease than their respective duplications,[45–48] trypsinogen locus triplication should be expected to cause a more severe phenotype than trypsinogen locus duplication (provided that each of the duplicated/triplicated copies is equally expressed). In this regard, it is worth mentioning that (1) the duplication was not found in any of our families with hereditary pancreatitis,[29] and (2) only the duplication was identified in group Ib of ICP patients (Table 4). Finally, although we previously suggested that early mortality of subjects with chromosome 7q duplication syndromes might preclude acute or chronic pancreatitis to get clinical attention, we cautioned that a threshold level of trypsinogen copy number might be important in causing the disease.[29] The new finding from this study provides support to the first theory. In short, gain of only one copy of the trypsinogen locus (ie, duplication) appears to be capable of causing the disease, although it might be associated with a less severe phenotype as compared with the gain of 2 copies of the trypsinogen locus (ie, triplication). When considered together with our negation of the previously described CNVs involving *PRSS1* and/or *PRSS2*, this further potentiated the importance of a tightly regulated trypsin activation and inhibition balance for pathophysiology.

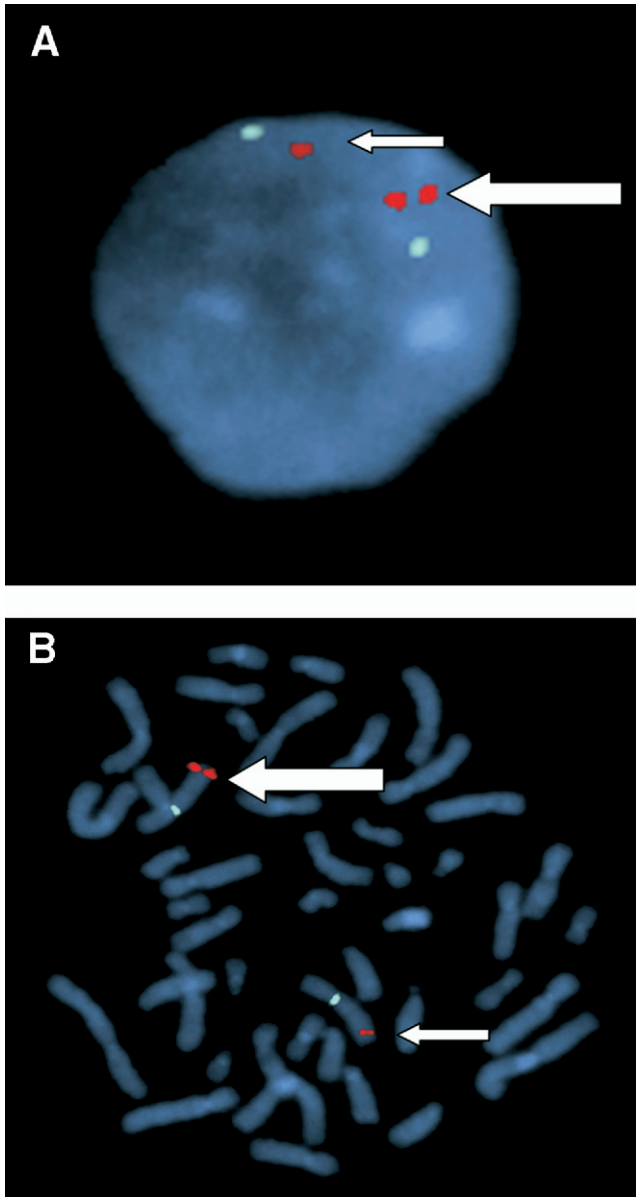

Figure 2. FISH analysis from cultured peripheral blood lymphocytes from one ICP patient carrying the trypsinogen duplication mutation. (*A*) Interphase cell showing duplication of RP11-771O19 in one chromosome (*arrows*). (*B*) Metaphase cell showing increased gene dosage of RP11-771O19 in one chromosome (*large arrow*) compared with normal chromosome 7 (*small arrow*). FISH was performed as previously described.[29]

All Five Trypsinogen Copy Nnmber Variations Are Apparent Artifacts

As shown in Table 1, the 5 trypsinogen CNVs involve both loss and gain of DNA sequence, and some of them appear to be quite frequent in the general population. As a matter of fact, these CNVs should have been detected by our QFM-PCR if they were really to exist in any of our analyzed French and Indian patients and controls. Nevertheless, on the basis of previous findings,[14,15] we surmised initially that trypsinogen CNV loss could potentially protect one from the disease in the respective carriers. But the detection of trypsinogen CNV gain in controls poses a serious challenge to our previous finding.[29]

To obtain further insights into this issue, we went on to screen additional 75 samples from different ethnic origins (25 Moroccans, 25 Ivorians, and 25 Vietnamese) by using our QFM-PCR. Again, neither loss nor gain of trypsinogen CNVs was identified in any of these samples. Given that both trypsinogen triplication and duplication have been confirmed independently by FISH and QFM-PCR has also been used successfully by us to identify large genomic deletions in the *CFTR*[49,50] and *SPINK1*[28] genes, it seems unlikely that the absence of trypsinogen CNVs in our control samples was due to any inherent problems with our analytical technique. Therefore, we speculated that all 5 previously reported trypsinogen CNVs might be artifacts.

Then we decided to investigate whether we could replicate the presence of these CNVs in the original samples by using our QFM-PCR. To this end, we purchased several relevant DNA samples from the Coriell Cell Repositories (Table 3). Not surprisingly, none of them was found to carry their respective CNV. That the common *TRY6* and *TRY7* deletion polymorphism[36] was found in most of these samples (lower panel, Figure 1*B*; Table 3) serves as an important internal control confirming that our analytical technique is highly reliable.

Conclusions and Perspectives

In summary, we have for the first time analyzed trypsinogen CNMs in a large cohort of chronic pancreatitis subjects in 2 contrasting populations. Although increased number of trypsinogen copies was found in 6% of French ICP patients whose age of disease onset was ≤20 years, they were not found in any of the 268 TCP patients from India, indicating an ethnic specificity of these mutations. In particular, we identified a novel trypsinogen duplication that was confirmed by FISH analysis. Our results have thus revealed the molecular basis of a subset (6%) of the so-called ICP and further demonstrated the view that chronic pancreatitis is a genomic disorder.[29] It is pertinent to emphasize 2 issues. First, the trypsinogen triplication and duplication mutations actually represent the second most frequent genetic factor predisposing to ICP in the French white population (the first being the *SPINK1* N34S polymorphism). Second, this kind of rare mutation, unlike the common disease-associated N34S polymorphism, is of significant importance; whenever such a mutation is detected in a patient, a genetic etiology can usually be assigned, and genetic counseling can be given to the patient and his/her relatives. The above notwithstanding, our finding raises more questions than answers. For example, why was the triplication mutation always found in patients with either hereditary pancreatitis or ICP but not in FCP? Is there any difference in terms of trypsinogen expression in vivo between the triplication found in ICP and that found in hereditary pancreatitis? Could trypsinogen CNMs also account for some unidentified patients with hereditary pancreatitis or ICP in other populations?

During the past 3 years, we have witnessed an explosion of data reporting CNVs in the human genome, with more than 6000 currently detailed in the Database of Genomic Variants. Although we have been cautioned to take extreme care with respect to the quality of these CNVs and their potential application to disease studies,[30] there is a scarcity of data describing the extent and impact of these potential problems. Here we have experimentally demonstrated that all 5 currently reported CNVs involving *PRSS1* and/or *PRSS2* on chromosome 7q34 are artifacts. To what extent this can be extrapolated to other genome regions is unknown, but our finding calls for a systematic and independent validation of all the CNVs currently reported in the human genome.

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