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Size heterogeneity of equine transferrin

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SUMMARY

It is interesting from the viewpoint of genetics, phylogeny and physiological function of transferrin (Tf), to know how the gene composition in equine *Tf* locus has been organized with the improvement and coming into existence of each horse breed. Accordingly, molecular weight (M.W.) differences (migrating distance), and amino acid sequences of N-terminal among Tf types and between two bands composing Tf types were analyzed biochemically to investigate at first, on construction of equine *Tf* locus, and to obtain phylogenetic information related to the size heterogeneity of horse Tf. The results obtained are as follows: 1) When two bands composing Tf type were analyzed by SDS-PAGE, the main component (T- component was strongly stained) was larger in M.W. than the T+ band. When this T- component was separated by isoelectric focusing, it was located on more acidic pI than T+ component. Migrating distance between T+ and T- bands in Tf•D and R types by SDS-PAGE was not changed by treatment of sugar chains with four enzymes. 2) The largest M.W. in equine Tf types was D variant (M.W. 76,000-78,000), followed in order by F, H₂, O (M.W. 75,000-78,000), R (M.W. 74,000-77,000) and X (74,500-76,000 M.W.) types. 3) From the diversity of M.W. differences (migrating distance) between T+ and T- components and the degree of staining of two bands composing Tf types, equine Tf types were basically classified into three groups (D, F and H₂ types, O and R types, and then X type). 4) As amino acid sequences of N-terminal 14 residues in Tf•D, F, H₂, O and R types, the T+ and T- components of Tf•D and R types were all the same, and each unique peak in Tf•D and R types fractionated by the HPLC method was evidenced that both Tf types had different amino acid sequences. From the diversity of size heterogeneity and the intensity of stained bands between T+ and T- components and among Tf types, the equine Tf type may be controlled by a few loci as haplotype rather than by single locus based on the diversity of splicing.

Key words : equine transferrin, size heterogeneity, amino acid sequence, haplotype, SDS-PAGE.

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INTRODUCTION

Transferrin (Tf) is plasma glycoprotein with a molecular weight (M.W.) of approx. 80,000 fractionated to the β -globulin region. Since Tf is associated with iron-carrying and inhibiting the growth of some bacteria *in vivo*, it is a very important component for physiological metabolism. In equine Tf locus, the homozygotic Tf types are composed of two components with different electrophoretic mobility. This phenomenon is recognized generally in other animal species.¹⁻⁶⁾ Since Tf protein shows a wider segregation region than other plasma proteins and has higher variety, it is difficult to think that Tf variants are controlled only by the combined amount of sialic acid, as has been considered.⁷⁾

In connection with the aforementioned, it is interesting from the viewpoint of genetics, phylogeny and physiological function of Tf, to know how the gene composition in Tf locus had been organized with the improvement and coming into existence of each horse breed. Accordingly, heterogeneity which is caused by the M.W. of each Tf type and two bands composing Tf types were analyzed biochemically to investigate at first, on construction of equine Tf locus and to obtain phylogenetic information related to the size heterogeneity of horse Tf.

MATERIALS AND METHODS

1. Serum samples

Serum samples were collected from a total of 28 horses; homozygous Tf variants D, F₁, F₂ types (five horses for each), H₂ types (three), O, R types (four each), and X types (two). And then, one Ass and Mongolian wild horse were also used for each one sample to compare with the horse Tf.

2. Isoelectric focusing (IEF)

The IEF to detect Tf type was performed as described previously.⁸⁾ The serum samples were treated with 1% ammonium ferrous sulfate in a 1:10 ratio overnight at 4°C, and carrier ampholytes used Servalyte AG 5-7 and AG 5-6.

3. Horizontal polyacrylamide gel electrophoresis (HPAGE)

The HPAGE was also carried out by method

which Yokohama *et al.* already reported.⁹⁾ The gel was three stepwise concentrations of 8%, 4% and 12%, and samples were prepared by the same method as case of the IEF.

4. Molecular weight

The molecular weight of every Tf type was estimated by comparing each Tf type protein with the molecular weight standard on SDS-PAGE gel. SDS-PAGE was carried out using 12.5% gel. Precipitate obtained after mixing equivalently polyclonal anti-equine Tf serum and horse serum, and following incubated for one hour at 37°C, one hour at room temp. and over night were prepared as Tf protein samples for SDS-PAGE.

5. Treatment of sugar chains

Tf components were digested by using four enzymes of N-glycanase, glycopeptidase A, β -galactosidase and sialidase in accordance with the method described by Takasaki.¹⁰⁾ Tf samples were used after purified in some degree by rivanol-ammonium sulphate fractionation, gel filtration and disc-electrophoresis improved for isolation. Tf patterns were detected by SDS-PAGE.

6. Sequencing of amino acid

The N-terminal amino acid residues of homozygotic Tf types were determined with a gas-phase protein sequencer (applied biosystems, ABI 477 A/120 A type) by using Tf component electroblotted from the SDS-PAGE gel to PVDF membrane. The unique peak components of Tf•D and R variants were fractionated respectively by high performance liquid chromatography (HPLC; TOSCH CCP and 8020 System) using a TSKgel Phenyl 5 PRRP 4.6 mmID×75 mm column, and the amino acid sequence was determined using the above method.

RESULTS AND DISCUSSION

Migration to anode in each equine Tf type band by the isoelectric focusing (IEF) was in the order Tf•D, F, H, O, R and X variants.¹¹⁾ However, when each Tf band was segregated according to molecular size characteristics, the mobility was changed to the order Tf•X, R, O, H₂, F and D variants, and was completely opposite in comparison with the case of segregation by the pI characteristics, as shown in Fig.1 and Table 1.

Fig.1. Comparison of electrophoretic mobilities of transferrin between equine Tf types, and donkey and Mongolian wild horse.

1. D type, 2. F₁ type, 3. F₂ type, 4. H₂ type, 5. O type, 6. R type and 7. X type in horses, Lane 8. and 9. are Tf types of donkey and Mongolian wild horse respectively. Region I; Tf components, Region II; IgG components. SDS-PAGE was carried out with 12.5% gel.

Table 1. Characteristic of equine Tf types based on molecular weight and staining degree.

Pheno- types and breeds	Number of analyzed gels	Number of animals	Distance between T+ and T- (mm)	Molecular weight ($\times 10^3$)		The intensity of stained bands of T+ and T- bands			
				T+	T-	SDS-PAGE		HPAGE and IEF	
						T+	T-	T+	T-
D	9	5	1-1.1	76	78	W(1/3 S)	S	S	W(1/3 S)
F ₁	9	5	1-1.1	76	78	W(1/3 S)	S	S	W(1/3 S)
F ₂	9	5	1-1.1	76	78	W(1/3 S)	S	S	W(1/3 S)
H ₂	7	3	1-1.1	76	78	W(1/3 S)	S	S	W(1/3 S)
O	12	4	1.5	75	78	W(2/3 S)	S	S	W(1/2 S)
R	8	4	1.5	74	77	W(2/3 S)	S	S	W(1/2 S)
X	5	2	1.0	74.5	75	T(<1/3 S)	S	S	T(<1/3 S)
Donkey	5	1	2.0 ?	74	78	S	W(2/3 S)	?	?
Prz.	2	1	?	75	79	?	S	?	?

T+ : Tf band of anode side, T- : Tf band of cathode side, ? : unknown, Prz. : Mongolian wild horse, S: strong, W: weak, T: trace.

Namely, the largest molecular weight (M.W.) in equine *Tf* locus was the D variant. A M.W. difference of approx. 2,000 was recognized between D and X variants. The migration to anode of the two components (T+ : anode side and T- : cathode side) which compose each Tf variant were opposite between IEF or HPAGE and SDS-PAGE. The result of SDS-PAGE analysis showed that the main component (T-)

had larger M.W. size than the minor band (T+).

When the order of electrophoretic mobility of the T+ and T- components was analyzed, the order was X, R, O, H₂, F and D types for the T- component, but in the case of the T+ component, the order was R, X, O, H₂, F and D types. The moving distance between the T+ and T- components of the Tf•X variant was the shortest among Tf types, and the Tf•X type showed only a

characteristic pattern in equine Tf variants.

Equine Tf types studied could be classified into three groups according to the diversity of M.W. differences (migrating distance) between the T+ and T- components. There was the X type (migrating distance; 1 mm), where the M.W. difference between the T+ and T- components was approx. 1,500, the group of D, F and H₂ types (migrating distance; 1-1.1 mm) with a M.W. difference of approx. 2,000, and then the group of O and R types (migrating distance; 1.5 mm) with a M.W. difference of approx. 3,000. We have already confirmed that moving distance between T+ and T- bands of R type was not even changed by treatment with neuraminidase (not published). Further, although sugar chains of Tf components in D and R types were dissociated by four enzymes in this studies, overlaps between the T+ and T- bands could be not recognized by the SDS-PAGE. Accordingly, it could be confirmed that M.W. differences between T+ and T- bands of each Tf type will be basically caused only by differences of Tf polypeptide structure. The moving distances between the T+ and T- components within each Tf type were all the same among individuals within the same group mentioned above. This grouping was similar to the results in which Kaminski *et al.* (1980)¹²⁾ had classified the equine Tf types into two group (groups of D, F, H₂ types and M, O, R types) by immunochemical analysis.

Equine Tf types could also be classified into three groups according to the density degree of the band stained with CBB between the T+ and T- components just like as in the case of M.W. difference. There was a group of O and R types with the T+ band stained at the ratio of approx. two thirds of the stained intensity of the T- band, a group of D, F and H₂ types with the T+ band stained at the ratio of approx. one third of the T- band, and the X type with a very weak T+ band.

Equine Tf locus could be basically classified into three groups according to the diversity of M.W. difference (migrating distance) and the intensity of the stained band between T+ and T- components of Tf types. Although the M.W. size in each Tf type was respectively different, Tf•F₁

and F₂ types, which have been classified as subtypes, had the same M.W. Tf•F₁ and F₂ types may be genetic variants determined only by the combined amount of sialic acid. Tf•D variant with the largest M.W., may be able to combine the most amount of sialic acid, consequently, this variant shows the most acidic pI in the IEF.

The existence of genetic information which controls the amount of Tf components could also be supposed according to the differences of intensity of stained bands between the T+ and T- bands (Table 1). In T- components analyzed by SDS-PAGE, the Tf•X type was the strongest band, the component in the group of D, F and H₂ types was in the middle, and the group of O and R types had the weakest bands. Reversely, T+ components of the Tf•X type had the weakest, relationship in direct opposition was observed between concentrations of T+ and T- components.

Tf types in donkey are also composed of two components, and the strongly stained components showed the fastest mobility by SDS-PAGE, the patterns differed from the cases of horses and Mongolian wild horses. The Tf types of the donkey studied in this time were heterozygote with four bands, and the M.W. was recognized within the segregation region of the D type and X type of horses. But, Tf types of Mongolian wild horses were heterozygote with larger M.W. than the D type of horses. By the way, the Tf•X type was a rare variant which was recognized in Noma native horses and Hokkaido native horses in Japan.¹¹⁾

Next, amino acid sequences of N-terminal 14 residues in Tf•D, F, H₂, O and R types, T+ and T- components in Tf•D and R types, and the amino acid sequence of each unique peak of D and R types fractionated by the HPLC method were analyzed (Table 2). Consequently, the N-terminal amino acid sequence was the same among each Tf type and T+ and T- components of the D and R types. But, it was clear that Tf•D and R types have respectively different components, because the unique peak (the 22nd peak in D type and 34th peak in R type) could be fractionated by the HPLC. Also, the 22nd and 34th peaks corresponded respectively at the 357-363rd and 438-

Table 2. Amino acid sequence of equine transferrin.

Tf types	Analyzed position		
	<i>N</i> -terminal	22nd peak	34th peak
D	EQTVRW ? TVSNHEV	GDEW?VN	SSSGPDLTWNSL
D T+ band	EQTVRW ? TVSN??V		
T- band	EQTVRW ? TVSN??V		
F	EQTVRW ? TVSNHEV		
F/-	EQTVRW ? TVSNHEV		
H ₂	EQTVRW ? TVSNHEV		
O	EQTVRW ? TVSNHEV		
R	EQTVRW ? TVSNHEV		
R T+ band	EQTVRW ? TVSN??V		
T- band	EQTVRW ? TVSN??V		

T+ : fast component of Tf by SDS-PAGE, T- : slow component of Tf by SDS-PAGE, F/- : Tf type with silent gene, 22nd and 34th peaks respectively show unique component in Tf•D and Tf•R variants. X type could not be analyzed, since the serum sample was a little amount.

460th amino acid fractions from the N-terminal in the data base on amino acid sequences of equine Tf.¹³⁾ The seventh amino acid which could be not determined in this study was cystine in the data base.

As above, equine Tf types could be classified into three basic groups according to diversity of the M.W. difference (migrating distance) and the stained intensity of the constructive band. Then, since the unique components of Tf•D and R types were fractionated respectively by the HPLC, it was thought that polymorphism of equine Tf types may also be basically caused by constructive differences of each Tf type component. From these results, the relationship between the three groups and establishment of horse breeds, and the functional differences between T+ and T- components of each Tf type were very interesting. The equine Tf type may be controlled by few loci as haplotype rather than by single locus based on diversity of splicing, because size heterogeneity was confirmed between T+ and T- components and among Tf variants, and differences of staining degree between T+ and T- bands in three Tf type groups were also observed.

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