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A. Harada · K. Umetsu · T. Suzuki

Inter-alpha-trypsin inhibitor polymorphism

An improved phenotyping procedure and two new alleles

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Abstract Serum samples treated with chondroitinase ABC and sialidase were investigated for the detection of inter-alpha-trypsin inhibitor (ITI) polymorphism. The improved phenotyping procedure has proved to be the most practical method for ITI phenotyping. The ITI allele frequencies were examined in 2 population samples from Japanese (n = 365) and Thais (n = 150). Three common alleles, ITI*1, ITI*2, and ITI*3 were identified in both populations, but the Thai population showed a higher frequency of ITI*1 and a lower frequency of ITI*3. Two new alleles were found, which were tentatively denoted ITI*Y and ITI*T. The ITI*T allele frequency in Thais was 0.047.

Key words Inter-alpha-trypsin inhibitor · ITI Polymorphism

Zusammenfassung Serumproben, welche mit Chondroitinase ABC und Neuraminidase behandelt wurden, wurden zum Nachweis des Inter-Alpha-Trypsin-Inhibitor (ITI)-Polymorphismus untersucht. Die verbesserte Methode der Typisierung hat sich als die praktischste Methode zum Nachweis der ITI-Phänotypen erwiesen. Die ITI-Allel-Frequenzen wurden in 2 Populationen von Japanern (n = 365) und Thais (n = 150) untersucht. Die drei häufigen Allele, ITI*1, ITI*2 und ITI*3, wurden in beiden Populationen identifiziert, aber die Thai-Population zeigte eine höhere Frequenz von ITI*1 und eine geringere von ITI*3. Zwei neue Allele wurden gefunden, welche vorsichtig als ITI*Y und ITI*T bezeichnet wurden. Die Häufigkeit von ITI*T bei Thais betrug 0,047

Schlüsselwörter Inter-Alpha-Trypsin-Inhibitor · ITI Polymorphismus

A. Harada (✉) · K. Umetsu · T. Suzuki
Department of Forensic Medicine,
Yamagata University School of Medicine,
Yamagata 990-23, Japan

Introduction

Human inter-alpha-trypsin inhibitor (ITI) is a serum glycoprotein with a molecular mass of about 220 kDa and is present at normal concentrations of 0.5 mg/ml. ITI is synthesized in the liver and consists of 3 kinds of subunits; a light (L) chain (30 kDa), also known as bikunin carrying the antiprotease activity [1], and 2 heavy chains, H1 (92 kDa) and H2 (98 kDa) [2]. The 3 subunits are bound together with a glycosaminogylcan (GAG) chain [3]. The GAG chain can be digested by hyaluronidase or chondroitinase, thereby allowing separation of H1, H2 and L chains.

The genetic polymorphism of ITI was first demonstrated by Vogt and Cleve [4] using agarose gel isoelectric focusing (IEF) followed by immunostaining with anti-human ITI antiserum. ITI is controlled by 3 common alleles ITI*1, ITI*2 and ITI*3. Recently, additional alleles ITI*4, ITI*5 and ITI*6 have been reported [5–7].

In ITI phenotyping, we have experienced that ITI bands become more intense in aged samples, while in fresh serum samples and samples stored at –20° C and –4° C, the intensity of ITI bands do not show such an increase. In the present paper, we describe an improved ITI phenotyping procedure using enzyme treatment with chondroitinase ABC and sialidase and report on the ITI phenotyping performed in 365 Japanese individuals and 150 Thais, in which 2 new alleles were found in addition to the 3 common alleles.

Materials and methods

Blood samples

Japanese serum samples (n = 365) were obtained from blood donors in Yamagata (northern Japan). Thai serum samples (n = 150) were from blood donors in Chiang Rai (northern Thailand). In connection with 2 new variant bands, serum samples were collected from 2 families. All samples were stored at –40° C until use.

Enzymatic treatment

Chondroitinase ABC from *Proteus vulgaris* (protease free, Seikagaku Kogyo, Japan) and sialidase from *Arthrobacter ureafaciens* (Nacalei Tesque, Japan) were used to digest the serum samples, as follows:

Method 1. Desialisation was performed by adding 10 µl of sialidase (1 U/ml) in 0.1 M sodium acetate buffer, pH 5.0 to 10 µl of serum followed by overnight incubation at 37° C.

Method 2. Digestion of the GAG chain was perfomed by adding 10 µl of chondroitinase ABC (1 U/ml) in 0.1 M Tris-HCl buffer, pH 8.0, containing 0.03 M sodium acetate to 10 µl of serum followed by overnight incubation at 37° C.

Method 3. Desialisation and digestion of the GAG chain were performed simultaneously by adding 10 µl of sialidase solution and 10 µl of chondroitinase solution to serum followed by overnight incubation at 37° C.

Isoelectric focusing (IEF)

Polyacrylamide gels (5% T, 3% C, 110 mm × 120 mm × 0.3 mm) were prepared containing 2.6% W/V carrier ampholyte (Pharmalyte, Pharmacia, Sweden) and 12% W/V glycerol. The pH ranges of carrier ampholyte were pH 4.5–8 and pH 5–8. The carrier ampholyte pH 4.5–8 was a 1:4 mixture of pH 4.5–5.4 and pH 5–8. The catholyte and the anolyte were 1 M NaOH and 1 M H_3PO_4, respectively. Samples were applied on filter papers (Whatman No. 3) about 1.5 cm from the anode. The gels were prerun for 30 min at a constant power supply of 2.5 W at 12° C. Once samples had been applied, focusing was continued for 2 h at a constant power of 5 W. The filter papers were removed after 30 min of focusing.

Immunoblotting

After IEF, proteins were passively transferred onto a Immobilon-P membrane (Millipore, USA). The membrane was treated with a blocking buffer (PBS containing 2% W/V skimmed milk and 0.01% V/V Tween-20) for 15 min and incubated for 30 min with rabbit anti-human ITI antiserum (Dako, Denmark) at a 1:500 dilution. After being washed three times for 5 min each with PBS contained 0.01% Tween-20, the membrane was incubated for 60 min with alkaline phosphatase conjugated goat anti-rabbit IgG antiserum (E·Y Lab., USA) diluted 1:500 with PBS, followed by the washing procedure described above. The ITI bands were visualized with 5-bromo-4-chloroindoxyl phosphate and nitroblue tetrazolium.

Results and discussion

Figure 1 shows the ITI band patterns of the aged native samples and the enzymically digested fresh samples by IEF with pH range 4.5–8. ITI phenotypes of the aged native samples were correclty decided (Fig. 1A). The simple band patterns of the fresh samples treated by method 1 were also correctly detected for ITI phenotype (Fig. 1C), although the bands were faint. The band patterns of the samples treated by method 1 shifted towards the cathode as a result of enzymatic cleavage of N-acetylneuraminic acid. The sialidase treatment facilitated the detection of some variant bands. The ITI phenotypes of the fresh samples treated by methods 2 or 3 were more easily detected, although a few minor bands appeared (Fig. 1B, D). In the samples treated by methods 2 or 3, the band intensity was increased by digestion of the GAG chain, because the dissociated ITI subunits (H1, H2 of L chain) which had originally appeared near the sample application area, shifted to the cathodal side and increased the band intensity for phenotyping.

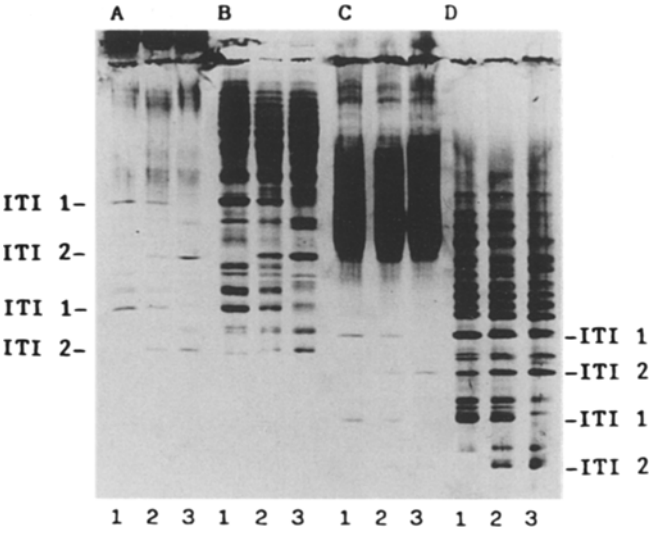

Fig. 1 Three common ITI phenotypes revealed by IEF (pH 4.5–8) of aged native samples *(A)*, and fresh samples treated by method 2 *(B)*, method 1 *(C)* and method 3 *(D)*. Anode is *at the top*. Lane (1) ITI 1, (2) ITI 2-1, (3) ITI 2

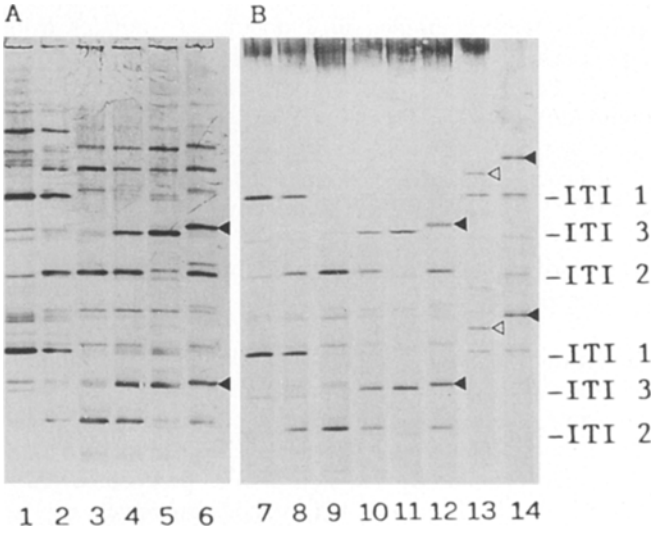

Fig. 2 ITI phenotypes revealed by IEF (pH5–8) of samples treated by method 3 *(A)* and method 1 *(B)*. Anode is *at the top*. *Filled triangles* indicate main new ITI variant bands and empty triangles indicate main ITI 4 bands. Lane (1,7) ITI 1, (2,8) ITI 2-1, (3,9) ITI 2, (4,10) ITI 3–2, (5,11) ITI 3, (6,12) ITI Y-2, (13) ITI 4-1, (14) ITI T-1

In ITI phenotyping, serum samples treated by method 3 proved to be most suitable for aged native samples. For this reason, the band intensity for ITI phenotyping and the reproducibility were improved using method 3, and the amount of serum sample could be reduced.

Figure 2 shows the immunoblot patterns of the different ITI phenotypes of the serum samples treated by method 3 (Fig. 2A) or by method 1 (Fig. 2B). In this study,

Table 1 Distribution of ITI phenotypes and allele frequencies in the Japanese and Thai populations

Japanese		Thai	
Phenotypes	Observed	Phenotypes	Observed
1	73	1	76
2-1	177	2-1	49
2	97	2	10
3-1	7	3-1	1
3-2	7	3-T	1
3	1	T-1	9
Y-1	1	T-2	4
Y-2	1	Others	0
T-2	1		
Others	0		
Total	365	Total	150
Allele frequencies			
ITI*1 = 0.4534		ITI*1 = 0.7033	
ITI*2 = 0.5205		ITI*2 = 0.2433	
ITI*3 = 0.0219		ITI*3 = 0.0067	
ITI*Y = 0.0027		ITI*T = 0.0467	
ITI*T = 0.0014			

Japanese: $\chi^2 = 4.1742$, $df = 3$, $0.3 > P > 0.2$
Thai: $\chi^2 = 0.7869$, $df = 3$, $0.9 > P > 0.8$

2 new variant bands were encountered, which were tentatively designated ITI Y and ITI T. The bands of ITI Y were located slightly anodal to the ITI 3 bands, and the ITI T bands were anodal to the ITI 4 bands. These 2 new variant bands are different from ITI 5 and ITI 6, as compared with the positions of the bands reported previously [6, 7]. The inheritance of ITI Y and ITI T was confirmed by the family study.

Table 1 shows the distribution of ITI phenotypes and the allele frequencies in a Japanese and a Thai population. The observed numbers of each phenotype gave a good fit with the expected ones calculated by assuming Hardy-Weinberg equilibrium for each population. In the Japanese population, 3 common alleles, ITI*1, ITI*2 and ITI*3, and 2 rare alleles, ITI*Y and ITI*T, were encountered, resulting in 9 phenotypes. In the Thai population, 4 alleles, ITI*1, ITI*2, ITI*3 and ITI*T were found showing 7 phenotypes. The ITI*3 and ITI*T were found at a polymorphic frequency in both Japanese and Thai population. The ITI*Y was found only in the Japanese population, while the ITI*T was found in both Japanese and Thai population. The ITI allele frequencies among the various populations are summarized in Table 2. The ITI*1 is the commenest allele in all populations studied except the Japanese. The Thai population possesses the highest ITI*1 allele frequency, 0.7033, among the previously studied populations. It is worthy of notice that the Thai population possesses polymorphic frequencies of ITI*T, like those of ITI*6 in Africa.

Commercially available polyclonal anit-human ITI antiserum from Dako reacts to H1, H2 and L chains of ITI [9]. It has not yet been shown which of the ITI subunits demonstrates the ITI polymorphism. To resolve the problem, we are trying to prepare the monoclonal antibodies specific to each ITI subunit.

In conclusion, the improved phenotyping procedure using 2 enzymes (chondroitinase ABC and sialidase) is the most practical method for detecting ITI polymorphism. The method can be applied to both fresh and aged samples and it is more sensitive.

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Table 2 ITI allele frequencies among the various populations

	n	ITI*1	ITI*2	ITI*3	ITI*4	Others	References
Japan							
Yamagata	365	0.4534	0.5205	0.0219	–	0.0041[a]	This study
Yamaguchi	400	0.4400	0.5263	0.0300	0.0038	–	[5]
Korea	200	0.5325	0.4225	0.0425	0.0025	–	[6]
China	308	0.5763	0.4107	0.0130	–	–	[8]
Thai	150	0.7033	0.2433	0.0067	–	0.0467[b]	This study
Iran	205	0.6122	0.3536	0.0293	–	0.0049[c]	[6]
German	468	0.5746	0.4173	0.0081	–	–	[4]
Austria	124	0.5766	0.4234	–	–	–	[4]
Ivory Coast	126	0.5635	0.0833	0.3373	0.0040	0.0119[d]	[7]

[a] ITI*Y and ITI*T
[b] ITI*T
[c] ITI*5
[d] ITI*6

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