A new polymorphism for the RI22H mutation in hereditary pancreatitis

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Abstract

Background and aims—Hereditary pancreatitis (HP) is a rare form of recurrent acute and chronic pancreatitis. Mutations in the cationic trypsinogen (protease serine 1, PRSS1) gene have been identified as causing HP. The R122H (previously known as R117H) mutation is the commonest and can be detected by a single and rapid polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) based technique using the AffIII enzyme. This test however may give a false negative result in the presence of a neutral polymorphism within the enzyme recognition site. The frequency of this event was examined by sequencing studies in patients with HP and in healthy controls.

Methods—Of 60 families identified by the UK and Ireland consortium of EUROPAC (European Registry for Hereditary Pancreatitis and Familial Pancreatic Cancer), 51 were screened for R122H, N29I, and A16V mutations using standard techniques, and by sequencing of all five exons of cationic trypsinogen.

Results—Twelve families had the N29I mutation, one family had A16V and, on standard testing, 15 families had the R122H mutation. An additional family with the R122H mutation was found on direct sequencing. The false negative result was due to a neutral polymorphism C→T at the third base of the codon, not affecting the amino acid coded for, destroying the AffIII restriction site. This polymorphism was not observed in 50 DNA specimens (100 chromosomes) from controls nor from 50 individuals from PRSS1 mutation negative HP families. A novel mutation specific PCR was developed to avoid this pitfall.

Conclusions—One of the 16 families with HP and an R122H mutation contained a polymorphism affecting the AffIII restriction site. Adoption of an alternative R122H assay is important for genetic studies in individuals with apparent HP.

Keywords: hereditary pancreatitis; R122H mutation

Hereditary pancreatitis (HP) represents a rare subgroup of chronic pancreatitis, characterised commonly at the outset by recurrent episodes of acute pancreatitis. Diagnosis is often delayed as symptoms are usually attributed to “idiopathic” recurrent acute pancreatitis or other causes of recurrent abdominal pain.

Most forms of HP are caused by one of two mutations of the cationic trypsinogen gene, located on chromosome 7. The first mutation described (R122H; previously known as R117H) is due to a G→A transition in exon 3, producing the amino acid substitution arginine to histidine at position 122. This alteration affects a trypsin sensitive hydrolysis site in the chain linking the two globular domains of the trypsin molecule. This eliminates an important fail safe mechanism against autoactivation of trypsin within the pancreas. The second mutation (N29I; previously known as N21I) is an A→T transversion in exon 2, resulting in an asparagine to isoleucine substitution at position 29. The mechanism of action of this mutation is unclear as the crystal structure of PRSS1 it appears that positively charged asparagine 29 and arginine 122 sandwich the negatively charged glutamate at position 32. Removal of the asparagine at position 29 would result in a shift in structure with the glutamate pulled towards the arginine and perhaps protecting the trypsin cleavage site. However, recent in vitro evidence suggests that the N29I mutation affects trypsinogen autoactivation rather than inactivation. A third mutation has recently been identified resulting in a change from an alanine to a valine at position 16 in PRSS1. Position 16 is the first amino acid of the trypsinogen molecule lying just beyond the signal peptide. This type of mutation may therefore affect transportation of trypsinogen.

In addition, two other causative mutations in PRSS1 have been suggested, the first causing a substitution of lysine at position 23 to an arginine (K23R), and the second a deletion of two base pairs in the 5'UTR of PRSS1 (−28ATCC).

In addition to PRSS1, a second candidate disease gene has recently been identified. The PSTI (SPINK1) gene located on chromosome 5 codes for a trypsin inhibitor and so its mutation would be predicted to increase basal trypsin activity. An A→G transition in this gene causes a substitution of asparagine to serine at position 34. Association analysis indicates a dependent relationship between the mutation and idiopathic chronic pancreatitis.

Abbreviations used in this paper: HP, hereditary pancreatitis; EUROPAC, The European Registry for Hereditary Pancreatitis and Familial Pancreatic Cancer; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.
Mutation screening can assist in diagnosis when the clinical picture is confusing and enable a better understanding of the natural history of the disease. The R122H mutation was originally detected by DNA sequencing and a simple screening system was developed by Whitcomb and colleagues.7 The technique is based on restriction fragment length polymorphism (RFLP) using the restriction enzyme AflIII. Utilisation of this assay has made the detection of HP easier, both for the diagnosis of affected individuals and for the identification of asymptomatic carriers. Nevertheless, there may be families that harbour a R122H mutation but who are not being recognised by the existing screening method. We report a family with an R122H mutation without an AflIII restriction site and describe an alternative polymerase chain reaction (PCR) based approach that will identify the mutation in all cases.

Materials and methods

RECRUITMENT OF FAMILIES

Families with HP based in the UK and Ireland were identified from the European Registry for Hereditary Pancreatitis and Familial Pancreatic Cancer (EUROPAC). Following informed consent from all patients over the age of 18 years, whole DNA was extracted from 10 ml of venous blood (Miniprep blood kit; Qiagen, Crawley, West Sussex, UK).

Detailed pedigrees were established and family members were questioned to determine their clinical symptoms (abdominal pain, nausea, vomiting, diarrhoea, and steatorrhoea), together with number and duration of attacks, age of onset of symptoms, and complications of pancreatitis. All information obtained from the families was corroborated by a questionnaire, completed by the referring clinician, who provided supporting radiological and biochemical investigations of chronic pancreatitis.

Study protocols, questionnaires, and consent forms were approved by the local research ethics committee.

STANDARD MUTATION ANALYSIS

Routine detection of the R122H mutation was carried out by PCR amplification of exon 3 followed by digestion using the restriction endonuclease AflIII which recognises a novel site created by the R122H mutation.7 Heterozygotes for the mutation were identified by the presence of the two fragments produced by digestion of the mutant sequence together with one of the 16 families with the A16V mutation. In this way an R122H mutation was identified which was not detected by the standard technique. Initially we used the Perkin Elmer Big Dye Sequencing Kit and the ABI PRISM 310 Genetic Analyser, but most of the sequencing was performed using the automated ABI 377 machine (ABI Warrington, Cheshire, UK). The frequency of the above polymorphism was also ascertained in 50 random unrelated donors, representing 100 different chromosomes. In addition, the presence of this polymorphism was ascertained in 50 individuals from PRSS1 mutation negative families with HP.

To avoid routine sequencing of exon 3 in R122H mutation negative families with HP, the following alternative method was developed.

ALTERNATIVE MUTATION ANALYSIS

This technique was designed to rapidly detect the R122H mutation, even in the absence of the AflIII mutation site. Exon 3 was amplified by PCR and the product was purified using the QIAquick PCR Purification Kit (Qiagen). This was used as a template for a second round PCR reaction using two reverse primers: HP R122H (5'–GCAGAGAGATGGTGACACTC-3') and NOR R122H (5'-AGTGCGTGGAGGCGCGTGGCAGAGATGTTGGA CACCTC-3') and a single forward primer HP CONS (5'-GAATGACGTTTACATG CAG-3'). Primer HP R122H is mutant specific and results in a shorter sequence than primer NOR R122H, which is specific for wild-type. Therefore, in a heterozygote, two products are produced while in a homozygous wild-type there is only one product.

Reaction conditions for this amplification consisted of 1.0 pmol/µl of each primer; 10 mM Tris HCl (pH 8.3); 50 mM KCl; 1.7 mM MgCl₂; 200 µM of each of the 4 dNTPs (dATP, dCTP, dGTP, and dTTP); and 1 U of Perkin-Elmer AmpliTaq Gold (ABI Warrington, Cheshire, UK). Cycling parameters consisted of an initial 10 minute denaturation step at 95°C, followed by 30 cycles of 95°C for one minute; 60°C for one minute; 72°C for one minute, with a final five minute elongation step at 72°C. PCR amplification was followed by agarose gel electrophoresis of the PCR products using a 1.5% (w/v) agarose gel (Syngene, New England Bio Labs, Hitchin, Hertfordshire, UK) run in 0.5×TBE stained with 0.5 µg/ml ethidium bromide.

Results

A total of 111 families with HP have been referred to EUROPAC, 60 of which have been referred by the UK and Ireland consortium. Of these latter 60 families, to date 51 (108 individuals) have been tested for mutations. The R122H mutation was found in 16 families (46 individuals); 13 families (32 individuals) had the N29I mutation, one family (two individuals) had the A16V mutation, and 21 (28 individuals) had no identified mutation (the PRSS1 gene from these 28 individuals was sequenced to check for novel PRSS1 mutations). We also sequenced the PRSS1 gene of the two individuals with the A16V mutation. In addition, one of the 16 families with the
R122H mutation was undetectable using the original technique and the mutation was originally identified only after sequencing all five exons of PRSS1. The proband of this family (pedigree III, fig 1) is a 15 year old female (IV-8) from a large Irish family who presented with abdominal pain, vomiting, and failure to thrive at the age of four years. Based on biochemical, clinical, and radiological features she was diagnosed at the age of 10 years with chronic pancreatitis. She experienced recurrent attacks of abdominal pain and vomiting but had no evidence of malabsorption. Following placement of a stent into the main pancreatic duct at 11 years of age she has remained asymptomatic. The stent has subsequently passed spontaneously. Detailed family enquiry revealed that the proband’s father (III-16) had been diagnosed with chronic pancreatitis and died from pancreatic ductal adenocarcinoma at 11 years of age. His father (I-1) also had symptoms compatible with chronic pancreatitis but no information was available as to the cause of his death.

Family members were negative on initial screening for the R122H mutation on the basis of AflIII digestion. On direct sequencing however both IV-8 and III-16 had inherited the R122H G→A transition. This was found to be adjacent to a C→T nucleotide change at position 366 of the PRSS1 cDNA. The C→T transition destroys the AflIII restriction site and was the reason for the previous false negative results. This change represented a neutral polymorphism. Using the nested PCR technique on exon 3, as described in materials and methods, we were able to detect the R122H mutation in the presence of the neutral polymorphism by gel electrophoresis (fig 2). This neutral polymorphism was not found in 50 random control samples from unrelated individuals (100 chromosomes) or in 50 individuals with HP from PRSS1 mutation negative families.

Our laboratory now routinely uses this test to detect the presence of the R122H mutation.

**Discussion**

The pattern of complications associated with HP is similar to other types of chronic pancreatitis and includes pseudocyst, steatorrhoea, ascites, portal hypertension, and diabetes mellitus. HP mutations have only 80% penetrance, with variable phenotype expression of the disease, even within the same family. There is added importance to the detection of HP families given the increased risk of pancreatic ductal adenocarcinoma that appears to be much greater than the increased risk found in non-hereditary chronic pancreatitis.

Clinical characteristics alone can be inadequate to diagnose HP, often leading to an alternative misdiagnosis. The ability to diagnose HP accurately by gene mutation analysis is therefore an important clinical tool for diagnosis and for identification of asymptomatic carriers. The EUROPAK reference laboratory routinely used the screening test for detecting the R122H mutation as described by Whitcomb and colleagues. A polymorphism adjacent to the R122H mutation was identified which had eliminated the AflIII restriction site. To our knowledge, this polymorphism has not been demonstrated by others.

The finding of a polymorphism adjacent to the R122H mutation has implications regarding the founder effect of the disease. If the polymorphism preceded the R122H mutation then we could confidently say that there were at least two founders for the disease mutation, one of whom had the polymorphism while the other carried the standard sequence. If this were the case, individuals would exist with the polymorphism but not the R122H mutation, but we can only speculate on the frequency of this polymorphism in the gen-

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**Figure 1** Family tree of pedigree III, illustrating multiple generations affected by hereditary pancreatitis and ductal adenocarcinoma of the pancreas.

**Figure 2** Gel electrophoresis of DNA fragments against pBR322 Hae111 molecular weight markers, following nested polymerase chain reaction (PCR) amplification of exon 3 of PRSS1. Lane 1, individual IV-8; lane 2, individual III-16; lane 3, R122H positive control; lane 4, R122H negative control. Nested PCR amplification of DNA in lanes 1 and 2 produced two PCR products which indicates that those individuals are heterozygous for the R122H mutation.
eral population. In contrast, if the R122H mutation preceded the polymorphism, individuals without HP would have had to acquire the polymorphism separately to those with the disease. Therefore, we would predict that the polymorphism would be rare in the general population. We have screened 50 individuals without HP and 50 individuals from 20 families where there is apparently HP but no PRSS1 mutations. We have found the polymorphism to be absent in all cases. If the polymorphism had been present in one or more of these individuals we could have concluded that it is highly probable that there are multiple founders for the R122H mutation, consistent with the data of Whitcomb et al where two distinct R122H haplotypes were described. From the present data we cannot offer firm support for or against the supposition that there is more than one founder.

From a clinical management viewpoint, adoption of the new assay avoids the potential failure of existing techniques in the detection of the R122H mutation in the presence of this and similar polymorphisms. Thus this assay together with those previously described provide a rapid and simple means of detecting the established mutations in PRSS1 without the requirement for sequencing.

We are grateful to all members of EUROPAC and in particular to those members of the UK and Ireland consortium of EUROPAC. Department of Clinical Genetics, Alder Hey Children's Hospital, Eaton Road, Liverpool L12 2AP, UK. EUROPAC is supported by the North West Cancer Research Fund, UK and Sölvay Healthcare, Hamburg, Germany.
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Gut 2001 48: 247-250
doi: 10.1136/gut.48.2.247

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