

Aged vervet monkeys developing transthyretin amyloidosis with the human disease-causing Ile122 allele: a valid pathological model of the human disease

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Mutant forms of transthyretin (TTR) cause the most common type of autosomal-dominant hereditary systemic amyloidosis. In addition, wild-type TTR causes senile systemic amyloidosis, a sporadic disease seen in the elderly. Although spontaneous development of TTR amyloidosis had not been reported in animals other than humans, we recently determined that two aged vervet monkeys (*Chlorocebus pygerythrus*) spontaneously developed systemic TTR amyloidosis. In this study here, we first determined that aged vervet monkeys developed TTR amyloidosis and showed cardiac dysfunction but other primates did not. We also found that vervet monkeys had the TTR Ile122 allele, which is well known as a frequent mutation-causing human TTR amyloidosis. Furthermore, we generated recombinant monkey TTRs and determined that the vervet monkey TTR had lower tetrameric stability and formed more amyloid fibrils than did cynomolgus monkey TTR, which had the Val122 allele. We thus propose that the Ile122 allele has an important role in TTR amyloidosis in the aged vervet monkey and that this monkey can serve as a valid pathological model of the human disease. Finally, from the viewpoint of molecular evolution of TTR in primates, we determined that human TTR mutations causing the leptomeningeal phenotype of TTR amyloidosis tended to occur in amino acid residues that showed no diversity throughout primate evolution. Those findings may be valuable for understanding the genotype–phenotype correlation in this inherited human disease.

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Amyloidosis is a protein conformational disorder characterized by extracellular accumulation of amyloid fibrils derived from various proteins.^{1,2} Thus far, 27 distinct protein precursors of amyloid fibrils have been identified as causing different kinds of amyloidosis, such as Alzheimer's disease, prion disease, and type 2 diabetes.³ In each type of amyloidosis, certain characteristics, such as aging, genetic mutation, inflammation, and tumors, can stimulate disease-causing polymerization of functional soluble proteins in the body.

Mutant forms of transthyretin (TTR) cause the most common type of autosomal-dominant hereditary systemic amyloidosis.^{4–6} In addition, wild-type TTR causes senile systemic amyloidosis, a sporadic disease seen in the elderly.⁷

TTR is mainly synthesized by the liver, forms a homotetramer that has a dimer of dimers configuration in the bloodstream, and acts as a plasma transport protein for thyroid hormone and retinol-binding protein with vitamin A.⁸ Destabilization of TTR tetramers is widely believed to be a critical step in amyloid formation.^{9,10} To date, 120 TTR mutations have been identified.^{6,7} Although 14 TTR mutations are nonamyloidogenic, other TTR mutations induce amyloidosis, which can be classified into several phenotypes such as neuropathy (peripheral neuropathy (PN) type), commonly known as familial amyloidotic polyneuropathy (FAP); cardiomyopathy (C type), also known as familial amyloidotic cardiomyopathy; vitreous opacity (V type); and

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leptomeningeal amyloidosis (LM type), which causes central nervous symptoms. Of those pathogenic TTR mutations, Val30Met was the first to be identified and is the most well-known mutation causing the PN type of amyloidosis found throughout the world, including Portugal, Sweden, and Japan.^{4,5} In the United States, however, the most prevalent TTR mutation is Val122Ile. Approximately 3–4% of African Americans have this mutation, which causes late-onset cardiac amyloidosis.¹¹

Various kinds of animals other than humans are well known to develop several kinds of amyloidosis, such as amyloid A, amyloid β (A β), and islet amyloid polypeptide (IAPP) amyloidosis.³ Although papers indicating that several transgenic rodents possessing mutant human TTRs developed human TTR deposits in different tissues have been published,^{12,13} spontaneous development of TTR amyloidosis in animals other than humans had not been reported. We recently determined, however, that two aged vervet monkeys (*Chlorocebus pygerythrus*) spontaneously developed systemic TTR amyloidosis.^{14,15} However, the pathogenesis of TTR amyloidosis in vervet monkeys was still unclear.

This study therefore investigated the clinicopathological features of TTR amyloidosis in vervet monkeys, elucidated the pathogenesis of TTR amyloidosis in these monkeys, and verified a pathological relationship between TTR amyloidosis in vervet monkeys and the disease in humans. The rationale for this study relates to our need in medical research for valid animal models of pathological conditions that mimic human diseases.

SUBJECTS AND METHODS

Animals

Vervet monkeys and cynomolgus monkeys were maintained at the Tsukuba Primate Research Center for Medical Science at the National Institute of Biomedical Innovation (NIBIO, Ibaraki, Japan). Those monkeys were individually housed in stainless steel cages under temperature conditions between 23 and 27 °C, humidity between 50 and 70%, 12 air changes/h, and a 12/12-h light/dark cycle, and were fed 70 g of commercial monkey chow (Type AS, Oriental Yeast, Chiba, Japan) and 200 g of fruits daily. Ketamine hydrochloride (Ketalar, 10 mg/kg; Daiichi-Sankyo, Tokyo, Japan) was used to induce anesthesia for clinical procedures such as blood sampling and clinical examinations. Tissue specimens were collected at necropsy immediately after their deaths and were fixed in 10% buffered formalin for histopathological examinations. Tissue specimens for evaluating mRNA levels were immediately frozen with liquid nitrogen and were kept at –80 °C. Necropsied cases in zoos were also employed for histopathological examinations (Supplementary Tables S1 and S2).

Histological and Immunohistochemical Studies

Tissue specimens from the primates studied (Supplementary Tables S1 and S2) stained with alkaline Congo red and hematoxylin. For immunohistochemistry, a polyclonal rabbit

anti-human TTR antibody (Dako, Glostrup, Denmark) (1:100 dilution), a monoclonal mouse anti-human IAPP antibody (Dako) (1:100 dilution), and a monoclonal mouse anti-human IAPP antibody (Abcam, Cambridge, UK) (1:100 dilution) were utilized, as described previously.¹³

Electron Microscopy

Post-mortem cardiac samples were fixed first in 4% paraformaldehyde and 1% glutaraldehyde solution and then in 1% OsO₄, after which they were embedded in Epon. Ultrathin sections were stained with 4% uranyl acetate and lead citrate and were examined with a transmission electron microscope (H-7500; Hitachi, Tokyo, Japan), as described previously.¹⁶

Extraction of Amyloid Proteins and Immunoblotting

We used the biochemical methods of Kaplan *et al*¹⁷ to extract amyloid proteins from 30 formalin-fixed, paraffin-embedded, amyloid-containing cardiac tissue specimens that had been sectioned at a thickness of 3 μ m. Isolated tissue pellets incubated at 90 °C for 15 min in Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) containing 8 M urea were subjected to SDS-PAGE and were then transferred to nitrocellulose membranes (Bio-Rad). Immunoblotting with the polyclonal rabbit anti-human TTR antibody (Dako) (1:1000 dilution) was performed as described previously.¹³

Echocardiography and Thoracic Radiography

All living vervet monkeys, which were kept in the Tsukuba Primate Research Center, and age- and sex-matched cynomolgus monkeys were used for echocardiographic studies. Echocardiographic images were obtained with the ProSound SSD-5000 (Aloka, Tokyo, Japan), as described previously.¹⁸ Thoracic radiographs were obtained by using the C-arm Radiography System (Siemens, Erlangen, Germany).

Measurements of TTR and Atrial Natriuretic Peptide Concentrations

Serum TTR concentrations were measured by means of nephelometry. Plasma atrial natriuretic peptide (ANP) concentrations were measured by a chemiluminescent enzyme immunoassay (Shionogi, Osaka, Japan).

TTR Gene Analyses in Primates

PCR amplification by direct DNA sequencing was accomplished with the use of ABI BigDye Terminator reagents (Applied Biosystems, Foster City, CA, USA) on the 3130 Genetic Analyzer (Applied Biosystems). We utilized specific primers to evaluate sequences of *TTR* genes in these primates (Supplementary Table S3). We inferred an evolutionary tree for the *TTR* gene by using the neighbor-joining method with the MEGA5 program.¹⁹

Mass Spectrometric Analysis of Serum TTR

Serum samples diluted 1:50 with 50 mM Tris-HCl pH 7.0 were used for evaluation. Surface-enhanced laser desorption/

ionization time-of-flight mass spectrometry (SELDI-TOF MS) (Bio-Rad) analyses with a strong anion exchange surface Q10 chip were performed as described previously.²⁰

Real-Time PCR to Evaluate TTR mRNA Levels in Tissues

Total RNA was isolated from frozen tissue specimens by use of the RNeasy Kit (Qiagen, Hilden, Germany). Then, reverse transcription (RT) was performed with the PrimeScript RT reagent Kit (Takara Bio, Shiga, Japan). Real-time PCR was performed with SYBR Premix DimerEraser (Takara Bio) and the LightCycler 480 system (Roche Diagnostics GmbH, Penzberg, Germany). The primers for detecting TTR and GAPDH are provided in Supplementary Table S3.

Expression and Purification of TTR in *Escherichia Coli*

Recombinant TTRs of vervet and cynomolgus monkeys were used in this study. We isolated and cloned full-length cDNAs of vervet and cynomolgus monkey TTR from liver samples and used these cDNAs for generating the pQE plasmid (Qiagen) containing cDNA for each TTR. The sequences of the inserted DNA segments were verified by DNA sequencing with a DNA sequencing kit (Applied Biosystems) with pQE30 sequencing primers (Qiagen). The plasmids were transformed into competent M15 cells (Qiagen). The recombinant TTRs were expressed and purified as described elsewhere.²¹

Evaluation of the Stability of TTR Tetramers

To evaluate the stability of TTR tetramers to urea denaturation, samples were incubated (25 °C, 12 h) with buffers containing concentrations of urea as described previously.²¹ TTR was detected by silver staining with the ProteoSilver Silver Stain Kit (Sigma, St Louis, MO, USA). Band intensities were quantified by using the NIH ImageJ program (<http://rsbweb.nih.gov/ij/>).²²

Amyloid Fibril Formation and Thioflavin T (ThT)-Binding Assay

Amyloid fibril formation of recombinant TTR was evaluated as described previously.²³ Recombinant proteins were diluted in 50 mM glycine-HCl (pH 3.0 or pH 4.0) containing 100 mM NaCl. TTR samples were prepared to a final concentration of 20 μ M and were then incubated at 37 °C. Aliquots were collected for measurement of the extent of ThT fluorescence, which was measured with a Hitachi F-2000 spectrofluorimeter (Hitachi). An excitation wavelength of 442 nm and an emission wavelength of 489 nm were used for all assays. The reaction mixture contained 5 mM ThT and 50 mM glycine-NaOH buffer (pH 9.5); 5 μ l of 20 μ M TTR solution was added to 1 ml of this reaction mixture.

Ethics

The studies described herein were performed in accordance with the recommendations of the Weatherall Report, 'The use of non-human primates in research' (<http://www.mrc.ac.uk/Utilities/Documentrecord/index.htm?d=MRC003440>), and

were approved by the ethics review committees of Kumamoto University, National Institute of Biomedical Innovation, and Azabu University.

Statistical Analysis

Data were evaluated with Pearson's χ^2 test or Student's *t*-test and ANOVA. All analyses were performed with JMP version 5.1 (SAS Institute Japan, Tokyo, Japan). *P*-values of <0.05 were regarded as statistically significant.

RESULTS

Amyloid Deposits in Various Nonhuman Primates: Histopathological Findings

Histopathological investigations using 124 post-mortem cardiac specimens obtained from nonhuman primates revealed that three of six aged vervet monkeys had amyloid deposits derived from TTR (Figures 1a and b, Supplementary Table S1), whereas the other primate species did not. Electron microscopy revealed haphazardly arranged rigid amyloid fibrils in the heart in these vervet monkeys (Figure 1c). Vervet monkeys also developed TTR amyloid deposits in systemic organs and tissues including the gastrointestinal tract (Figure 1d), subcutaneous adipose tissue (Figure 1e), lung, tongue, and vascular walls (Figures 1g and h). Amyloid deposits were not found in the kidney glomerulus (Figures 1g and h), however, a site with severe amyloid deposits in most types of systemic amyloidosis. We also confirmed by means of immunoblotting that amyloid proteins extracted from cardiac specimens reacted with anti-TTR antibody (Figure 1f). In this study, we did not find lesions in monkey tissues that were positive for TTR and negative for Congo red. In addition, A β amyloid deposits in the brain and IAPP amyloid deposits in the pancreatic islet developed with age in both vervet monkeys and cynomolgus monkeys (Figures 1i and j, Supplementary Table S2), but TTR amyloid deposits developed only in vervet monkeys.

Cardiac Function of Living Vervet Monkeys and Cynomolgus Monkeys

We investigated the functioning of the heart in living vervet monkeys and cynomolgus monkeys that lived in captivity in an experimental facility. As Table 1 shows, almost half of the aged vervet monkeys had abnormal cardiac findings, such as arrhythmia, increased cardiothoracic ratio, increased plasma ANP concentrations, and reduced ejection fraction (EF) (Figures 2a–c), but cynomolgus monkeys did not have those abnormal findings. Cardiac diastolic function could not be evaluated by means of echocardiography, because these monkeys generally had rapid heart rates and much smaller hearts than humans. In vervet monkeys, the occurrence of cardiac symptoms correlated with sex (*P* = 0.016) (Table 1).

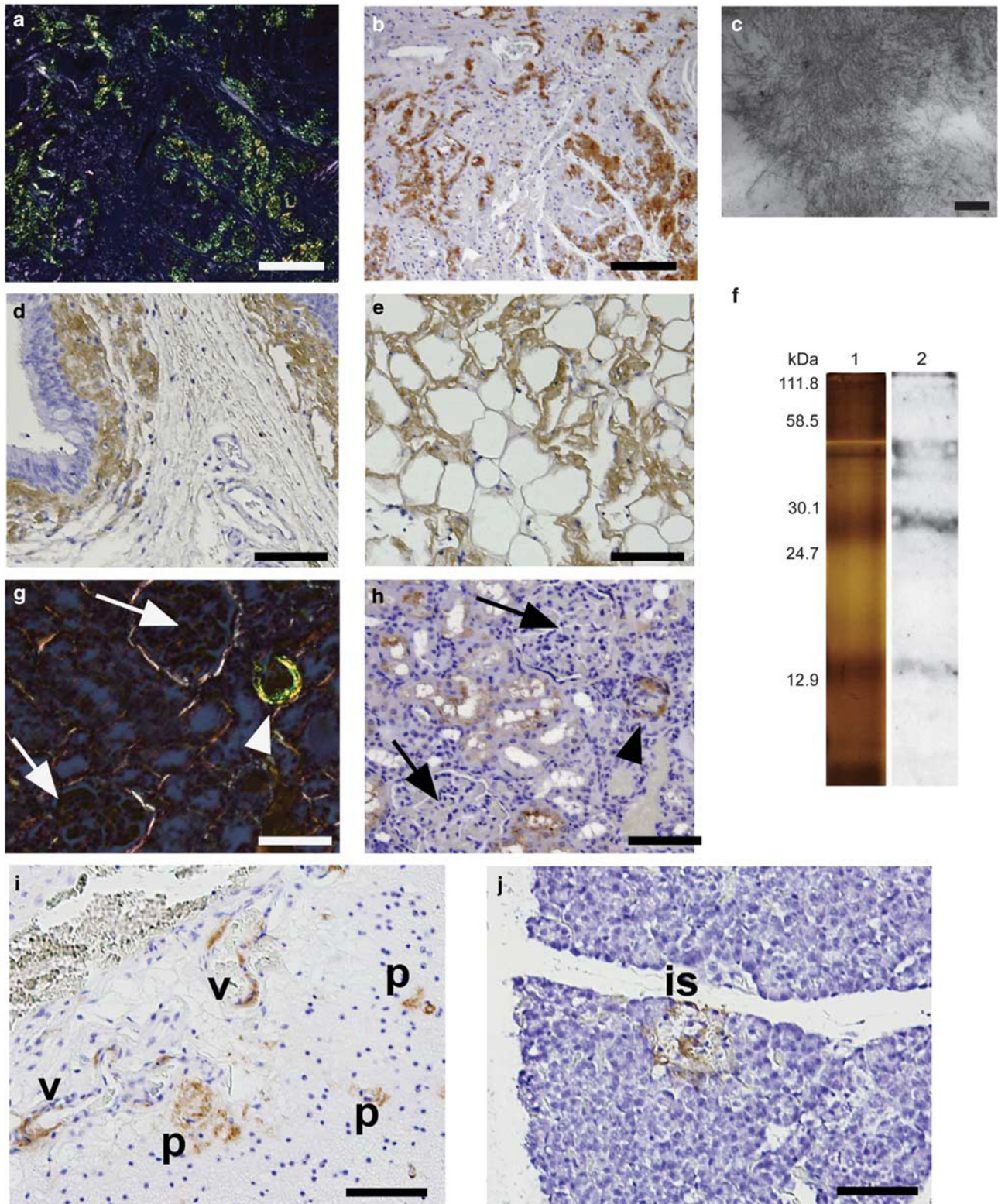


Figure 1 Histopathological features of aged vervet monkeys. TTR amyloid deposits in the heart (a–c), duodenum (d), subcutaneous fat (e), and kidney (g, h) of an aged vervet monkey. (a, g) Congo red staining under polarized light; (b, d, e, h) immunohistochemical staining using anti-TTR antibody; and (c) electron micrograph of amyloid fibrils in the cardiac specimen. Arrowheads: amyloid deposits in the vascular wall; arrows: absence of amyloid deposits in the kidney glomerulus. (f) Silver stain (lane 1) and immunoblotting using anti-TTR antibody (lane 2) of amyloid proteins extracted from cardiac amyloid deposits. Aged monkeys also developed amyloid β (A β) amyloid deposits in the brain (i; immunohistochemical staining using anti-A β antibody) and IAPP amyloid deposits in the pancreas (j; immunohistochemical staining using anti-IAPP antibody). is, islet IAPP amyloid deposit; p, A β amyloid deposits in senile plaques; v, vascular A β amyloid deposits.

Table 1 Clinical cardiac and laboratory findings for aged vervet monkeys and cynomolgus monkeys

Case no.	Age (years)	Sex	Living conditions	BW (kg)	Clinical findings	IVSd (mm)	LVPWd (mm)	EF (%)	FS (%)	Plasma ANP concentrations (pg/ml)	TTR amyloid deposits
V1	8	F	E	2.85	Normal cardiac function, CTR: 51%	0.44	0.38	78.4	44.1	19.0	NE
V2	14	F	E	3.35	Heart enlargement, reduced ventricular wall motion	0.67	0.43	48.8	23.2	41.0	NE
V3	15	F	E	3.10	Normal cardiac function	0.28	0.40	69.9	35.8	9.3	NE
V4	15	F	E	2.95	Normal cardiac function	0.45	0.66	81.6	46.3	16.1	NE
V5	15	F	E	2.70	Arrhythmia, heart enlargement, reduced ventricular wall motion, CTR: 67%	0.57	0.52	32.5	14.2	133.0	NE
V6	17	M	E	4.80	Arrhythmia	0.29	1.12	58.3	27.9	10.9	NE
V7	17	M	E	4.40	Arrhythmia, heart enlargement, reduced ventricular wall motion, CTR: 64%	0.44	0.84	45.8	20.9	50.4	NE
V8	17	F	E	NE	Normal cardiac function, uterine myoma	0.63	0.44	78.2	43.0	<5.0	—
V9	17	F	E	4.00	Normal cardiac function	0.43	0.35	67.8	34.9	11.1	NE
V10	18	F	E	2.75	Normal cardiac function	0.61	0.73	69.9	35.9	33.5	NE
V11	20	M	E	5.00	Heart enlargement, reduced ventricular wall motion, CTR: 63%	0.37	0.70	35.4	15.9	14.6	NE
V12	20	F	E	4.45	Arrhythmia	0.70	0.55	64.3	32.5	19.2	NE
V13	25	M	E	NE	NE	NE	NE	NE	NE	NE	—
V14 ^a	26	M	Z	3.70	Cardiac failure (shortness of breath)	NE	NE	NE	NE	NE	+
V15	27	M	E	3.95	Arrhythmia, thoracic tumor	0.71	0.61	65.0	32.9	64.2	+
V16 ^b	29	M	E	NE	Arrhythmia, CTR: 68%	NE	NE	NE	NE	44.7	+
V17	>30	F	E	NE	NE	NE	NE	NE	NE	NE	—
C1	1	F	E	1.50	Normal cardiac function	0.46	0.51	77.6	42.0	NE	NE
C2	1	F	E	2.12	Normal cardiac function	0.32	0.46	68.1	34.5	NE	NE
C3	4	M	E	NE	Normal cardiac function	0.36	0.68	60.6	28.9	NE	—
C4	4	M	E	4.53	Normal cardiac function	0.52	0.60	65.6	33.4	NE	NE
C5	6	M	E	NE	Normal cardiac function	0.45	0.63	61.7	30.7	NE	—
C6	7	F	E	NE	Normal cardiac function	0.48	0.61	65.9	33.3	NE	—
C7	9	F	E	3.34	Normal cardiac function	0.36	0.40	63.4	31.6	NE	NE
C8	9	F	E	3.22	Normal cardiac function	0.38	0.38	74.8	41.9	NE	NE
C9	10	M	E	6.21	Normal cardiac function	0.49	0.67	64.8	33.3	NE	NE
C10	16	F	E	2.89	Normal cardiac function	0.57	0.75	79.9	44.3	NE	NE
C11	20	M	E	5.00	Normal cardiac function	0.39	0.53	81.3	45.7	NE	—
C12	21	F	E	3.16	Normal cardiac function	0.44	0.58	84.7	49.2	NE	NE
C13	22	M	E	5.78	Normal cardiac function	0.37	0.47	74.4	39.8	NE	—
C14	23	F	E	5.77	Normal cardiac function	0.28	0.28	71.0	38.4	NE	NE
C15	24	M	E	5.68	Normal cardiac function	0.67	0.78	77.5	43.3	NE	—
C16	24	M	E	6.54	Normal cardiac function	0.75	0.80	76.1	41.6	NE	—
C17	25	M	E	7.12	Normal cardiac function	0.61	0.72	60.7	30.2	NE	—

Abbreviations: BW, body weight; C, cynomolgus monkey; CTR, cardiothoracic ratio; E, living in captivity in an experimental facility (the Tsukuba Primate Research Center for Medical Science at the National Institute of Biomedical Innovation); EF, ejection fraction; F, female; FS, fractional shortening; IVSd, interventricular septal thickness in diastole; LVPWd, left ventricular posterior wall thickness in diastole; M, male; NE, not examined; V, vervet monkey; Z, living in captivity in a zoo.

^aWe previously reported details of case no. V14.¹⁵

^bWe also previously reported details of case no. V16.¹⁴
Underlined bold type indicates abnormal data.

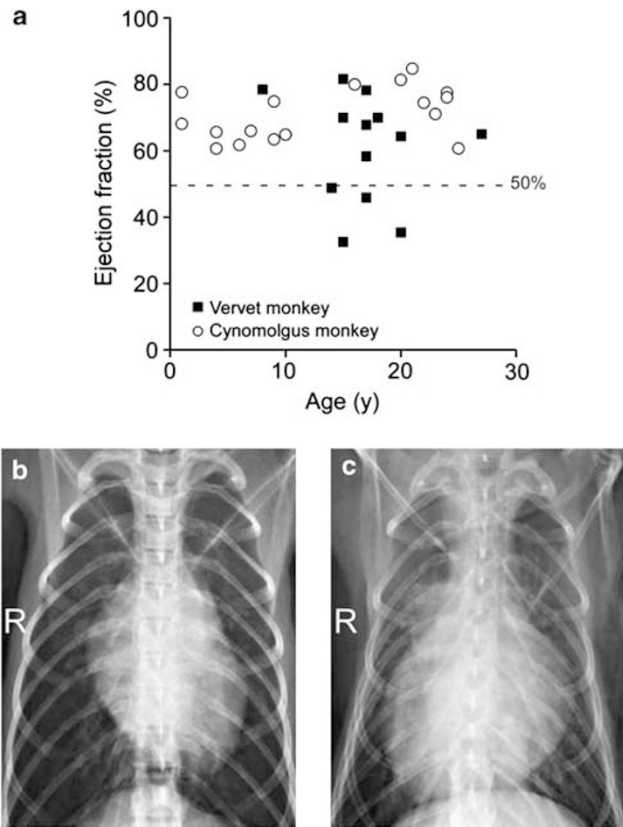


Figure 2 Cardiac functions of monkeys. (a) EF of vervet monkeys and cynomolgus monkeys. Four aged vervet monkeys showed EF values <50%. (b, c) Ventrodorsal thoracic radiographs of vervet monkeys without (b, case no. V1) and with (c, case no. V11) abnormal cardiac findings.

TTR Sequences in the Vervet Monkey and Other Primates

All of the vervet monkeys and one malbrouck monkey (*Chlorocebus cynosuros*), which belongs to the same genus as the vervet monkey, had the TTR Ile122 allele (Figures 3 and 4), which is well known as a mutation-causing TTR amyloidosis in humans, whereas other primates had the TTR Val122 allele (Figure 3). Except for the change at position 122, the other amino acid sequences of vervet monkey TTR were identical to TTR sequences in three other species of Old World monkeys: the cynomolgus monkey, rhesus macaque, and hamadryas baboon (Figure 3). Studies utilizing SELDI-TOF MS revealed that the measured mass of serum TTR was almost identical to the theoretical mass of TTR calculated on the basis of the TTR sequences in each monkey species (Figures 4c and d). We also investigated TTR gene sequences in 17 vervet monkeys and 15 cynomolgus monkeys and found the polymorphism Val/Ile at position 5 in both monkey species (Figure 4e), but this polymorphism had no correlation with TTR amyloidosis. In addition, cynomolgus monkeys possessed several other TTR gene polymorphisms (Figure 4e).

Genetic analyses also revealed that TTRs of apes, Old World monkeys, and New World monkeys had 95–98%,

93–94%, and 76–81% amino acid homologies to human TTR, respectively (Figure 3). Furthermore, we found that all five species of New World monkeys showed a deficiency of three amino acids in the N-terminal side of mature TTR (from position 4 to position 6) compared with TTRs of the other primates. An evolutionary tree based on these primate TTR gene sequences was also developed (Supplementary Figure S1).

Expression and Serum Concentration of TTR in Vervet Monkeys and Cynomolgus Monkeys

Real-time PCR revealed that vervet monkeys expressed the TTR gene in the liver, choroid plexus, eye, and pancreas (Figure 5a), which are locations of TTR gene expression in humans and rodents. Cynomolgus monkeys also expressed the TTR gene in the liver to the same degree as did vervet monkeys (Figure 5a). However, serum TTR concentrations in vervet monkeys were less than half of those in cynomolgus monkeys (Figure 5b). Serum TTR concentrations in vervet monkeys with and without cardiac dysfunction did not differ (Figure 5c). Serum TTR concentrations also showed no significant sex differences (male: 75.2 ± 6.8 , female: 82.3 ± 16.8 mg/l, $P = 0.494$) in this study.

Stability and Amyloid Formation of Recombinant Monkey TTRs

To investigate the role of the TTR Ile122 allele on TTR stability and amyloid formation in the vervet monkey, we generated recombinant monkey TTRs. The recombinant TTR of the vervet monkey showed a significantly greater tendency to dissociate to monomers under the influence of urea-mediated denaturation stress than recombinant TTR of the cynomolgus monkey, which had the Val122 allele (Figures 6a–c). Furthermore, the ThT-binding assay revealed that the recombinant TTR protein of the vervet monkey formed more amyloid fibrils *in vitro* than did that of the cynomolgus monkey (Figure 6d).

Human TTR Mutations and the Diversity of TTR Amino Acid Sequences in Primates

In Figure 3, we provided human TTR mutations and compared those mutations with the TTR sequences of nonhuman primates. Although as many as 120 TTR mutations have been reported in humans, nonhuman primates evidenced only three amyloidogenic TTR alleles—Thr81 in two New World monkeys (common marmoset (*Callithrix jacchus*) and black-bearded saki (*Chiropotes satanas*)), Val107 in all New World monkeys, and Ile122 in the vervet monkey—and one non-amyloidogenic TTR allele—His104 in all Old and New World monkeys.

In addition, to determine the genotype–phenotype correlation for human hereditary TTR amyloidosis, we investigated the relationship between the distribution of pathogenic mutations in human TTR and the diversity of amino acid sequences in primate TTRs (Figure 3). As

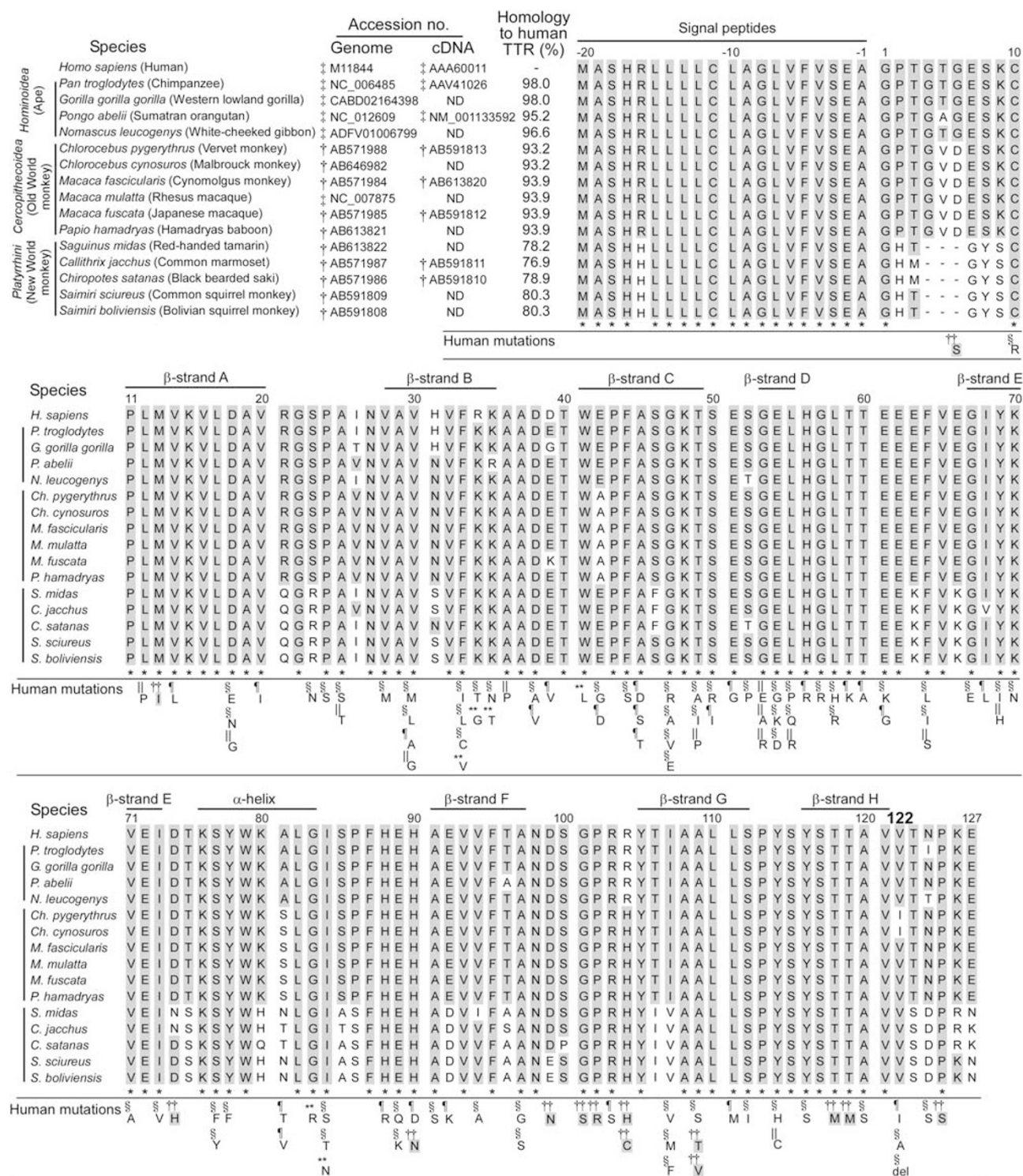


Figure 3 TTR amino acid sequence alignment for the 15 primate species, and human TTR mutations. The shaded area indicates conserved sequences in the primates. *Conserved amino acid residues in all 15 primates; †sequence data examined in this study; ‡sequence data cited from GenBank; ND, not determined. §Mutation-causing amyloid PN type; †mutation-causing amyloid C type; ‡mutation developing LM amyloid deposits causing CNS symptoms; **mutation developing V-type amyloid deposits causing visual disturbance; and ††with shading: nonamyloidogenic mutation. Del, deletion of amino acid residue.

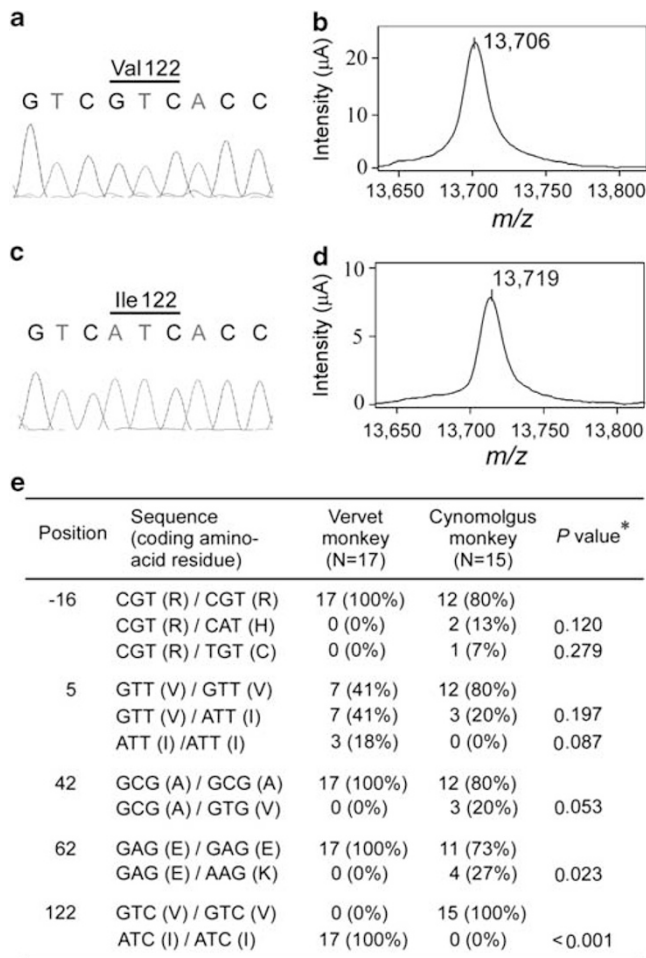


Figure 4 Analyses of TTR gene sequences and serum TTR in monkeys. (a, c) TTR gene sequence at position 122. Cynomolgus monkeys had the TTR Val122 allele coded by GTC, which was identical to the allele in healthy humans, but vervet monkeys had the TTR Ile122 allele coded by ATC, which is a well-known mutation-causing TTR amyloidosis in humans. (b, d) SELDI-TOF MS analyses of serum TTR in the cynomolgus monkey (c) and the vervet monkey (d). (e) Polymorphism of TTR sequences in vervet monkeys and cynomolgus monkeys. *According to Pearson's χ^2 test, comparing each group with the other groups.

Figure 7a demonstrates, the ratios of the number of human TTR mutations causing two amyloidosis phenotypes, PN and LM, to the number of uniform TTR amino acid residues were higher than the ratios for diverse amino acid residues, whereas for the other phenotypes, these ratios were almost equal. All mutations causing LM amyloidosis were found only in uniform amino acid residues in the primates (Figure 7b).

DISCUSSION

To our knowledge, the vervet monkey is the first species other than humans to develop TTR amyloidosis spontaneously. We initially determined that only vervet monkeys developed TTR amyloidosis and that almost half of the aged vervet monkeys showed cardiac dysfunctions, such as arrhythmia, increased

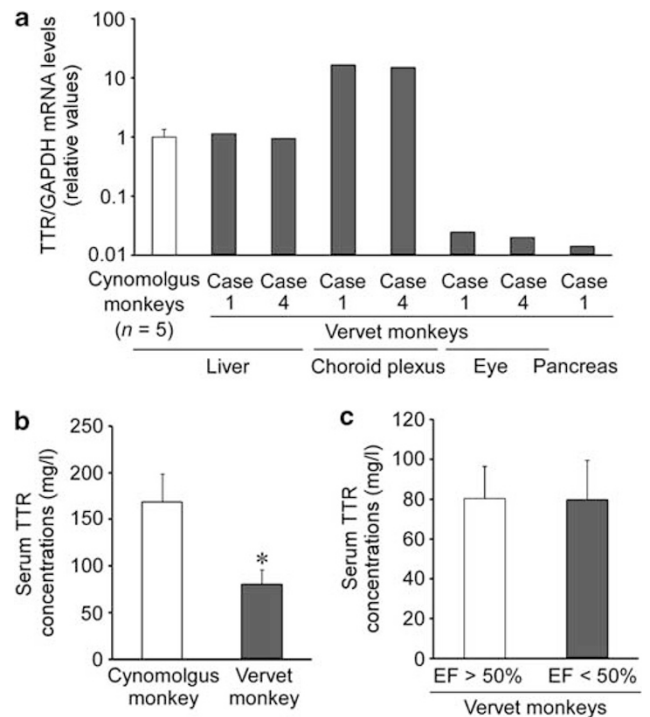


Figure 5 TTR gene expression and serum TTR concentrations in cynomolgus monkeys and vervet monkeys. (a) TTR gene expression in cynomolgus monkeys in the liver (n=5) and in vervet monkeys in various tissues including the liver. (b, c) Serum TTR concentrations measured by means of nephelometry. (b) Comparison of serum TTR concentrations in 15 cynomolgus monkeys (male: n=6, female: n=9) and 13 vervet monkeys (male: n=4, female: n=9). * $P < 0.0001$. (c) Serum TTR concentrations of vervet monkeys with (EF < 50%, n=4) and without (EF > 50%, n=9) cardiac dysfunction. Error bars show the s.d.

cardiothoracic ratio, increased plasma ANP concentration, and reduced EF. The occurrence of cardiac symptoms in the aged vervet monkeys correlated with sex ($P = 0.016$), as is the case in human TTR amyloidosis.^{7,24} In addition, all the vervet monkeys studied had the TTR Ile122 allele, which is known to be the mutation that causes TTR amyloidosis in humans, whereas other primates had the TTR Val122 allele. Except for Ile122, the amino acid sequence of vervet monkey TTR was identical to that of the cynomolgus monkey, which did not have TTR amyloid deposits or cardiac symptoms. These results suggest that the Ile122 allele has a critical role in TTR amyloidosis in the vervet monkey.

With regard to amyloidosis in humans, that 3–4% of African Americans have a heterozygous amino acid substitution from valine to isoleucine at position 122 (Val122Ile) of TTR is well known, and elderly individuals with this substitution are at high risk of developing TTR amyloidosis that affects mainly cardiac function.¹¹ Several patients with a homozygous Val122Ile mutation reportedly showed more severe clinical symptoms of amyloidosis than did patients with the heterozygous mutation.^{24,25} Although the reason why the TTR Ile122 allele occurs only in vervet monkeys

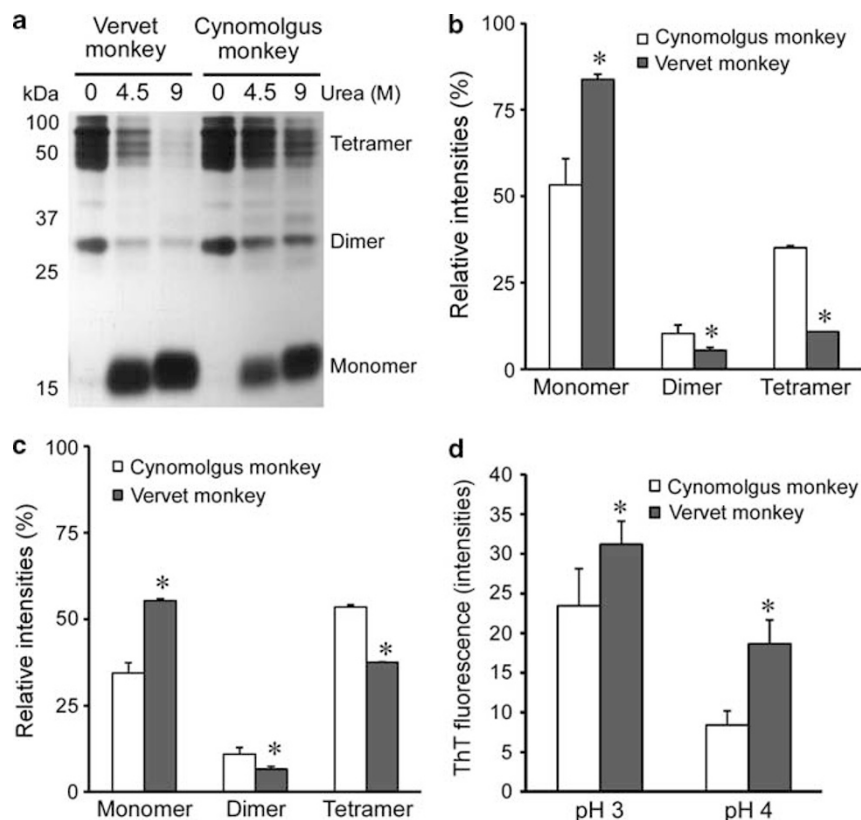


Figure 6 Stability and amyloid fibril formation of recombinant monkey TTRs. **(a–c)** Stability of the tertiary structure of recombinant monkey TTRs to urea-mediated denaturation stress. **(a)** Silver staining of recombinant monkey TTRs cross-linked by glutaraldehyde after denaturation stress induced by different urea concentrations (0, 4.5, and 9 M). **(b)** Relative intensities of each molecular form of TTR after denaturation stress induced by 9 M urea. **(c)** Relative intensities of each molecular form of TTR after denaturation stress induced by 4.5 M urea. **(d)** *In vitro* fibril formation of recombinant monkey TTRs. Samples were incubated for 120 h at pH 3.0 or pH 4.0. * $P < 0.05$ (vs cynomolgus monkey). Error bars show the s.d.

remains to be determined, we suggest that this allele may be subjected to positive selection through the evolution—both vervet monkeys, which originated in Africa and African Americans—and may provide certain functional advantages for living in Africa. Further studies should be undertaken to answer this question.

We also determined that the recombinant TTR of the vervet monkey carrying Ile122 allele was less stable and tended to form more amyloid fibrils than that of the cynomolgus monkey carrying Val122 allele. In the human TTR, residue Ile122 is located on the periphery of the H strand, which makes an antiparallel-sheet interaction with strand H of another monomer, stabilizing the dimer interface. It is also one of the residues involved in dimer-dimer interactions. Based on results from X-ray crystallography, Damas *et al*²⁶ reported that the human TTR Val122Ile variant indicated an elongation of the dimer-dimer interactions present in the tetrameric molecule and consequently less stable molecules, with a higher propensity to dissociation. Jiang *et al*²⁷ also reported that the human TTR Val122Ile mutation enhanced the rate of dissociation from tetramer to monomer, thus enhancing amyloid fibril formation, *in vitro*. Together, all these findings strongly support our working hypothesis that

the Ile residue at position 122 has a critical role in TTR amyloid formation in the vervet monkey.

In addition, we determined that, compared with cynomolgus monkeys, vervet monkeys had lower TTR concentrations in the blood, whereas both species had identical TTR gene expression in the liver, which is the major organ that secretes TTR entering the bloodstream. Buxbaum *et al*²⁸ reported that human TTR Val122Ile carriers had significantly lower serum TTR concentrations than did age-matched controls although the majority of such individuals did not have clinical symptoms. A recent *in vitro* study also indicated that highly destabilized mutant TTRs are subject to processing by intracellular quality control systems and are not efficiently secreted into the bloodstream.²⁹ In view of these findings, we speculate that the TTR in vervet monkeys may also be degraded by intracellular quality control systems. Together, all these findings led us to conclude that aged vervet monkeys developed TTR amyloidosis because of the Ile122 allele and had pathological conditions mimicking those of patients with TTR Val122Ile amyloidosis.

Furthermore, we investigated a relationship between the diversity of amino acid sequences of TTR in primates and the distribution of human TTR mutations, and we determined

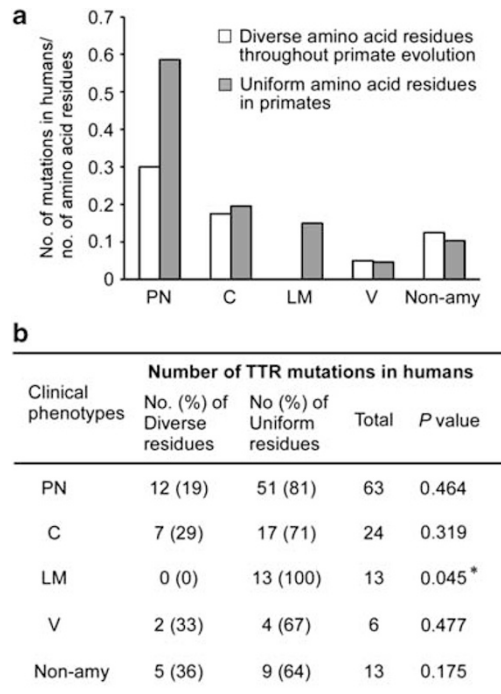


Figure 7 Difference in occurrence of human pathogenic TTR mutations in diverse and uniform amino acid residues in primates. **(a)** Difference in the ratio of the number of TTR mutations causing various human phenotypes to the number of diverse and uniform TTR amino acid residues. **(b)** TTR mutations causing different human phenotypes as related to diverse and uniform TTR amino acid residues. Non-amy, nonamyloidogenic. *P*-values according to Pearson's χ^2 test, comparing each group with the other groups. **P* < 0.05.

that all TTR mutations causing the LM amyloidosis phenotype occurred in uniform amino acid residues in these primates. Certain studies suggested that some TTR mutations causing the LM amyloidosis phenotype were more unstable than were the other TTR mutations.^{29,30} Also from the viewpoint of molecular evolution of primates, human TTR mutations causing the LM phenotype may occur in functionally and/or conformationally important amino acid residues, because those amino acid residues are maintained throughout primate evolution. These findings may be valuable for understanding the genotype–phenotype correlation in this inherited human disease.

In conclusion, we propose that vervet monkeys develop TTR amyloidosis because of the Ile122 allele and serve as a valid animal model of this inherited human disease. Moreover, we suggest that the genotype–phenotype correlation in TTR amyloidosis can be investigated from the viewpoint of molecular evolution in primates.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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