Association between the defective Pro369Ser mutation and in vivo intrahepatic α 1-antitrypsin accumulation

Susana Seixas, Ana Isabel Lopes, Jorge Rocha, Lúcia Silva, Carlos Salgueiro, Jaime Salazar-de-Sousa, Amélia Batista

EDITOR— α 1-antitrypsin (PI), the major inhibitor of neutrophil elastase in the lower respiratory tract, is a highly polymorphic glycoprotein synthesised in the liver that has several rare gene products in which serum protein levels are reduced or even undetectable.¹ Early onset pulmonary emphysema, resulting from unopposed elastase activity, and neonatal cholestasis probably resulting from the retention of the defective protein in the liver,² are the two most common clinical manifestations of PI deficiency and are mainly associated with PI*Z, the most common deficient allele. In addition, other rare alleles occasionally associated with liver injury have been shown to share with PI*Z an increased tendency for intracellular accumulation. Recently, a complete intracellular transport block has been reported for a newly identified^{3,4} defective Pro369Ser allele (Mwürzburg) by in vitro expression studies in human cell cultures. Adenovirus mediated transfer of the mutant gene into the mouse reproduced the consequences of this block and no traceable amounts of the variant protein could be detected in the plasma after in vivo recombinant expression.³ However, no detectable intrahepatocytic PI inclusions were found in the mice expressing the Mwürzburg mutant³

and no liver biopsy material has yet been presented from patients with this defective allele.

Case report

We report a carrier of the Mwürzburg allele with evidence for in vivo intrahepatic accumulation of PI. The patient is a white Portuguese boy who presented at the age of 1.5 months with cholestasis associated with a recent cytomegalovirus (CMV) infection. A percutaneous liver biopsy performed at the age of 2.5 months showed significant portal fibrosis with porto-portal bridging, giant cell transformation, moderate cholestasis, and an intense portal-acinar inflammatory infiltrate. Periodic acid-Schiff staining after diastase treatment (PAS-D) additionally showed the presence of positive, diastase resistant, intracellular inclusions. Immunoperoxidase staining specific for PI was positive (fig 1). Serum PI concentration, determined by automated nephelometry (Behring), was found to be 92% of normal on admission and dropped to 45% of normal at the age of 24 months, after CMV serology (IgM) and antigens became negative and following progressive decline of transaminase levels to their normal upper limit. The PI

J Med Genet
2001;38:472–474

Instituto de Patologia e Imunologia Molecular, Universidade do Porto (IPATIMUP), R Dr Roberto Frias s/n, 4200 Porto, Portugal
S Seixas
J Rocha

Faculdade de Ciências, Universidade do Porto, Portugal
S Seixas
J Rocha

Unidade de Gastroenterologia Pediátrica, Hospital de Santa Maria, Lisboa, Portugal
I A Lopes
J Salazar de Sousa

Serviço de Pediatria, Hospital do Barreiro, Barreiro, Portugal
L Silva
C Salgueiro

Serviço de Anatomia Patológica, Hospital de Santa Maria, Lisboa, Portugal
A Batista

Correspondence to:
Dr Rocha,
jrocha@ipatimup.pt

Figure 1 Tissue section from the patient's percutaneous liver biopsy. PAS positive diastase resistant inclusions were found in the cytoplasm of several hepatocytes (PAS-D). PI is identified by immunoperoxidase in inset (immunoperoxidase staining).

Figure 2 PI patterns after polyacrylamide gel hybrid isoelectric focusing (pH range of 4.3 to 4.8) and Coomassie blue staining.⁵ From left (1) M2/S = patient's mother, (2) Mwürzburg/S = patient, (3) Mwürzburg/M3 = patient's father, (4) M1 = control. The major band corresponding to the Mwürzburg gene product is indicated with an arrow.

concentration in the patient's father was 49% of normal.

Isoelectric focusing analysis of PI types showed that the patient was heterozygous for the S allele and for a deficient variant gene product, inherited from his father, which has an isoelectric point identical to M1 and a band with decreased intensity (fig 2). These patterns were confirmed by print immunofixation (not shown). DNA sequencing of all PI coding exons (II-V), performed as previously described,^{6,7} has shown that the patient and his father shared a C to T transition leading to a 369Pro (CCC)→Ser (TCC) substitution in the common M1Val213 allele, as in the variant Mwürzburg.^{3,4} The presence of the Mwürzburg allele was also confirmed by partial PCR amplification of exon V with a mismatched primer that generates an 118 bp fragment: 5'-CCCGAGGTCAAGTTCAA CAG-3' (bases 10049-10069, mismatched base in lower case); 5'- GAGGAGCGAGAGG CAGTTATT-3' (bases 10166-10146). Thirty five cycles of PCR were performed for one minute at 94°C, one minute at 58°C, and one minute at 72°C. The mismatched primer artificially introduces a *Nde*II restriction site in the mutated Pro369Ser allele during PCR amplification (fig 3, lanes 1 and 2). In addition, the primer also generates a further *Hinf*I restriction site in the presence of the Pro369Leu mutation (fig 3, lanes 3 and 4), which characterises the severely deficient variant Mheerlen.⁸

Discussion

To our knowledge the present case represents the first reported association between the defective α 1-antitrypsin Pro369Ser mutation and in vivo intrahepatic protein accumulation. Although the S variant has been found to show increased intracellular retention and the ability to form heteropolymers with Z,⁹⁻¹¹ this increase is only marginal and no evident inclusions of S α 1-antitrypsin have been found in most pathology samples observed so far. Therefore, the observation of PI liver inclusions in the Mwürzburg/S patient is most likely to be predominantly caused by the Mwürzburg variant and provides further in vivo evidence that the severe deficiency resulting from the Pro369Ser mutation is caused by protein accumulation, as in the case of the Z allele.

Since the patient's PI type would be expected to be similar to SZ, which is not associated with increased risk of liver disease in infancy, his liver injury is probably related more to the CMV infection than to the Mwürzburg variant. However, the similar behaviour of Mwürzburg and Z both in vitro and in vivo indicates that it may lead to liver disease in Mwürzburg homozygotes or in Mwürzburg/Z heterozygous combinations.

Contrary to previous observations,³ the present detection of a faint PI band with the same isoelectric point of M1, both in the patient and his father, indicates that Mwürzburg can still be secreted in limited amounts into the plasma. However, since the variant will remain unidentified in combination with the common normal M1 allele, the PCR introduction of a *Nde*II restriction site is a simple alternative tool

Figure 3 Detection of the Pro369Ser and Pro369Leu mutations after PCR amplification with a mismatched primer. (Lanes 1 and 2) *Nde*II digested fragments from the Mwürzburg/S patient (1) and from a normal M1 control (2). The Pro369Ser mutated sequence generates one 98 bp fragment and one 20 bp fragment (not visible). (Lanes 3 and 4) *Hinf*I digested fragments from a M2/Mheerlen subject (3) and from a normal M1 control (4). The normal sequence produces one 84 bp fragment and one 34 bp fragment. The Pro369Leu mutated sequence generates an additional restriction site and 64 bp, 34 bp, and 20 bp (not visible) fragments are produced. MW = molecular weight marker. DNA fragments were visualised by silver staining after non-denaturing electrophoresis on 9% polyacrylamide gels.

to detect the Pro369Ser mutation, especially in cases where no unusual isoelectric focusing patterns are associated with decreased PI serum levels or with intrahepatic protein accumulation.

We thank Ms Lúcia Ramires and Ms Piedade Mendonça for technical assistance. This work was supported in part by Conselho de Prevenção do Tabagismo. Susana Seixas is supported by grant BD/13885/97 from Praxis XXI.

- Cox DW. α 1-antitrypsin deficiency. In: Scriver CH, Beaudet AL, Sly WS, Valle D, eds. *The metabolic and molecular bases of inherited disease*. Vol 3. New York: McGraw-Hill, 1995:4125-58.
- Permlutter DH. The cellular basis for liver injury in α 1-antitrypsin deficiency. *Hepatology* 1991;13:172-85.
- Poller W, Merklein F, Schneider-Rasp S, Haack A, Fechner H, Wang H, Anagnostopoulos I, Weidinger S. Molecular characterisation of the defective α 1-antitrypsin alleles PI Mwürzburg (Pro369Ser), Mheerlen (Pro369Leu), and Qölsbom (Thr68Ile). *Eur J Hum Genet* 1999;7:321-31.
- Rocha J, Seixas S, Lopes AI, Silva L, Salgueiro C, Salazar-de-Sousa J, Batista A. Human gene mutation report. *Hum Genet* 1999;104:114.
- Rocha J, Pinto D, Santos MT, Amorim A, Amil-Dias J, Cardoso-Rodrigues F, Aguiar A. Analysis of the allelic diversity of a (CA)_n repeat polymorphism among α 1-antitrypsin gene products from northern Portugal. *Hum Genet* 1997;99:194-8.
- Graham A, Kalsheker NA, Bamforth FJ, Newton CR, Markham AF. Molecular characterization of two α 1-antitrypsin deficiency variants: proteinase inhibitor (Pi) Nullnewport (Gly115→Ser) and (Pi) Zwexham (Ser19→Leu). *Hum Genet* 1990;85:537-40.
- Seixas S, Garcia O, Amorim A, Rocha J. A novel α 1-antitrypsin R281del variant found in a population sample from the Basque Country. *Hum Mutat* 2000;15:121-2.

- 8 Hofker MH, Nukiwa T, van Paassen, Nelen M, Kramps JA, Klasen EC, Frants RR, Crystal RG. A Pro→Leu substitution in codon 369 of the alpha-1-antitrypsin deficiency variant PI Mheerlen. *Hum Genet* 1989;81:264-8.
- 9 Teckman JH, Permuter DH. The endoplasmic reticulum degradation pathway for mutant secretory proteins α 1-antitrypsin Z and S is distinct from that for an unassembled membrane protein. *J Biol Chem* 1996;271:13215-20.
- 10 Elliot PR, Stein PE, Bilton D, Carrell RW, Lomas DA. Structural explanation for the deficiency of S α 1-antitrypsin. *Nat Struct Biol* 1996;3:910-11.
- 11 Mahadeva R, Chang WSW, Dafforn TR, Oakley DJ, Foreman RC, Calvin J, Wight DGD, Lomas DA. Heteropolymerization of S, I and Z α 1-antitrypsin and liver cirrhosis. *J Clin Invest* 1999;103:999-1006.

The androgen receptor and DXS15-134 markers show a high rate of discordance for germline X chromosome inactivation

Vikas Mahavni, Seung C Kim, Teresa A Benda, Linda Sanders, Richard E Buller

EDITOR—The process of X chromosome inactivation was identified as early as 1960 when Ohno and Hauschka¹ described the presence of a pyknotic X chromosome in both benign and malignant cell lines. Mary Lyon formalised the role of X inactivation and its relationship to dosage compensation of X chromosome genetic material in a letter to *Nature* in 1961.² This phenomenon, now known as the Lyon hypothesis, states that only one X chromosome is transcriptionally active in a given female cell. While the Lyon hypothesis dictates that the X inactivation process is random, skewing of this process to the point of non-random X chromosome inactivation is a known mechanism associated with the development of X linked genetic diseases in females.³

Our laboratory has been interested in the association of non-random X chromosome inactivation (NRXI) with ovarian cancer.⁴ Not only does this process violate a basic biological principle, the Lyon hypothesis, but it also provides a mechanism to bypass one of two steps generally accepted as necessary for the development of a cancer phenotype, dictated by the Knudson two hit model.⁵ The process of X inactivation silences one of two alleles for a particular gene and hence creates a state of functional loss of heterozygosity. Thus, X linked tumour suppressor genes can require only a single mutational event (or "hit") to contribute to the process of carcinogenesis. Furthermore, with NRXI, hypothetical germline mutation of tumour suppressor genes could contribute to early onset disease. We have hypothesised that the process of NRXI may provide a mechanism for some hereditary breast or ovarian cancers independent of *BRCA1/BRCA2* associated disease because of its strong association with invasive ovarian cancers.⁴ More recently, data from our group suggest that this association extends to breast and endometrial, but not cervical or vulvar cancers.⁶

The gold standard for assessment of X chromosome inactivation status has been an evaluation of the androgen receptor (AR) gene polymorphism(s). The AR gene, localised to Xq13, is characterised by a highly polymorphic trinucleotide repeat (CAG)_n in the coding

region of its first exon. This repeat sequence is preceded by target sequences for the methylation sensitive restriction enzymes *HhaI* and *HpaII*. The X chromosome inactivation mechanism results in site specific cytosine methylation of the inactive chromosome. This renders the methylated chromosome resistant to restriction enzyme digestion. In contrast, the active (unmethylated) X chromosome is sensitive to digestion by methylation specific restriction enzymes. Polymerase chain reaction (PCR) amplification of restriction digest can therefore be used to identify the active X chromosome. The majority of papers relating to X chromosome inactivation use the AR model. While other loci have been proposed, other potentially useful loci have primarily been studied only in small cohorts and these studies have been lacking in comparative analysis between the AR and the test locus.

In order better to understand NRXI and the extent to which it may occur throughout a given X chromosome, it would be useful to evaluate the degree of methylation of multiple X chromosome markers. In 1998, Okamoto *et al*⁷ published a report of 110 Japanese females evaluated for mononuclear cell clonality, using a unique short tandem repeat (STR) site at DXS15-134.⁷ This marker has been localised distal to the AR locus at Xq28 and is characterised by a pentameric repeat pattern flanked by *HhaI* and *HpaII* restriction enzyme target sequences. These authors reported the results of AR and DXS15-134 PCR analysis of germline DNA in 91 women. Eighteen of these 91 females were heterozygous at both loci. The results of the AR and DXS15-134 PCR analysis were completely concordant. We have therefore chosen to perform a similar comparative analysis at the AR and DXS loci using germline DNA samples from probands with breast or ovarian cancer and a cohort of healthy cancer free controls.

Material and methods

Blood samples were obtained in accordance with guidelines set forth by the Committee on Human Subjects at the University of Iowa Hospitals and Clinics (UIHC). Case selection was from the Surgical (breast cancer) and

J Med Genet
2001;38:474-478

**Division of
Gynecologic Oncology,
Department of
Obstetrics and
Gynecology, The
University of Iowa
Hospitals and Clinics
(UIHC), 200 Hawkins
Drive, 4630 JCP, Iowa
City, Iowa 52242-1009,
USA**

V Mahavni
S C Kim
T A Benda
L Sanders
R E Buller

Correspondence to:
Dr Buller,
richard-buller@uiowa.edu