TECHNICAL NOTE

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Genetic Polymorphism of the Inter-Alpha-Trypsin Inhibitor (ITI) in Cádiz Province, Southern Spain

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ABSTRACT: The results of a study of Inter-Alpha-Trypsin Inhibitor (ITI) polymorphism in 281 blood samples are reported in this paper. These samples were taken from healthy individuals of both sexes, unrelated and resident in the Province of Cadiz. The frequency of ITI*1 was 0.617 and of ITI*2 was 0.383. The probability of exclusion in paternity testing was 0.18.

KEYWORDS: forensic science, paternity testing, genetic, polymorphism, inter-alpha-trypsin inhibitor, Cádiz Province, Spain

The inter-alpha-trypsin protein was discovered by Steinbuch and Loeb (1) by means of electrophoresis in starch. Later, Heide et al. (2) succeeded in isolating it in its pure form and named it ITI.

ITI is a plasma protein that belongs to a group of protease inhibiting proteins denoted "Kunitz type," as it possesses certain dominions ("kunin") that are characteristic of this group of proteins in their active regions (Hochstrasser et al. (3)). It is found in human plasma in small quantities, approximately 0.5 mg/mL (Steinbuch and Loeb, (4)) while its synthesis takes place in the liver (Diarra-Mephour et al. (5)). Its molecular weight has been determined by various authors who give values that range from 180 Kdaltons (Yuasa et al. (6); Kaumeyer et al. (7); Vogt and Cleve, (8)) to 225 Kdaltons (Enghild et al. (9)).

Electrophoretically it is situated in the intermediate region between the alpha 1 and alpha 2 globulins. Its physiological role is for the most part unknown. According to Traboni et al. (10), its functions are probably related to the regulation of immunologic and inflammatory response.

ITI genetic polymorphism was first described by Vogt and Cleve (8) who demonstrated the existence of three common phenotypes (1-1, 2-2 and 1-2) along with two rarer or less frequent phenotypes (1-3 and 2-3).

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The homozygous patterns consist of 2 main bands (thicker) and additional minor bands. The most frequent heterozygous phenotype (1-2) consists of a combination of protein bands that correspond to the sum of both homozygous phenotypes.

Materials and Methods

Sample Selection

Blood samples were taken from healthy individuals of both sexes, unrelated and resident in the Province of Cádiz. Five mL were extracted from each individual by means of venous puncture. The blood was then transferred to sterile tubes with EDTA as anticoagulant.

Sample Preparation

The separation of the plasma and cellular fractions was carried out by means of centrifugation at 3600 rpm for 10 min. They were stored separately in Eppendorf tubes that were kept in a freezer at 20°C until the time they were used.

To carry out isoelectricfocusing it was necessary to use desialized protein. To obtain the latter, the sera were diluted with a solution of Neuraminidase type V (1 IU/mL) in a proportion of 1:2 and then incubated at 4°C overnight. The volume of sample applied to gels was 0.5 μL (sample applicator 8/0.5).

Isoelectric Focusing Conditions

The isoelectricfocusing was carried out by Phastsystem™ (Pharmacia LKB). Commercial gels Phastgel™ were used (gel matrix 5% T, 3% C; dimensions 43 × 50 × 0.35 mm; pH 5–8). The program used for the analysis of ITI polymorphism is shown in Table 1.

TABLE 1—*Isoelectric focusing conditions for programming in the PhastSystem.*

Sample appl. down at 1.2					0 Vh
Sample appl. up at 1.3					0 Vh
Extra alarm to sound 1.1					70 Vh
Step 1.1	2000 V	2.0 mA	3.5 W	15°C	75 Vh
Step 1.2	200 V	2.0 mA	3.5 W	15°C	15 Vh
Step 1.3	2000 V	5.0 mA	3.5 W	15°C	610 Vh

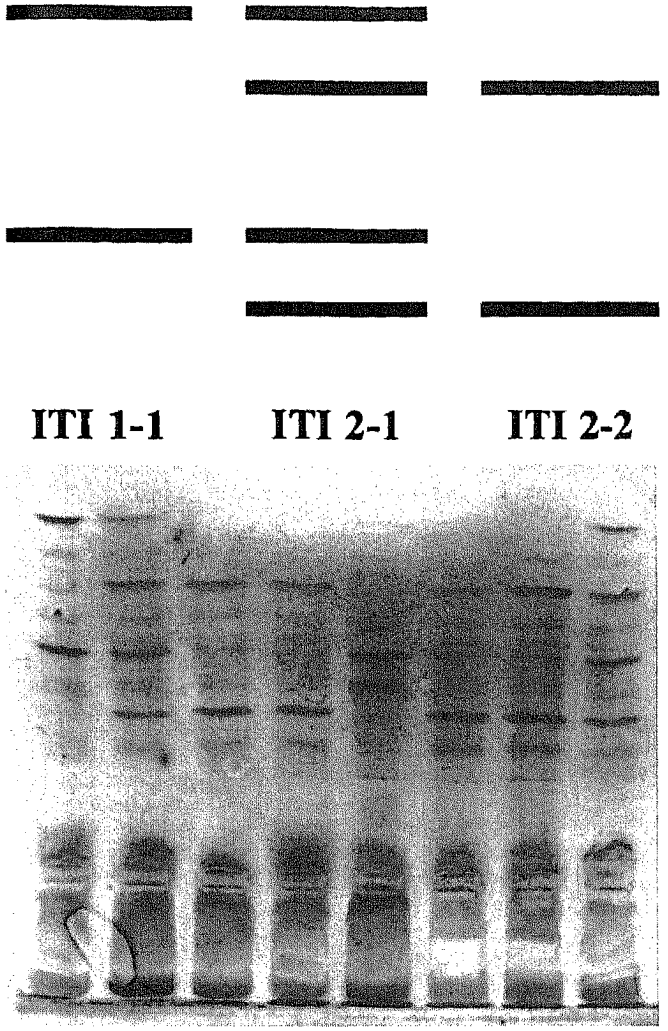

FIG. 1—*Band pattern of the common phenotypes of ITI* (top). *ITI phenotypes in PhastGel IEF 5-8* (bottom). *Anode is at the* top. *Phenotypes are from left to right: 2-2; 2-2; 2-1; 1-1; 2-2; 2-1; 1-1; 2-1.*

TABLE 3—*ITI phenotypes and gene frequencies in a population sample in Cádiz Province, Southern Spain.*

	Observed Values		Calculated Values	
Phenotypes	*N*	%	*N*	%
1-1	107	38.0783	107.13	38.1229
2-1	133	47.3310	132.75	47.2417
2-2	41	14.5907	41.13	14.6354
Total	281	100.000	281.00	100.000

NOTE: $\chi^2 = 1.003{,}697 \times 10^{-4}$; d.f. = 2; $0.975 > P > 0.950$.
Frequency of allele ITI*1 = 0.6174.
Frequency of allele ITI*2 = 0.3826.

Once isoelectricfocusing had been carried out the ITI was subjected to immunofixation. The gels were immunofixed with 20 μL of anti-ITI specific antiserum (rabbit immunoglobin to human inter-alpha-trypsin inhibitor, Dakopatts A/S, Denmark), diluted 1:2 in saline. A micropipette was used to apply the diluted ITI antiserum directly onto the gel and it was then distributed homogeneously with a glass rod bent into a right angle. The gels were then incubated in a water bath for 30 min at 37°C. They were finally washed in saline overnight.

After immunofixation, the gels were then stained with silver salts as described by Heukeshoven and Derwick (11). The automatic staining program applied is shown in Table 2.

Results and Discussion

Figure 1 shows a schematic representation of the ITI phenotypes and an actual stained gel observed in this work.

The system described by Vogt and Cleve (8), has been followed for the nomenclature of different Inter-alpha-trypsin phenotypes. They employed a numeric classification system to designate the different ITI phenotypes (1-1, 2-2, 2-1).

The observed phenotype frequencies are shown in Table 3 as well as the values of the genic frequencies of the ITI system. It was observed that the population of the Cádiz Province is in Hardy-Weinberg equilibrium for this marker.

The genotypic frequencies found in the population of the Province of Cádiz are not significantly different compared to those

TABLE 2—*Staining method.*

Step	Solution	In	Out	Time	Temp.
1	Trichloroacetic 20%	1	1	5 min	20°C
2	Methanol 50% + Acetic Acid 10%	2	2	2 min	50°C
3	Wash with ethanol 10% + Acetic Acid 5%	3	0	2 min	50°C
4	Wash with ethanol 10% + Acetic Acid 5%	3	0	4 min	50°C
5	Glutardialdehyde 8.33%	4	0	6 min	50°C
6	Glutardialdehyde 8.33%	4	0	3 min	50°C
7	Glutardialdehyde 8.33%	4	0	5 min	50°C
8	Milli-Q water	5	0	2 min	50°C
9	Milli-Q water	5	0	2 min	50°C
10	Silver Nitrate 0.5%	6	0	10 min	50°C
11	Milli-Q water	5	0	30 min	30°C
12	Milli-Q water	5	0	30 min	30°C
13	Sodium Carbonate 0.5% + Formaldehyde 0.004%	7	0	30 min	30°C
14	Sodium Carbonate 0.5% + Formaldehyde 0.004%	7	0	3 min	30°C
15	Acetic Acid 5%	8	0	5 min	50°C

values determined in the Basque country, Northern Spain (García et al. (12)). The frequency obtained in Cádiz for the allele ITI*1 (0.617) is the highest obtained for the studies that have been published up to the present, and only close to those studies by Vogt et al. (13), Luckemback et al. (14), and Martin et al. (15), with values of 0.612, 0.607 and 0.615, respectively. However, it is necessary to point out that the studies of population carried out for this marker are scarce. With regard to application in the cases of paternity testing, the "a priori" probability of exclusion was of the order of 18%.

References

(1) Steinbuch M, Loeb J. Isolation of an alpha-2-globulin from human plasma. Nature 1961;192:1196.

(2) Heide K, Heimburger N, Haupt H. An inter-alpha-trypsin inhibitor of human serum. Clin Chim Acta 1965;11:82–85.

(3) Hochstrasser K, Watcher E, Albrecht GJ, Reisinger P. Kunitz-type proteinase inhibitors derived by limited proteolysis of the ITI. X. The aminoacid sequences of the trypsin-released inhibitors from horse and pig inter alpha-trypsin inhibitors. Biol Chem Hoppe Seyler 1985;366(5):473–78.

(4) Steinbuch M. Inter-alpha-trypsin inhibitor. Meth Enzymol 1976;45: 760–72.

(5) Diarra-Mephour M, Bourguignon J, Sesboüe R, Mattei MG, Passage E, Salier JP, Martin JP. Human plasma inter-alpha-trypsin inhibitor is encoded buy four genes on three chromosomes. Eur J Biochem 1989;179(1):147–54.

(6) Yuasa I, Suenaga K, Saneshige Y, Tamaki N, Ito K, Ohada K. Inter-alpha-trypsin inhibitor (ITI): A useful genetic system in paternity testing. Evidence for polymorphic occurrence of ITI*3 and existence of a new allele, ITI*4. Int J Legal Med 1991;104:197–99.

(7) Kaumeyer JF, Polazzi JO, Kotick MP. The RNAm for proteinase inhibitor related to the HI-30 domain of inter-alpha-trypsin inhibitor also encodes alpha-1-macroglobulin (protein HC). Nucleic Acids Res 1986;14(20):7839–50.

(8) Vogt U, Cleve H. A new genetic polymorphism of a human serum protein: inter-alpha-trypsin inhibitor. Hum Genet 1990;84:151–54.

(9) Enghild JJ, Thogersen IB, Pizzo SV, Salvesen G. Analysis of inter-alpha-trypsin inhibitor and a novel trypsin inhibitor, pre-alpha-trypsin inhibitor, from human plasma. J Biol Chem 1989;264:15975–81.

(10) Traboni C, Tosini F, Covone A. The gene coding for proteins HC and HI-30 of inter-alpha-Trypsin inhibitor maps to 9q 22.3 → q33. Cytogene Cell Genet 1989;50(1):46–48.

(11) Heukeshoven J, Derwick R. Simplified method for silver staining in polyacrilamide gels and the mechanism of silver staining. Electrophoresis 1985;6:103–12.

(12) García O, Alonso A, Aguirre A, De La Rua C, Manzano C. Genetic polymorphism of inter-alpha-trypsin inhibitor (ITI) in the Basque Country (Northern Spain). Int J Legal Med 1993;106:129–31.

(13) Vogt U, Cleve H, Farhud DD, Goedde HW. The ITI system in South Koreans and Iranians analysed by an improved classification procedure. Distribution of alleles and description of a "new" phenotypes. Hum Genet 1991;87(6):677–79.

(14) Luckembach C, Kömpf J, Titter H. Genetic polymorphism of inter-alpha-trypsin inhibitor (ITI): Formal genetic and linkage analysis. Hum Genet 1991;89(1):89–90.

(15) Martin A, Corens A, Otremba P, Geserick G. Simple detection of the inter-alpha-trypsin-inhibitor (ITI) polymorphism by isoelectric focusing with direct immunofixation. Forensic Sci Int 1995;73: 15–18.

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