Discussion

One-stage clotting assays based on the APTT are generally used for the measurement of FVIII:C, and coagulation factor levels generally correlate well with the clinical phenotypes of patients with haemophilia A. Assays of this type, however, only partially reflect coagulation in a non-physiological environment, and the limited sensitivity at lower levels of FVIII:C (<2-3%) could compromise the accuracy between these specific laboratory assays and clinical status. Furthermore, discrepancies in one-stage and two-stage clotting assays of FVIII:C are observed in haemophiliacs with point mutations at Ala^{284}/Ser^{289} in A1, $Arg^{527}/Arg^{531}/Arg^{698}$ in A2, and His^{1954} in A3, which are functionally crucial residues for the interactive surfaces in the FVIII A domains (20). One-stage clotting assays may have limited use, therefore, in the evaluation of coagulation function *in vivo* in haemophiliacs. In consequence, a number of global coagulation functional assays have been established and are utilised for clinical diagnosis (6-8). We have previously reported that both clot waveform and thrombin generation assays are useful for clinical assessment in patients with very low levels of FVIII (<1%) (7, 8). In the present investigation, global coagulation assays demonstrated that the R1781H mutant exhibited enhanced coagulation potential, in accordance with clinical phenotype, whilst one-stage clotting assays failed to identify this association. The findings suggested that the global tests rather than the one-stage clotting assays reflected coagulation potential in the patient with FVIII R1781H. It seems likely that comprehensive appraisal of coagulation function in haemophilia A could depend on both conventional clotting assays and modern global coagulation assessment.

The results of the different coagulation measurements appear likely to depend on the final concentration of the substrate FX in the assay systems. In the current investigations, ROTEM, FXa and thrombin generation assays were performed at near physiological concentrations of FX (~120 nM). These FX concentrations were significantly greater than the K_m values of FX for tenase, comprising FVIII wild type and R1781H (by 2.1- and 3.7-fold, respectively), and were near the saturating level of FX for FVIIIa binding (FVIII; ~1 nM in plasma). In the one-stage assay, however, the plasma is diluted at least four-fold, and the limiting amount of FX is lower than that for the K_m for FX (by 0.50- and 0.93-fold, respectively), and provides a limiting level of FX. This markedly depresses both the rate of tenase activity and the subsequent reactions leading to clot formation. The differences in K_m for FX and FVIII-variant association could account for the discrepancies between coagulation activities seen using different methodologies, and may be similar to the mechanism governing the dampening of tenase activity observed when comparing FVIII:C levels of FVIIIa and inactivated FVIIIa lacking the $A1^{337-372}$ region. In these circumstances, significant differences are found between one-stage clotting assays and FXa generation assays (21).

The addition of incremental amounts of exogenous FX, but not FIXa, to control, FVIII-deficient plasma (FVIII:C 0.9 IU/dl) mediated a pattern of clot waveform similar to that seen with native R1781H plasma. It could be that the concentration of FVIII in plasma in these experiments was very low (~1 nM), and that the physiological amount of FX in plasma was insufficient for interaction with FVIIIa. In the presence of excess amounts of FX, however, FVIIIa would have been completely saturated, and the clot waveform obtained under these conditions was indeed similar to that observed with R1781H. It seems likely, therefore, that the relatively high affinity of R1781H for FX in plasma compared to that of wild-type FVIII could have accounted for the enhanced interaction between these two components of the tenase complex, and hence mediated the greater coagulation potential of R1781H.

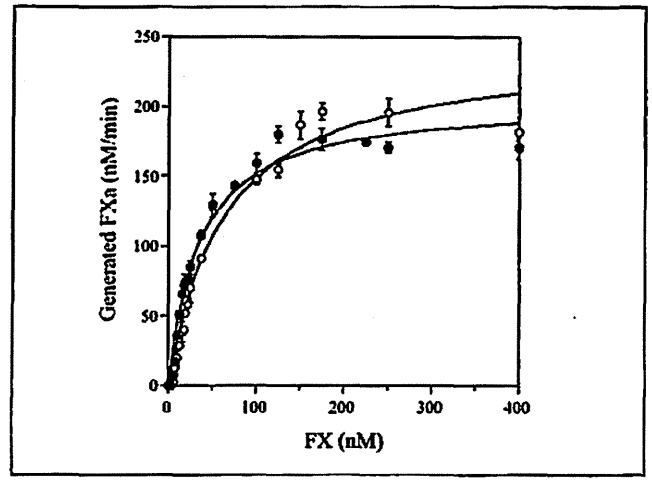

Figure 5: Michaelis-Menten analysis of the tenase complex formed using R1781H. FVIII preparations (5 nM) were activated with thrombin (30 nM) for 1 min in the presence of PL vesicles (20 μM). FXa generation was initiated by the addition of FIXa (0.5 nM) and various concentrations of FX (0-400 nM) as described in *Methods*. The symbols used are as follows: FVIII wild type (open circles), R1781H (closed circles). Initial rates of FXa generation are plotted as a function of FX concentration and fitted to the Michaelis-Menten equation by non-linear least squares regression. All experiments were performed at least four separate times, and the average values and standard deviations were calculated.

A FX-interactive site on FVIII is localised within the acidic region in the A1 domain, especially in the clustered acidic residues $Asp^{361-363}$ (22). This region interacts with the heparin-binding exosite (in particular Arg^{240}) in the catalytic domain of FX. In addition, Fay and colleagues have recently reported (23) that FX interacts with the residues 2007-2016 (in particular Thr^{2012} and Phe^{2014}) in the A3 domain, which contributes to the K_m of tenase complex for FX. Studies on the X-ray crystal structure of the FVIII molecule (24) indicated that Arg^{1781} is unlikely to be in close proximity to the 2007-2016 region, although the 337-372 acidic region in A1 is not defined in this model. It might be, therefore, that Arg^{1781} represents a yet unidentified FX-interactive site in FVIII. Alternatively, Arg^{1781} might not directly participate in FX binding, but rather might indirectly affect the interaction of FVIII with FX through a possible conformational change by the substitution of Arg^{1781} to His.

To date, 28 haemophilia A patients, indentified with the R1781H mutation, have demonstrated clinical heterogeneity, rang-

What is known about this topic?

- The clinical severity in haemophilia A patients with similar FVIII:C levels may be different. A mild bleeding phenotype has been reported in ~10% of individuals with severe haemophilia A.
- Non-null mutations of *F8* may represent the main determinant for bleeding tendency. Other factors associated with other coagulation abnormalities appear to be negligible as modulators of disease severity. The heterogeneity of clinical symptoms in severe haemophilia A remains to be fully explained.
- Some severe haemophilia A patients with the specific R1781H gene mutation present with a mild/moderate clinical phenotype, but the mechanism remains to be fully investigated.

What does this paper add?

- The plasma FVIII:C in a patient with haemophilia A and the R1781H mutation was ~0.9 IU/dl in one-stage clotting assays. Comprehensive global coagulation tests showed significantly greater haemostatic potential, corresponding to a FVIII:C level of 5~10 IU/dL
- Thrombin generation tests demonstrated that haemostatic function in the presence of recombinant R1781H FVIII (~0.9 IU/dl) was comparable to that observed at concentrations of ~5 IU/dl native FVIII:C.
- The recombinant R1781H mutant demonstrated an ~1.9-fold decrease in K_m for FX in the tenase complex compared to FVIII wild type. This enhancement of binding affinity of FVIII R1781H for FX appeared to reflect the severity of the haemophilia A phenotype.

ing from a severe to mild/moderate phenotype (13). Among these patients, only three patients with FVIII:C ≤1 IU/dl have been classed as severe. Some of these cases seem to contradict our conclusion based on enhanced coagulation potential of R1781H relative to wild-type FVIII. Complete profiles of these patients (type and/or degree of life activity and arthropathy, etc) have not been reported, however, and further clarification is required. In addition, 17 patients with this mutation had FVIII:C >1 IU/dl and presented with a mild/moderate clinical phenotype. Our findings indicate that enhanced coagulation potential mediated by the R1781H mutant would contribute to the phenotype of these individuals, although it is well known that in general patients with FVIII:C >1 IU/dl have mild/moderate haemorrhagic symptoms.

A mutation of the Arg residue to His is a relatively conservative amino acid change. Interestingly, another causative mutation (R1781C) described in several reports, also seems to mediate variable phenotypes, ranging from severe to mild/moderate. It may be, therefore, that the mutation of Arg at 1781 by itself, and not the nature of the amino acid substitution, influences the mechanisms governing the clinical presentation. Experiments using a range of amino acid substitutions at this site might help to clarify if the bulkier imidazole side chain of the His residue is a fundamental contributor to the structural change in the FVIII molecule.

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Conflicts of interest

None declared.

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ORIGINAL ARTICLE

Genotypic and phenotypic features of Japanese patients with mild to moderate hemophilia A

Hiroshi Inaba · Keiko Shinozawa · Ikuo Seita · Manabu Otaki · Takashi Suzuki · Takeshi Hagiwara · Kagehiro Amano · Katsuyuki Fukutake

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Abstract Hemophilia A is the most common inherited bleeding disorder. To better understand the genotypic and phenotypic features of Japanese patients with mild to moderate hemophilia A, we studied 29 unrelated patients with more than 1 % FVIII activity (FVIII:C). Differences were observed in nine of 21 patients in measured FVIII:C levels between the one-stage clotting and chromogenic assays. We identified a mutation in *F8* in 28 of the 29 patients. Mutations in two amino acids, Y492 and R550, were detected at a much higher frequency in our patients than in the international hemophilia A mutation database. We demonstrated that all five patients with the Y492C mutation have an identical *F8* haplotype that is unique to them, suggesting that the mutation may have originated from a common ancestor. Because non-severe, moderate to mild, hemophilia patients have a longer lifespan, mutations that cause non-severe phenotypes tend to persist in the population. We believe that the Y492C mutation is a distinctive feature of Japanese patients with mild hemophilia A. The identification of a high frequency of R550 mutation that underlies the discrepancies in FVIII:C measurements in the present study suggests that Japanese patients with mild hemophilia may require careful characterization.

Keywords Hemophilia A · Founder effect · Mutation · Discrepancy

H. Inaba (✉) · I. Seita · M. Otaki · T. Suzuki · T. Hagiwara · K. Amano · K. Fukutake
Department of Laboratory Medicine, Tokyo Medical University, 6-7-1 Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan
e-mail: hrsinb@tokyo-med.ac.jp

K. Shinozawa · K. Amano · K. Fukutake
Department of Molecular Genetics of Coagulation Disorders, Tokyo Medical University, 6-7-1 Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan

Introduction

Hemophilia A (MIM +306700) is the most common inherited bleeding disorder caused by a quantitative or qualitative abnormality of the blood coagulation factor VIII (FVIII). The clinical severity of hemophilia A correlates well with the residual factor VIII activity (FVIII:C) in circulating blood, and the presence of small amounts of FVIII:C markedly reduces the clinical severity. Mild to moderate hemophilia A, generally presenting with mild or negligible bleeding symptoms, is defined as FVIII:C of more than 1 % [1]. About 60 % of hemophilia A patients are classified into this category [2]. The factor VIII gene (*F8*) is located on the most distal band of chromosome X (Xq28) and spans over 186 kb. This large gene consists of 26 exons and 25 introns, and encodes 2,351 amino acids. Since the cloning of *F8* in 1984 [3–6], numerous analyses regarding *F8* have been performed to identify the mutation(s) responsible for hemophilia A. To date, about 1,500 unique mutations have been identified and registered in a worldwide mutation database [HADB; http://hadb.org.uk, also known as HAMSTeRS (The Haemophilia A Mutation, Structure, Test and Resource Site)]. According to HADB, almost all the mutations that cause a mild to moderate hemophilia A are a missense mutation.

There are some racial differences in the allele frequency of single nucleotide polymorphisms (SNPs) and the frequency of mutations that cause hemophilia A. For example, the frequency of *F8* inversion, which is known as the most common mutation responsible for severe hemophilia A, is possibly lower in the Japanese population than in the Caucasian population [7]. Some founder effects detected at a high frequency only in a limited geographical area have been reported thus far [8–10]. Therefore, it is important to understand the features of genetic abnormality of

hemophilia A in a particular region or race. In the present study, we analyzed and characterized the genotypic and phenotypic features of 29 Japanese patients with mild to moderate hemophilia A.

Materials and methods

Patients

We analyzed 29 unrelated Japanese patients with moderate to mild (FVIII:C > 1 %) hemophilia A. All the patients characterized in this study live in the Tokyo metropolitan area. The study was approved by the ethical committee of Tokyo Medical University, and written informed consent was obtained from all patients. This study was conducted in accordance with the ethical principles of the declaration of Helsinki, revised at Seoul in 2008.

Coagulation assay

FVIII:C assay

We measured the FVIII:C level by one-stage clotting assay and chromogenic assay.

The one-stage clotting assay was performed on an ACL-9000 automatic coagulation analyzer (Instrumentation Laboratory, Bedford, MA, USA) using 2 commercially available APTT reagents, HemosIL^{TM} APTT-SP reagent (Instrumentation Laboratory) and Thrombo-Check APTT(S) (Sysmex, Kobe, Japan).

The chromogenic assay was carried out using a Coatest SP FVIII kit (Chromogenix, Milan, Italy) according to the manufacturer's instructions.

Factor VIII antigen (FVIII:Ag) assay

We measured the FVIII:Ag level using an Asserachrom VIII:Ag kit (Diagnostica Stago, Asnieres, France) according to the manufacturer's instructions.

Gene analysis

Genomic DNA was extracted from peripheral blood cells according to the standard methods with proteinase K and phenol/chloroform, or using an EZ1 DNA Blood 350 μL kit (Qiagen, Hilden, Germany) on a BioRobot EZ1 workstation (Qiagen). *F8*, including the entire coding sequence, exon–intron junctions, and part of the 5′ and 3′ untranslated regions were amplified by PCR using 36 sets of primers. Although we designed most PCR primers used in this study, some were designed as described previously [11]. The M13 consensus sequence was added to the 5′ end of all primers for direct sequencing. The amplified products were electrophoresed on a 3 % agarose gel and were extracted using a QIAquick Gel Extraction kit (Qiagen). The purified PCR products were directly sequenced by the dideoxy chain termination method using the M13 consensus sequence as the primer, and analyzed with an SQ-5500 or SQ-5500E sequencer (Hitachi, Tokyo, Japan). Nucleotide sequences obtained by sequencing were compared with the *F8* reference sequence (ENSG00000185010) in the Ensembl.

F8 haplotype analysis

Seven different SNPs [rs6649625 (C/T), rs1470586 (C/T), rs1800291 (C/G), rs4898352 (A/T), rs4074307 (C/T), rs1050705 (A/G), and rs6571266 (A/G)] in *F8* were analyzed for 79 unrelated Japanese male patients (51 hemophilia A patients including 29 patients in this study, 26 hemophilia B patients, and 2 normal individuals). The regions including each SNP were amplified by PCR using specific primer pairs that we designed and the products were digested by appropriate restriction endonucleases. The location of the SNPs, nucleotide primer sequences, and restriction endonucleases used in this study are shown in Table 1. The short tandem repeats (STR) in IVS-13 of *F8* [12] were also analyzed. The region including the STR was amplified by PCR using specific primer pairs (fwd: 5′-CAC TTT AAA AAT GCC GCT CC-3′; rev: 5′-CAA GAG CTG TGT GAC AAA ATT GA-3′) and the number of the CA repeats was determined by direct sequencing of the PCR products.

Bioinformatic analysis

The effects of missense mutations were predicted using the PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and the SIFT (http://sift.jcvi.org/). The 3D structure of factor VIII was obtained from the crystallographic data (3CDZ) [13] registered in the Protein Data Bank (http://www.rcsb.org/pdb/) and was built with the UCSF Chimera program.

Results

Coagulation study

In this study, we examined the FVIII:C level in 21 of the 29 patients (Table 2). Because an appropriate plasma sample was not obtained to evaluate FVIII:C level in this study, the phenotypes of the remaining 8 patients were decided by the historically lowest FVIII:C level in the medical record. No significant differences were observed between the FVIII:C

Table 1 SNPs used for haplotype analysis of *F8*

SNP	Location	Direction	Primer sequence (5′→3′)	Restriction enzyme	Informative
rs6649625	IVS-1	Fwd	ATC TGG TGG GTG AAA GCA AT	*Alu*I	Yes
		Rev	GCA TCA CAC TTA TAA AAT ACA CAG AGA		
rsl470586	IVS-1	Fwd	GAT TCA ATA TAG AAA TCC TGC CAA A	*Aci*I	Yes
		Rev	ATG CTG ATT AAC AGG ATA AGC TGA C		
rsl800291	Exon 14	Fwd	GCC TCA GAT ACA TAC AGT GAC	*Mnl*I	Yes
		Rev	CTG CTG GCT TGT ATT AGG AG		
rs4898352	IVS-18	Fwd	ATG GTC TAG GCA CTG GGA AC	*Bcl*I	No
		Rev	GTG CCC TAT GGG ATT TGA GA		
rs4074307	IVS-19	Fwd	TTC CTT TCT GGA ATG GTT GC	*Hind*III	No
		Rev	TTC GAG CTT TAC CAA GTT GTG A		
rsl050705	3′-UTR	Fwd	CCC TGT GAA GTT CTT AAA GT	*Bcl*I	No
		Rev	GGG AAA AGA ATG CCA AAA TAA GAT GAT		
rs6571266	3′-UTR	Fwd	GCC TCA ATC CAG GAG AAC AG	*Bsl*I	No
		Rev	TTA TTA GCG ACG GGA TTT CG		

Seven SNPs located in a wide range of *F8* were analyzed. However, only 3 SNPs (i.e., rs6649625, rs1470586 and rs1800291) were informative for the analysis. There is a strong linkage disequilibrium between rs1470586 and 4 other SNPs (i.e., rs4898352, rs4074307, rs1050705, and rs6571266) in this analysis

levels obtained by the one-stage clotting assay using HemosIL^{TM} APTT-SP, which we usually use in our laboratory as the APTT reagent, and those obtained by the chromogenic assay. However, the FVIII:C levels obtained by the one-stage clotting assay using Thrombo-Check APTT(S) as the APTT reagent were clearly higher than those obtained by the chromogenic assay in some of the 21 patients. Six patients (1 case of A303P, 1 case of G498R, 2 cases of R550C, 1 case of R550H, and 1 case of W707L) who showed TC/Coa ratios higher than 2.0 (Table 2) were concluded to have FVIII:C discrepancy. Three additional patients (patients #12, 14 and 16 in Table 2) had TC/Coa ratios of <2.0, but they showed the same R550C and R550H mutations; thus, we conclude that these three patients similarly have the FVIII:C discrepancy.

Gene analysis

We identified mutations in *F8* in 28 of the 29 patients (Table 2). In the remaining patient who showed a moderately severe phenotype, we could not find any genetic abnormality that may be responsible for hemophilia A. The mutations identified in this study were all missense mutations. Five mutations (W707L, T1793N, H1878P, S1907N, and Q2106H) were novel and not yet registered in the HADB database as of August 2012.

The missense mutations were distributed over the entire length of *F8*. However, there was a high frequency of mutations in 2 amino acid residues: 5 (17.2 %) mutations were found in Y492 in exon 10, and 6 (20.7 %) in R550 in exon 11.

An intragenic analysis of 7 SNPs and 1 STR was carried out to determine the haplotype of patients with the Y492C mutation. Seven SNPs that were considered to work effectively for haplotype analysis by referring to the allelic frequency from the HapMap project data were selected. However, there was a strong linkage disequilibrium between rs1470586 (C/T) SNP and 4 other SNPs: rs4898352 (A/T), rs4074307 (C/T), rs1050705 (A/G), and rs6571266 (A/G). Therefore, only 3 SNPs [rs6649625 (C/T), rs1470586 (C/T), and rs1800291 (C/G)] were informative for the analysis. *F8* from 79 Japanese males were classified into 4 haplotypes using 3 informative SNPs (Table 3) and 9 of them showed the CTC haplotype. All 5 *F8* with the Y492C mutation showed the CTC haplotype. IVS-13 STR analysis was additionally performed in the 9 *F8* with the CTC haplotype. These were divided into 2 groups: 4 with 21 CA repeats, and 5 with 22 CA repeats. The latter group is composed of only *F8* with the Y492C mutation. Taken together, all 5 *F8* with the Y492C mutation showed the same unique haplotype (CTC and 22 CA repeats).

Discussion

We set out to study 29 unrelated Japanese patients with mild to moderate hemophilia A to analyze their genotypic and phenotypic features.

We have identified a high frequency of mutations in R550 and Y492 in these patients. The R550 residue includes the CpG dinucleotide in the codon. The CpG

Table 2 Phenotype and genotype of 29 Japanese patients with mild to moderate hemophilia A

No.	One-stage clotting (IU/mL)			FVIII:C Coa (IU/mL)	TC/ Coa	Discrepancy	FVIIIAg (IU/mL)	Severity	Exon	Mutation		HADB
	FVIII:C SP	FVIII:C TC	Medical record							Nucleotide change[a]	Amino acid change[b]	
1	0.080	0.085		0.084	1.01		0.238	Mild	3	c.328A > G	p.M110V (91)	Yes
2	NA	NA	0.031	NA				Moderate	5	c.606T > G	p.S202R (183)	Yes
3	0.054	0.051		0.073	0.7		0.060	Mild	5	c.606T > G	p.S202R (183)	Yes
4	0.180	0.265		0.109	2.43	Yes	0.218	Mild	7	c.907G > C	p.A303P (284)	Yes
5	NA	NA	0.088	NA				Mild	10	c.1475A > G	p.Y492C (473)	Yes
6	0.079	0.082		0.082	1		0.086	Mild	10	c.1475A > G	p.Y492C (473)	Yes
7	0.078	0.087		0.095	0.92		0.100	Mild	10	c.1475A > G	p.Y492C (473)	Yes
8	NA	NA	0.110	NA				Mild	10	c.1475A > G	p.Y492C (473)	Yes
9	0.063	0.084		0.088	0.95		0.094	Mild	10	c.1475A > G	p.Y492C (473)	Yes
10	0.053	0.109		0.037	2.95	Yes	0.234	Moderate	10	c.1492G > A	p.G498R (479)	Yes
11	0.080	0.119		0.045	2.64	Yes	0.186	Mild	11	c.1648C > T	p.R550C (531)	Yes
12	0.084	0.133		0.074	1.8	Yes	0.182	Mild	11	c.1648C > T	p.R550C (531)	Yes
13	0.053	0.110		0.047	2.34	Yes	0.170	Mild	11	c.1648C > T	p.R550C (531)	Yes
14	0.272	0.362		0.225	1.61	Yes	0.402	Mild	11	c.1648C > T	p.R550C (531)	Yes
15	0.199	0.320		0.134	2.39	Yes	0.481	Mild	11	c.1649G > A	p.R550H (531)	Yes
16	0.232	0.366		0.250	1.46	Yes	0.464	Mild	11	c.1649G > A	p.R550H (531)	Yes
17	0.083	0.209		0.096	2.18	Yes	0.372	Mild	14	c.2120G > T	p.W707L (688)	No
18	0.408	0.448		0.417	1.07		0.638	Mild	14	c.2149C > T	p.R717W (698)	Yes
19	0.298	0.364		0.295	1.23		0.262	Mild	14	c.2149C > T	p.R717W (698)	Yes
20	NA	NA	0.034	NA				Moderate	14	c.2167G > A	p.A723T (704)	Yes
21	NA	NA	0.070	NA				Mild	14	c.5122C > T	p.R1708C (1689)	Yes
22	NA	NA	0.074	NA				Mild	14	c.5122C > T	p.R1708C (1689)	Yes
23	0.178	0.196		0.214	0.92		0.211	Mild	16	c.5378C > A	p.T1793N (1774)	No
24	NA	NA	0.060	NA				Mild	17	c.5633A > C	p.H1878P (1859)	No
25	0.159	0.165		0.162	1.02		0.106	Mild	17	c.5720G > A	p.S1907N (1888)	No
26	0.372	0.310		0.425	0.73		0.302	Mild	22	c.6318G > C	p.Q2106H (2087)	No
27	0.070	0.055		0.062	0.89		0.216	Mild	23	c.6506G > A	p.R2169H (2150)	Yes
28	0.052	0.052		0.058	0.9		0.080	Mild	26	c.6977G > A	p.R2326Q (2307)	Yes
29	NA	NA	0.034	NA	1.01			Moderate				

FVIII:C SP, FVIII activity obtained from a one-stage clotting assay using the HemosIL™ APTT-SP reagent as the APTT reagent; FVIII:C TC, FVIII activity obtained from a one-stage clotting assay using the Thrombo-Check APTT(S) as the APTT reagent; FVIII:C Coa, FVIII activity obtained from a chromogenic assay using the Coatest SP FVIII kit. Eight patients (# 2, 5, 8, 20, 21, 22, 24 and 29) from whom an appropriate plasma sample was not obtained to evaluate FVIII:C level in this study provided the historically lowest FVIII:C level in the medical record

NA not available

[a] The nomenclature of mutation is based on the cDNA sequence with nucleotide +1 corresponding to A of the ATG initiation codon

[b] Amino acids are numbered from the initial Methionine 1. The numbers in parentheses indicate amino acid numbers of mature processed FVIII

dinucleotide is a well-known hotspot for mutations [14] and a large number of missense and nonsense mutations were identified in the Arg(CGN) residues in the *F8* of hemophilia A patients. The mutation of C-to-T transition in Arg(CGN) accounted for 990 (35 %) of 2,830 point mutations in the HADB database. The mutation of C-to-T transition in the R550 residue is one of the most frequent point mutations in mild to moderate hemophilia A and accounted for 2.5 % in the HADB database. The R550 mutation was identified with a high frequency (20.7 %) in this study and this frequency was considerably higher than the frequency of R550 mutation found in the international database. When analyzing the mutations of the R550 residue, it is necessary to note that the measured FVIII:C values differ significantly among assay methods, particularly between the one-stage clotting assay and either the

Table 3 Haplotype analysis of *F8* in 79 Japanese male patients

SNP (1,2,3) haplotype[a]	Frequency	IVS-13 VNTR	Y473C
CCC	54/79 (69 %)	NE	0/54
CTC	9/79 (11 %)	21	0/4
		22	5/5
CTG	7/79 (9 %)	NE	0/7
TCC	9/79 (11 %)	NE	0/9

NE not examined

[a] Haplotype: rs6649625 (C/T), rs1470586 (C/T), rs1800291 (C/G)

chromogenic assay or the two-stage assay [15, 16]. It has been reported that the dissociation rate of the A2 domain from the FVIII molecule, which leads to the inactivation of the active FVIII, may be involved in the measurement discrepancies [17, 18], and that the dissociation rate of the A2 domain may be related to some reaction conditions, particularly the pH [19, 20]. We previously reported that there is a weak negative correlation between the pH of the APTT reagent and the measured FVIII:C values in the one-stage clotting assay [21]. In that report, we analyzed FVIII:C of the R550H mutation identified from a hemophilia A patient with a very mild phenotype. When this patient's FVIII:C was measured by the one-stage clotting assay using 14 different APTT reagents, the results were markedly different with each reagent. The highest and the lowest values for FVIII:C were about 50 and 25 %, respectively, showing almost twice the difference. Although all the FVIII:C levels obtained with a variety of APTT reagents reflect a mild phenotype, there is a possibility that the patient cannot receive an optimized treatment based on the numerical value. Furthermore, this may lead to a misdiagnosis of mild hemophilia. The identification of a high frequency of R550 mutation in this study suggests that Japanese patients with mild hemophilia might require more attention and care than the Caucasian population.

Y492 does not include the CpG dinucleotide in the codon. Y492C mutation registers only 6 (0.21 %) of point mutations in the HADB [22–25]. One of these registered cases was from the analysis of a Japanese patient [24], and 2 out of the 5 remaining cases were possibly Japanese subjects [23]. Surprisingly, we have identified 5 cases (17.2 %) of Y492C mutation in this study. Although it is not possible to declare with certainty because of the large difference in the number of patients between this study and the HADB, it is possible that one of the major genotypic features of Japanese patients with mild to moderate hemophilia A is the high frequency of Y492C mutation. The patients identified with Y492C mutation were apparently unrelated Japanese individuals. However, the results of the 3 SNPs and IVS-13 STR analysis indicated that all 5 patients with this mutation had an identical *F8* haplotype that is unique to them. These results strongly suggest a founder effect. The Y492C mutation possibly originated from a common ancestor and then spread into the Japanese population.

Five novel missense mutations were identified in this study. As for 4 out of these 5 mutations, the possibility that they may be the causes of mild to moderate hemophilia A was predicted by either the PolyPhen-2 or SIFT prediction method. However, T1793N was not predicted by either methods (Table 4) although, this mutation was also detected from another Japanese hemophilia A patient. An expression study is necessary to clarify that this mutation is also an etiology of mild to moderate hemophilia A.

We could not identify any mutation from 1 patient in this study. Because some genetic abnormality probably exists in the patient's *F8*, we should perform a different type of analysis such as multiplex ligation-dependent probe amplification.

Measurement of FVIII:C is necessary to determine the phenotype of a hemophilia A patient. However, we must be aware of the characteristics of the method that is used to assay FVIII:C. In some particular cases, such as a mutation identified in R550 as described above, the FVIII:C level possibly differs markedly according to the measurement methods. Mild cases of hemophilia A particularly require more attention [17, 26]. Furthermore, in the case of a mutation located in the interface of each of the A domains,

Table 4 Novel missense mutations identified in the present study

Mutation	Conservation[a] (h/p/m/c)	Polyphen-2[b] (HumDiv score)	SIFT[c] (score)
p.W707(688)L	W/W/W/W	Probably damaging (1.000)	Damaging (0.01)
p.T1793(1774)N	T/T/T/T	Benign (0.061)	Tolerated (0.36)
p.H1878(1859)P	H/R/H/R	Probably damaging (0.988)	Damaging (0.03)
p.S1907(1888)N	S/S/S/S	Benign (0.189)	Damaging (0.00)
p.Q2106(2087)H	Q/Q/Q/Q	Probably damaging (1.000)	Damaging (0.00)

[a] Factor VIII amino acid line up for human, porcine, murine and canine

[b] PolyPhen-2 prediction (http://genetics.bwh.harvard.edu/pph/)

[c] SIFT prediction (http://sift.jcvi.org/)

there is a great possibility of discrepant FVIII:C levels. In the present study, 9 cases showed clearly different FVIII:C levels between the one-stage clotting assay using Thrombo-Check APTT(S) and the chromogenic assay or the one-stage clotting assay using HemosIL^{TM} APTT-SP. The amino acid residues of all 9 cases (1 case of A303P, 1 case of G498R, 4 cases of R550C, 2 cases of R550H, and 1 case of W707L) were previously reported as residues which show different FVIII:C levels.

The discrepancy in FVIII:C measurement was originally reported as the difference between the one-stage clotting assay and the chromogenic assay. Subsequently, it was reported that the discrepancy was also observed within the one-stage clotting assays depending on the assay conditions, particularly when different APTT reagents were used [27]. The discrepancy leads to difficulties in understanding the exact phenotype of the patient. However, this can also bring useful information to the analysis of relationships between phenotypes and genotypes. That is, if the discrepancy can be confirmed before gene analysis, the type of mutation that is likely to exist in the interface of A domains can be predicted. Moreover, if it is a case where a novel mutation is identified, the importance of A2 domain dissociation with respect to the mutation site can also be grasped by confirming the discrepancy. Therefore, intentional confirmation of the discrepancy may become an effective analytical tool. We usually measure the FVIII:C using ACL9000 and an APTT reagent (HemosIL^{TM} APTT-SP). In this analysis, the results obtained by the one-stage clotting assay were almost the same as those obtained by the chromogenic method (Coatest SP FVIII), and it was suggested that the methods can be substituted interchangeably. Because the information about A2 domain dissociation is easily obtained by the one-stage clotting assay using an appropriate APTT reagent even if a chromogenic method is not performed, we recommend to attempt the one-stage clotting assay with two or more APTT reagents to analyze patients, except for a severe hemophilia case.

In conclusion, we have described the genotypic and phenotypic features of Japanese patients with mild to moderate hemophilia A. The identification of a high frequency of Y492C, which is thought to be the founder effect of the Japanese population, is one of the genetic features of Japanese patients with mild to moderate hemophilia A. The identification of a high frequency of R550 mutation that causes the discrepancy in FVIII:C measurement in this study suggests that Japanese patients with mild hemophilia might need more attention and care than the Caucasian population.

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ORIGINAL ARTICLE *Musculoskeletal*

The risk of elective orthopaedic surgery for haemophilia patients: Japanese single-centre experience

J. HIROSE,*† H. TAKEDANI† and T. KOIBUCHI‡
**Faculty of Medicine, Department of Orthopaedic Surgery The University of Tokyo; †Department of Joint Surgery; and ‡Department of Infectious Diseases and Applied Immunology, Research Hospital, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan*

Summary. Haemophilic arthropathy causes pain and a severely restricted range of motion, and results in a significant reduction in quality of life. When conservative treatments have failed, orthopaedic surgery is recommended for these patients with severe haemophilic arthropathy. However, surgery for haemophilia patients is challenging due to high complication rate such as infection, delayed wound healing and mortality. The aim of this study was to evaluate the incidence of early complications and identify preoperative risk factors of surgery for haemophilia patients. We report a series of haemophilia patients undergoing elective orthopaedic surgery between 2006 and 2012. During this period, 119 surgeries in 81 patients were prepared and 118 surgeries in 80 patients were actually performed. Four deep bacterial infections and four delayed wound healings occurred within 6 months postoperatively. One patient died preoperatively and four patients died postoperatively. Only the presence of inhibitor was a significant risk factor for infection. We found no risk factor related to delayed wound healing. Our data revealed alkaline phosphatase, albumin, platelet, alpha-fetoprotein, presence of ascites and child classification C as predictors of perioperative mortality following elective orthopaedic surgery. Our role is to identify potential patients who present with risk factors for complications and attempt to seek the best determination of treatment strategy for these people.

Keywords: complication, elective orthopaedic surgery, haemophilia, Japanese, risk factor, single centre

Introduction

Haemophilia was a life-threatening disease until the discovery of factor concentrates. The development of factor concentrates resulted in prolonged life expectancy in haemophilia patients and increased orthopaedic surgery for haemophilic arthropathy. However, hepatitis C virus (HCV) and human immunodeficiency virus (HIV), which had been mediated by unheated plasma-derived factor concentrates, discouraged orthopaedic surgeons from performing surgery for haemophilia patients. Since 1990s, development of antiretroviral therapy (ART), pegylated interferon and ribavirin [1] has made it possible to control the status of viral-infected haemophilia patients and enabled orthopaedic surgeons to perform elective surgery in increasing number of patients.

However, ART is known to increase the risk of metabolic syndrome, diabetes, renal insufficiency and cardiovascular disease [2]. Furthermore, some of the HCV-infected patients fail to achieve a sustained virological response with pegylated interferon and ribavirin, and most of these patients eventually develop liver cirrhosis and subsequent hepatocellular carcinoma. The elevated risk of bacterial infections and other perioperative complications of these patients [3] are major concerns of orthopaedic surgeons and many of them still hesitate to perform operations on these viral-infected haemophilia patients. To perform orthopaedic surgeries safely on such patients, it is important to have knowledge of the risk factors that may be involved.

The purpose of this study was to identify the risk factors for perioperative complications of elective orthopaedic surgeries and to establish the proper preoperative assessment of haemophilia patients.

Correspondence: Jun Hirose, MD, PhD, Department of Joint Surgery, Research Hospital, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan.
Tel.: +81 3 3443 8111, ext. 75099; fax: +81 3 6409 2402;
e-mail: j.hirose513@gmail.com

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Materials and methods

Patients

From July 2006 to July 2012, we prepared to perform 119 orthopaedic surgeries for 81 patients with haemophilia and other coagulation disorders at IMSUT Hospital of the Institute of Medical Science in Japan. Because one haemophilia A patient died before surgery, we actually performed 118 orthopaedic surgeries for 80 patients.

Preoperative evaluation

Medical evaluation prior to surgery included blood examinations, abdominal ultrasound scans, abdominal computed tomography and upper gastrointestinal endoscopy within 3 months before surgery. We additionally measured CD4 counts and plasma HIV-1-RNA in patients with HIV, and HCV-RNA in patients with HCV. We assessed the size of liver and spleen, the presence of abdominal fluid and hepatocellular carcinoma by abdominal ultrasound and computed tomography. Upper gastro-intestine was scoped to check the oesophageal varices indicating portal hypertension.

Statistical analysis

Surgical complications within 6 months after final surgery were recorded. To analyse the relationship between preoperative clinical status and postoperative complications, all parameters were divided into two categories. HBs antigen, HCV antibody and HIV antibody were divided into positive or negative. Parameters that have reference value, such as blood examination results, were divided as to whether the result was within or outside the reference values. Imaging findings, such as abdominal fluid or hepatocellular carcinoma, were divided into presence or absence. Child classification was divided into A/B or C.

Statistical analyses were performed with the chi-squared test using SPSS version 15.0 (SPSS Inc., Chicago, IL, USA). All tests were two-sided and a $P < 0.05$ was considered statistically significant. Results are expressed as mean ± SD and odds ratios (OR) with 95% confidence intervals (95% CI).

Results

Eight salvage surgeries, which were performed within 3 months after primary surgery, were included: six irrigations, one wound refreshment and one amputation above the knee. One haemophilia A patient died before total joint arthroplasty (TJA). This case was included in this study as death case. Finally, a total of 118 surgeries were performed in 80 patients: For 63 haemophilia A patients, 62 TJAs, 10 arthroscopic synovectomies (ASs), and 18 other surgical interventions were performed. For 16 haemophilia B patients, 17 TJAs, 5 ASs and 4 other surgical interventions were performed. TJA was performed for one factor VII deficiency patient and one von Willebrand's disease (VWD) patient. Table 1 summarizes the baseline characteristics of patients and surgical procedure conducted. The mean patient age was 40.9 years (range: 13–70 years). All haemophilia patients were male, and factor VII deficiency and VWD patients were female. Eighteen surgeries for 12 haemophilia patients with inhibitor were included: seven were haemophilia A and five were haemophilia B.

Human immunodeficiency virus and HCV infection status of patients are shown in Table 2. One hundred and six cases (89.1%) were HCV-seropositive and 39 cases (32.8%) were HIV-seropositive. All HIV-seropositive cases, but one was HCV-seropositive.

Table 1. Demographics of patients included in this study.

	Patients/surgical cases
Gender	
Male	79/117
Female	2/2
Age	
Years	40.9 (13–70)
Diagnosis	
Haemophilia A	63/91
Haemophilia B	16/26
FVII deficiency	1/1
von Willebrand disease	1/1
Surgery	
THA	15/16
TKA	43/59
TEA	1/1
TAA	1/1
AS	13/15
Others	24/27
Inhibitor	
Negative	69/101
Positive	12/18
Child classification	
A	43/65
B	5/8
C	1/1

THA, total hip arthroplasty; TKA, total knee arthroplasty; TEA, total elbow arthroplasty; TAA, total ankle arthroplasty; AS, arthroscopic synovectomy.

Table 2. Viral infection status of patients.

Patients/surgical cases	HAE-A	HAE-B	FVII def	VWD	Total
HCV(−)/HIV(−)	6/7 (2/2)	3/5 (3/5)	0	0	9/12
HCV(−)/HIV(+)	1/1	0	0	0	1/1
HCV(+)/HIV(−)	37/26 (3/4)	6/10 (2/4)	0	1/1	44/68
HCV(+)/HIV(+)	19/26 (2/3)	1/1	1/1	0	27/38
Total	63/91 (7/9)	16/26 (5/9)	1/1	1/1	81/119

HCV(−/+), anti HCV antigen negative/positive; HIV(−/+), anti-HIV-1 antigen negative/positive; HAE-A, haemophilia A; HAE-B, haemophilia B; FVII def, factor VII deficiency; VWD, von Willedbrand disease; (*N*), inhibitor case.

HCV-RNA was measured in 100 HCV-seropositive cases: 38 cases (38%) still had HCV virus and 62 cases (62%) had achieved sustained virological response or their HCV-RNA disappeared spontaneously. HIV-RNA was measured in 39 HIV-seropositive cases: 14 cases (35.9%) were positive and 25 cases (64.1%) were at undetectable level for HIV-RNA. CD4 cells were counted in 35 HIV-seropositive cases: there was no case whose CD4 cell count was <50 cells mm^{-3}, two cases (5.7%) had 50-200 CD4 cells mm^{-3}, 17 cases (48.6%) had 200–400 cells mm^{-3} and 16 cases (45.7%) had more than 400 cells mm^{-3}.

Twenty-seven abdominal ultrasound scans, 17 computed tomographies and 17 upper gastrointestinal endoscopies were performed. Eight abdominal fluids and five splenomegary were detected, but oesophageal varix was not detected. Seventy-four cases were divided into three groups according to child classification: 65 cases (87.8%) were in Child A, 8 cases (10.8%) were in Child B and 1 case (1.4%) was in Child C.

Deep bacterial infections occurred in four cases (3.4%): two were after total knee arthroplasty (TKA) and two were after other surgery. Three infections were managed with irrigation of the wound and one was managed with amputation above the knee. Delayed wound healing occurred in four cases (3.4%): one after total ankle arthroplasty (TAA), one after TKA and two after other surgery. One patient died before surgery and four patients died after surgery. Average age of patients who died was 48.2 years (range: 38–51 years). All patients were HCV-seropositive and three patients (60%) were HIV-seropositive. Overall, mortality rate was 6.2%. Symptomatic deep venous thrombosis and pulmonary embolism did not occur.

Complications and their risk factors are shown in Table 3. Surgeries for patients with inhibitor showed significantly higher incidence of deep bacterial infection (11% vs. 2%). The relative risk (RR) of infection in inhibitor patients was 6.13 (95% CI: 0.80–46.6, $P = 0.05$). On the other hand, infection rate of HIV-seropositive and HIV-seronegative patients were 2.6% and 3.8% respectively ($P = 0.75$). There was no factor which significantly increased the rate of delayed wound healing. Life prognosis was significantly related to abnormal values of alkaline phosphatase (ALP) (RR: 9.13, 95% CI: 0.98–87.89), albumin (ALB) (RR: 11.63, 95% CI: 1.76–76.76), platelet (PLT) (RR: 7.75, 95% CI: 1.13–53.36), alpha-fetoprotein (AFP) (RR: 7.25, 95% CI: 1.13–53.36), presence of ascites (RR:2, 95% CI: 0.5–8.0) and Child C classification (RR: 11.83, 95% CI: 5.50–25.45). All these factors represent advanced liver disease.

Discussion

Chronic haemophilic synovitis of joint results in haemophilic arthropathy, which causes pain and a severely restricted range of motion [4,5]. Development of clotting factor concentrates has improved the life expectancy of haemophilia patients and enabled orthopaedic surgeons to perform elective surgery for haemophilic arthropathy. However, many orthopaedic surgeons still hesitate to perform surgeries for haemophilia patients due to high complication rate. To know the preoperative factors which affect the incidence of perioperative complications is crucial to

Table 3. Risk factors for complications.

			Infection			Delayed wound healing			Life prognosis		
Factor	Category	*n*	*P* value	Odds	95% CI	*P* value	Odds	95% CI	*P* value	Odds	95% CI
Diagnosis	HAE-A/HAE-B	116	0.9	1.16	0.12–11.65	0.9	1.16	0.12–11.65	0.34	2.42	0.38–15.3
Operation	TJA/others	118	0.41	2.26	0.31–16.68	0.41	2.26	0.31–16.68	0.42	0.45	0.06–3.32
HBs	Positive/negative	118	0.74	0.97	0.95–1	0.74	0.97	0.95–1.00	0.71	1.03	1.00–1.06
HCV-III	Positive/negative	118	0.47	0.89	0.83–0.95	0.47	0.89	0.83–0.95	0.42	1.13	1.06–1.21
HIV	Positive/negative	118	0.75	0.69	0.7–6.70	0.75	0.69	0.07–7.90	0.19	0.31	0.50–1.95
GOT	Within/outside the RV	110	0.23	0.74	0.66–0.83	0.98	1.03	0.10–10.28	0.08	4.56	0.72–28.79
GPT	Within/outside the RV	110	0.23	0.74	0.66–0.83	0.98	1.03	0.10–10.28	0.74	0.69	0.74–6.42
ALP	Within/outside the RV	110	0.74	1.48	0.15–14.74	0.74	1.48	0.15–14.74	0.02	9.13	0.98–87.89
TP	Within/outside the RV	110	0.16	0.26	0.04–1.97	0.16	0.26	0.04–1.97	0.35	2.38	0.37–15.09
ALB	Within/outside the RV	110	0.45	0.42	0.04–4.34	0.44	0.87	0.81–0.94	<0.005	11.63	1.76–76.76
CD4 counts	200< or 200≧	35	0.76	1.03	0.97–1.1	0.76	1.03	0.97–1.10	0.66	1.07	0.98–1.16
HIV-RNA	Positive/negative	38	0.44	0.62	0.48–0.78	0.44	0.62	0.48–0.80	0.92	1.13	0.09–13.70
HCV-RNA	Positive/negative	99	0.12	0.61	0.52–0.72	0.6	1.83	0.18–18.27	0.86	0.8	0.70–9.11
PLT	Within/outside the RV	106	0.54	0.91	0.86–0.97	0.54	0.91	0.86–0.97	0.02	7.75	1.13–53.36
Hyalronic acid	Within/outside the RV	61	0.75	0.04	0.75–1.00	0.75	1.57	0.93–26.28	0.73	0.61	0.04–10.27
AFP	Within/outside the RV	70	0.5	0.87	0.79–0.95	0.5	0.87	0.79–0.95	0.04	7.25	0.89–58.89
Ascites	Presence/absence	43	0.82	0.98	0.93–1.02	0.82	0.98	0.93–1.02	<0.005	2	0.5–8.0
Splenomegaly	Presence/absence	43	0.22	0.56	0.43–0.74	0.22	0.56	0.43–0.74	0.22	1.78	1.36–2.34
Child classification	A/B or C	73	0.47	0.88	0.81–0.96	0.54	0.89	0.81–0.96	<0.005	11.83	5.50–25.45
Inhibitor	Positive/negative	118	0.05	6.13	0.80–46.6	0.39	0.84	0.78–0.91	0.33	1.19	1.1–1.29

TJA, total joint arthroplasty; RV, reference value.

perform surgeries safely on haemophilia patients. This study included a large series of elective orthopaedic surgeries consecutively performed over 6 years at a single institution mainly by the same primary surgeon.

In this study, deep bacterial infections occurred in four cases (3.4%), two after primary TKA and two after other surgery. When it comes to TKA, infection rate was 3.4% (2/59), which was higher than 1–2% in non-haemophilic population [6,7]. However, it was lower than the reported infection rate of TKA in haemophilic patients (0–16%; median 7.1%) [8–13]. Until the end of 1990s, CD4 cell counts of <200 cells mm^{-3} were thought to be one of the risk factors of deep bacterial infection [14]. Contrary to this, a more recent study of 53 TKA performed in 38 patients reported that infection was unrelated to HIV infection or CD4 counts [15]. Powell *et al.* [9] also reported that the presence of HIV infection did not increase the incidence of prosthetic joint infection. Our data also showed no significant difference in infection rate between HIV-positive and -negative groups. This result may reflect the stable immunological status of HIV-positive patients treated with ART. In this study, the only factor that significantly increased the infection rate was the presence of an inhibitor. Orthopaedic surgery started to be performed internationally for haemophilia patients with inhibitors after the development of bypassing agents, such as activated prothrombin complex concentrates and recombinant factor VIIa. As Solimeno *et al.* [11] reported, elevated risk of infection in patients with inhibitor may be attributed to prolonged operative time due to severe destruction of joints, massive bleeding and/or frequent infusion of clotting factors. Limitation of this study is that we only evaluated infections within relatively short period after operations. Because not only early infections but also late infections are critical issues following TJA in haemophilic patients [13], we should carefully follow-up these patients continuously.

Proper wound healing requires coagulation with strong thrombin generation resulting in fibrin formation. Therefore, it has been demonstrated in animal models that a number of bleeding disorders may affect wound healing, including haemophilia [16]. In this study, delayed wound healing occurred in four cases; one after TAA, one after TKA and two after other surgeries. Poor haemostasis, renal or liver disease and immunodeficiency were reported to be systemic factors that may compromise wound healing in TJAs [17]. However, we did not find preoperative factors which affect the incidence of delayed wound healing. It is difficult to explain the exact reason for this, but it may be attributed to good haemostasis in our patients especially with inhibitor and stable immunological status of HIV-seropositive patients.

Five perioperative deaths occurred in our patients. Preoperative factors significantly influencing mortality rate were abnormal values of ALP, ALB, PLT, AFP, presence of ascites and Child C classification. All these factors were associated with severe liver disorders due to HCV hepatitis. Until the development of viral inactivation procedures in the mid-1980s, almost all haemophilia patients who had received plasma-derived factor concentrates were infected with HCV. A large proportion of these patients were also infected with HIV. Before the introduction of the ART in the mid-1990s, HIV was considered to be the main viral infection in HIV- and HCV-coinfected haemophiliacs. However, as HIV infection has become effectively controllable, HCV liver disease has become a major cause of death in HIV- and HCV-coinfected patients [9,18,19]. At present, the treatment choice for HCV is combination therapy with pegylated interferon and rivabirin. Although this regimen can achieve HCV eradication rates of over 40% in patients with HCV genotype 1, there still are non-responders and the response rates are in the range 20–35% in HIV-positive patients [20]. Recently, anti-HCV drug telaprevir, which is NS3 protease inhibitor was developed and clinical studies demonstrated significantly better sustained virological response rates with telaprevir + Peg-IFN-α + RBV in comparison with Peg-IFN-α + RBV in treatment-naive as well as in treatment-experienced patients with HCV genotype 1 [21]. Although several issues are still pending, telaprevir will be included in future treatment recommendations. Because viral infection status of haemophilia patients is complicated and treatment options become more and more specialized, cooperation with a gastroenterologist or specialist for infectious disease will become increasingly essential. Therefore, surgical procedures for haemophilia patients are preferably performed in a specialized centre that has experience in treating haemophiliacs and where involvement of a multidisciplinary team is possible.

In conclusion, our results confirmed that risk factor related to deep bacterial infection was the presence of inhibitor and decompensated liver function was associated with high mortality rate.

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Author contributions

JH and HT designed the research study and wrote the manuscript. TK provided statistical interpretation of the results.

Disclosures

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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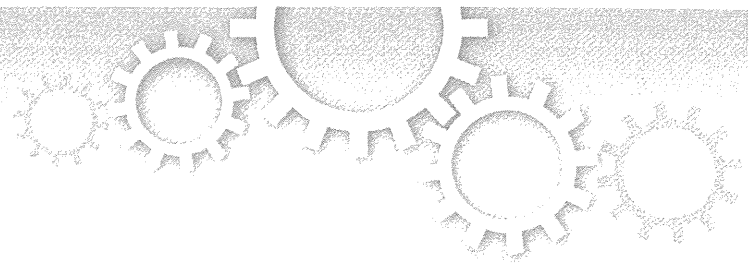

SUBJECT AREAS:
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Correspondence and requests for materials should be addressed to N.I. (iwata-n@nagasaki-u.ac.jp) or T.C.S. (saido@brain.riken.jp)

Global brain delivery of neprilysin gene by intravascular administration of AAV vector in mice

Nobuhisa Iwata[1,2], Misaki Sekiguchi[1], Yoshino Hattori[2], Akane Takahashi[2], Masashi Asai[2], Bin Ji[3], Makoto Higuchi[3], Matthias Staufenbiel[4], Shin-ichi Muramatsu[5] & Takaomi C. Saido[1]

[1]Laboratory for Proteolytic Neuroscience, RIKEN Brain Science Institute, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan, [2]Laboratory of Molecular Biology and Biotechnology, Department of Molecular Medicinal Sciences, Graduate School of Biomedical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki-shi, Nagasaki 852-8521, Japan, [3]Molecular Imaging Center, National Institute of Radiological Sciences, 4-9-1 Anagawa, Inage-ku, Chiba-shi, Chiba 263-8555, Japan, [4]Novartis Institutes for Biomedical Research, 4002 Basel, Switzerland, [5]Division of Neurology, Department of Medicine, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke-shi, Tochigi 329-0498, Japan.

Accumulation of amyloid-β peptide (Aβ) in the brain is closely associated with cognitive decline in Alzheimer's disease (AD). Stereotaxic infusion of neprilysin-encoding viral vectors into the hippocampus has been shown to decrease Aβ in AD-model mice, but more efficient and global delivery is necessary to treat the broadly distributed burden in AD. Here we developed an adeno-associated virus (AAV) vector capable of providing neuronal gene expression throughout the brains after peripheral administration. A single intracardiac administration of the vector carrying neprilysin gene in AD-model mice elevated neprilysin activity broadly in the brain, and reduced Aβ oligomers, with concurrent alleviation of abnormal learning and memory function and improvement of amyloid burden. The exogenous neprilysin was localized mainly in endosomes, thereby effectively excluding Aβ oligomers from the brain. AAV vector-mediated gene transfer may provide a therapeutic strategy for neurodegenerative diseases, where global transduction of a therapeutic gene into the brain is necessary.

Aggregation and deposition of amyloid-β peptide (Aβ) in the brain are triggering events of the long-term pathological cascade of Alzheimer's disease (AD), and are closely associated with the metabolic balance between Aβ anabolic and catabolic activities[1,2]. As almost all familial AD mutations cause an increase in the anabolism of a particular form of Aβ, $Aβ_{1-42}$, leading to Aβ deposition and accelerating AD pathology, a chronic reduction in the catabolic activity would also promote Aβ deposition[1,2]. Neprilysin (EC 3.4.24.11) is a rate-limiting peptidase involved in brain Aβ catabolism, as proven by *in vivo* experiments tracing the catabolism of radiolabeled Aβ in brain and by reverse genetics studies for candidate peptidases in mice[3,4]. Neprilysin gene-disruption caused a gene dosage-dependent elevation of endogenous Aβ levels in mouse brain, suggesting that a subtle but long-term reduction in neprilysin activity would contribute to AD development by promoting accumulation of Aβ[2].

Mounting evidence that expression levels of neprilysin are decreased in the hippocampus and cerebral cortex of AD patients from the early stages of disease development and also with aging in humans, as well as mice, suggests a close association of neprilysin with the etiology and pathogenesis of AD[2]. Indeed, reduced activity of neprilysin in mouse brain elevates the levels of highly toxic Aβ oligomers at the synapses, and leads to impaired hippocampal synaptic plasticity and cognitive function even before apparent amyloid deposition is observed in the brain[5]. Thus, a decline in neprilysin activity appears to be at least partly responsible for the memory-related symptoms of AD, and up-regulation of neprilysin is considered to be a promising strategy for therapy and prevention of AD.

Experimental gene therapy to transfer neprilysin gene into the brains of AD model mice has been reported, and for this purpose various kinds of recombinant viral vectors carrying wild-type neprilysin or its variants that are truncated at the transmembrane region and can be released to extracellular space have been utilized[6,7]. Viral vector-mediated delivery of neprilysin gene successfully retarded amyloid deposition in the brains of AD model mice[6,7]. Beneficial potential of gene therapy has also been shown in other neurodegenerative diseases, including Parkinson's disease (PD). Gene transfer of dopamine-synthesizing enzymes into the putamen alleviated motor

symptoms in PD patients[8,9]. However, infusion of viral vectors via stereotaxic surgery is not necessarily appropriate if the therapeutic gene should be delivered to broad areas of the brain.

In this study we have successfully developed a new gene delivery system by employing the combination of rAAV9 with a neuron-specific promoter, and we have shown that this system can provide functional gene expression throughout the brains of mice after intracardiac administration. The AAV vector can achieve comprehensive gene expression of neprilysin in the brain of young neprilysin-deficient mice, eventually decelerating Aβ accumulation and alleviating cognitive dysfunction based on a water maze test in aged APP transgenic (tg) mice. We show that the majority of the exogenous neprilysin is localized in late and early endosomes, where newly generated Aβ is concentrated, and this may be the reason why Aβ can be effectively excluded from the brain.

Results

Expression profile of neprilysin in the brain after AAV-mediated gene transfer. To deliver an AAV vector from circulating blood to the brain, we employed intracardiac administration, i.e., injection into the left ventricle of the heart, because this provides a direct route to the brain. To evaluate gene expression of neprilysin, we injected rAAV9 vectors that encode either an active or an inactive form of neprilysin in neprilysin-deficient mice[10] and examined the outcome by means of specific immunochemical staining for neprilysin. This staining generated specific signals of endogenous neprilysin in wild-type mice, but not in neprilysin-deficient mice without vector treatment (Fig. 1a,b). Expression of exogenous neprilysin after a single injection of rAAV9-NEP vector (4×10^{11} vector genome [v.g.]) into the left ventricle of the heart of neprilysin-deficient mice was spread over the limbic region on the neprilysin-null background (Fig. 1c,d), and presented a scattered distribution, but with locally intense signals. The total amount of exogenous neprilysin expression was dependent on amount of vector injected into the mice over a range of $0.5–4.0 \times 10^{11}$ v.g., as far as we examined (data not shown). On the other hand, intracardiac administration of rAAV9-NEP vector did not cause prominent gene expression of neprilysin in heart, lung, kidney or liver (Supplementary Fig. 1).

Next, we examined the localization of neprilysin in the brain by confocal double immunostaining for neprilyin and several marker proteins, after the injection of rAAV9-NEP vector into neprilysin-deficient mice. Neprilysin was present in vesicular structures of NeuN-positive neurons (Fig. 2a–c), but not in glial fibrillary acidic protein (GFAP)-positive astrocytes (data not shown). In addition, we found that exogenous neprilysin is colocalized with late endosomal marker proteins Ras-related protein 7 (Rab7) (Fig. 2d–f) and Rab9 (Fig. 2g–i), and also in part with early endosomal markers Rab5 (Fig. 2j–l) and early endosome antigen 1 protein (EEA1) (Fig. 2m–o), but not with presynaptic markers SV2 (Fig. 2p–r) and syntaxin 1, secretory vesicle marker Rab3a, clathrin-coated vesicle marker clathrin heavy chain, somato-dendritic marker microtubule-associated protein 2 (MAP2), or postsynaptic marker PSD-95 (data not shown).

Functional expression of neprilysin. We investigated functional expression of neprilysin and subsequent reduction of Aβ levels in the brain. Four weeks after the single intracardiac injection of rAAV-NEP_{WT} vector into neprilysin-deficient mice, neprilysin activity in the limbic region including the neocortex and hippocampus was significantly increased compared to that after injection of rAAV-NEP_{MT} vector, although the increased level of neprilysin activity was less than 10% of the level observed in intact wild-type mice (Fig. 3). The injection of rAAV-NEP_{WT} vector into neprilysin-deficient mice significantly reduced Aβ40, Aβ42 and total Aβ levels in the limbic region compared to those in the mice injected with rAAV-NEP_{MT}. The partially compensated neprilysin activity was sufficient to achieve a 50% reduction of the elevated Aβ levels in the neprilysin-deficient mice.

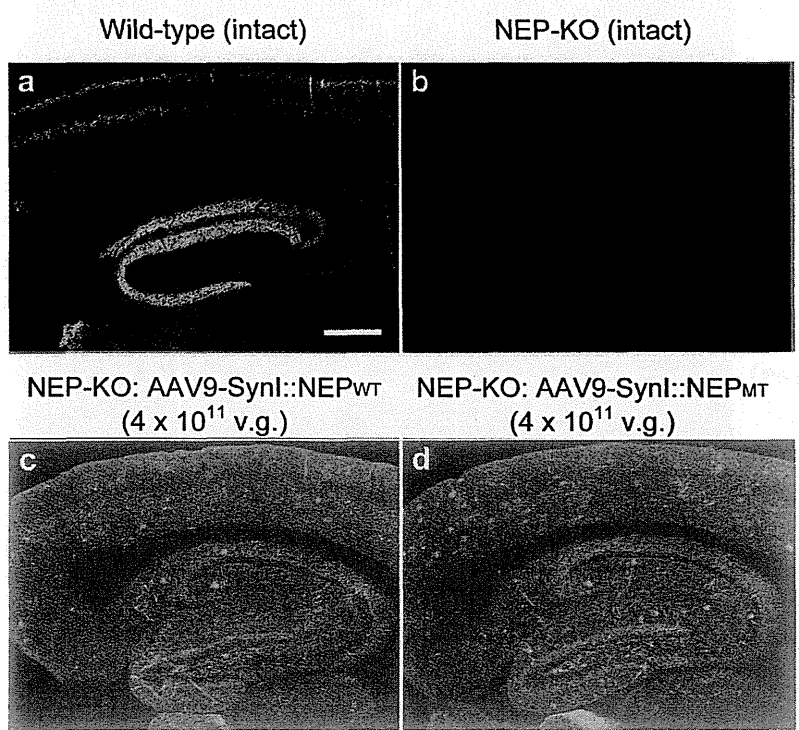

Figure 1 | Intracardiac injection of rAAV9 with SynI promoter leads to widespread gene transduction of neprilysin in the brain. Brain sections from intact wild-type mice (a), intact neprilysin-deficient mice (b), and neprilysin-deficient mice 14 days after intracardiac injection of 4×10^{11} genome vectors of rAAV9-SynI::NEP_{WT} (c) or rAAV9-SynI::NEP_{MT} (d). Scale bars, 200 μm.

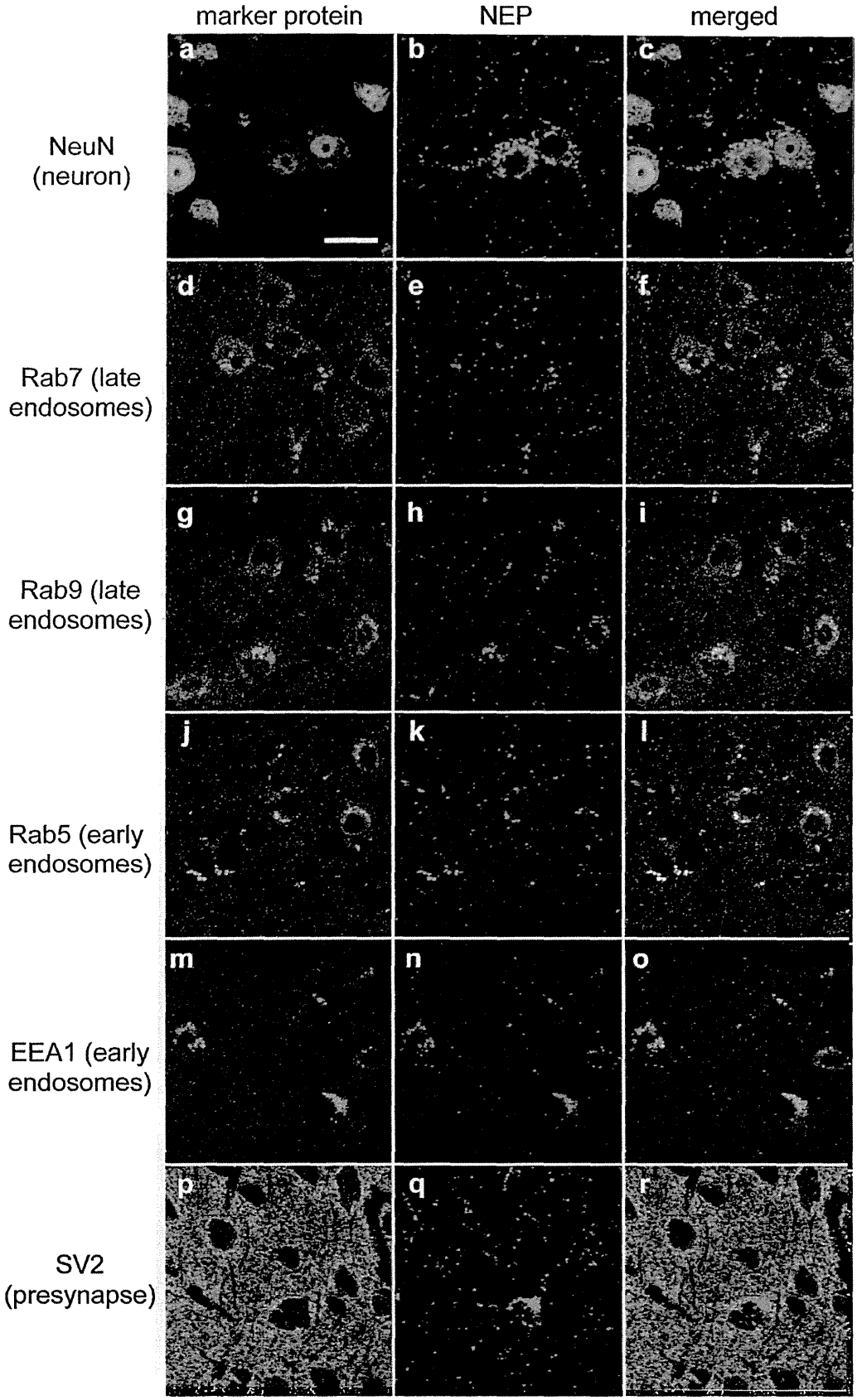

Figure 2 | Localization of the exogenous neprilysin in the brain. Brain sections from neprilysin-knockout mice 14 days after intracardiac injection of 4×10^{11} genome vectors of rAAV9-SynI::NEP_{WT}. Exogenous neprilysin was localized in NeuN-positive neurons (a–c), and was also observed in endosomes as confirmed by colocalization with Rab7 (d–f), Rab9 (g–i), Rab5 (j–l), EEA1 (m–o), and SV2 (p–r). Scale bars, 20 μm.

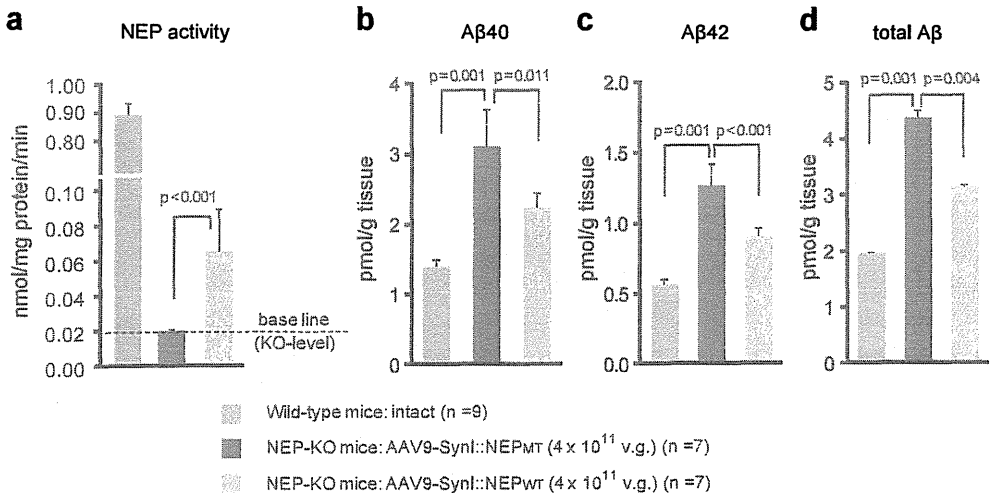

Figure 3 | Functional expression of NEP and Aβ levels in the limbic region after gene transfer. (a) Levels of neprilysin-dependent endopeptidase activity in the limbic regions of intact wild-type, rAAV9-SynI::NEP_{MT}- and rAAV-SynI::NEP_{WT}-injected NEP-KO mice 14 days after intracardiac injection. Data represent mean ± s.e.m. of 7–9 mice. (b–d) Levels of Aβ40, Aβ42 and total Aβs in the limbic regions after the gene transfer were determined by sandwich ELISA. A non-specific reaction of the ELISA system was excluded by subtracting values obtained in the brain tissue from APP-KO mice. Data represent mean ± s.e.m. of 7–9 mice.

Rescue of aged mutant APP tg mice from Aβ accumulation and subsequent impairment of memory and learning function. We next investigated the potential of intracardiac injection of rAAV9 vector to reverse the impaired memory and learning function in mutant APP tg mice (APP23)[11]. Since Aβ production in APP23 mice is about 10-fold higher than that of wild-type mice, a nearly 4-fold higher dose of vector was used than in the above-mentioned treatment of neprilysin-deficient mice. We examined reference memory as an indication of spatial memory and learning function, using a Morris water maze task. Under our experimental conditions, impairment of reference memory function of mutant APP tg mice was detected at the age of 15 months (Fig. 4a), and so we randomly divided mice of this age into two groups, which were given intracardiac administration of rAAV9-NEP_{MT} or rAAV-NEP_{WT}. Five months after the gene transfer, we re-examined their memory functions. The escape latency of rAAV9-NEP_{WT}-injected APP tg mice was significantly shorter than that of rAAV9-NEP_{MT}-injected mice ($^*p < 0.05$), in which the escape latency was not shortened at all and the learning and memory function remained impaired (Fig. 4b). The cognitive function of the rAAV9-NEP_{WT}-injected APP tg mice was restored nearly to the level of intact wild-type mice. In addition, it is reported that anxiety-like behaviors affect performance in spatial learning tasks[12], and we cannot rule out the possibility that the effect of neprilysin gene transfer involved, at least in part, alleviation of anxiety-like behaviors that might have been exacerbated by the amyloid burden.

Next, we assessed plaque deposition and glial activation in the brains of rAAV9-injected APP tg mice by positron emission tomography (PET) with radioligands for amyloid (Pittsburgh Compound-B [^{11}C]PIB) and 18-kDa translocator protein (TSPO) ([^{11}C]Ac5216), respectively (Fig. 4c,d)[13,14]. Plaque deposition in both the hippocampus and neocortex was clearly reduced in the rAAV9-NEP_{WT}-injected mice compared to rAAV9-NEP_{MT}-injected mice ($^*p < 0.05$). Mice treated with rAAV9-NEP_{WT} showed a tendency of enhanced TSPO upregulation, and the TSPO/amyloid burden ratio was significantly different between the two treatment groups ($^*p < 0.05$). This observation is attributable to reinforcement of TSPO-positive, neuroprotective astrogliosis surrounding Aβ plaques[15]. Thus, *in vivo* assessments supported the potential effectiveness of gene therapy by intracardiac administration of rAAV9-NEP_{WT} in a pathological animal model.

Therapeutic effects of NEP gene transfer on Aβ pathologies in the brain. After PET imaging analysis (i.e., 6 months after the gene transfer), functional expression of neprilysin was estimated by measurement of enzyme activity using a standard fluorescent substrate, and amyloid deposition was assessed by immunohistochemical staining using specific antibodies against either the unmodified amino-terminus of Aβ, N1D, or the modified amino-terminus of Aβ, N3pE[16]. Neprilysin-dependent endopeptidase activity in both the hippocampus and the neocortex maintained a 1.5-fold increase in the rAAV9-NEP_{WT}-injected mice compared to that in rAAV9-NEP_{MT}-injected mice ($^*p < 0.05$) (Fig. 5a), and both N1D- and N3pE-positive amyloid deposits were consistently and significantly decreased ($^*p < 0.05$) (Fig. 5b,c).

We further investigated membrane-associated Aβ oligomers, which were extracted with Triton X-100 from the membrane fraction, using western blotting (Fig. 5d,e). Membrane-associated Aβs were detected as oligomers, consisting mainly of trimers/tetramers, followed by dimer, and with only a trace of monomer. The Aβ trimers/tetramers, which were not detected from non-tg (wild-type) mouse brain, were significantly decreased by the rAAV9-NEP_{WT} administration (20% reduction; $^*p < 0.05$), compared to that in the rAAV9-NEP_{MT} group. According to the current hypothesis that Aβ oligomers are the primary molecules responsible for cognitive dysfunction, rather than Aβ fibrils[17–19], the reduction of Aβ oligomers following rAAV9-NEP_{WT} administration may contribute directly to the alleviation of abnormal spatial learning and memory function in aged mutant APP tg mice.

Discussion

Recombinant AAV vectors are among the most promising vehicles for gene delivery to the central nervous system. Stereotaxic infusion of AAV vector carrying neprilysin gene into the hippocampus has been shown to decrease Aβ in AD model mice[6,7]. However, when it was infused into the neocortex or hippocampal formation, expression of exogenous neprilysin and its effects on Aβ degradation were locally restricted[6,7]. Since the extent of the Aβ burden is broad in AD, a more efficient and widespread delivery technology is necessary. Among more than one hundred primate AAVs, AAV9 has gained much attention, showing high efficiency of gene transduction in neurons after intravenous administration in neonatal mice[20]. Here

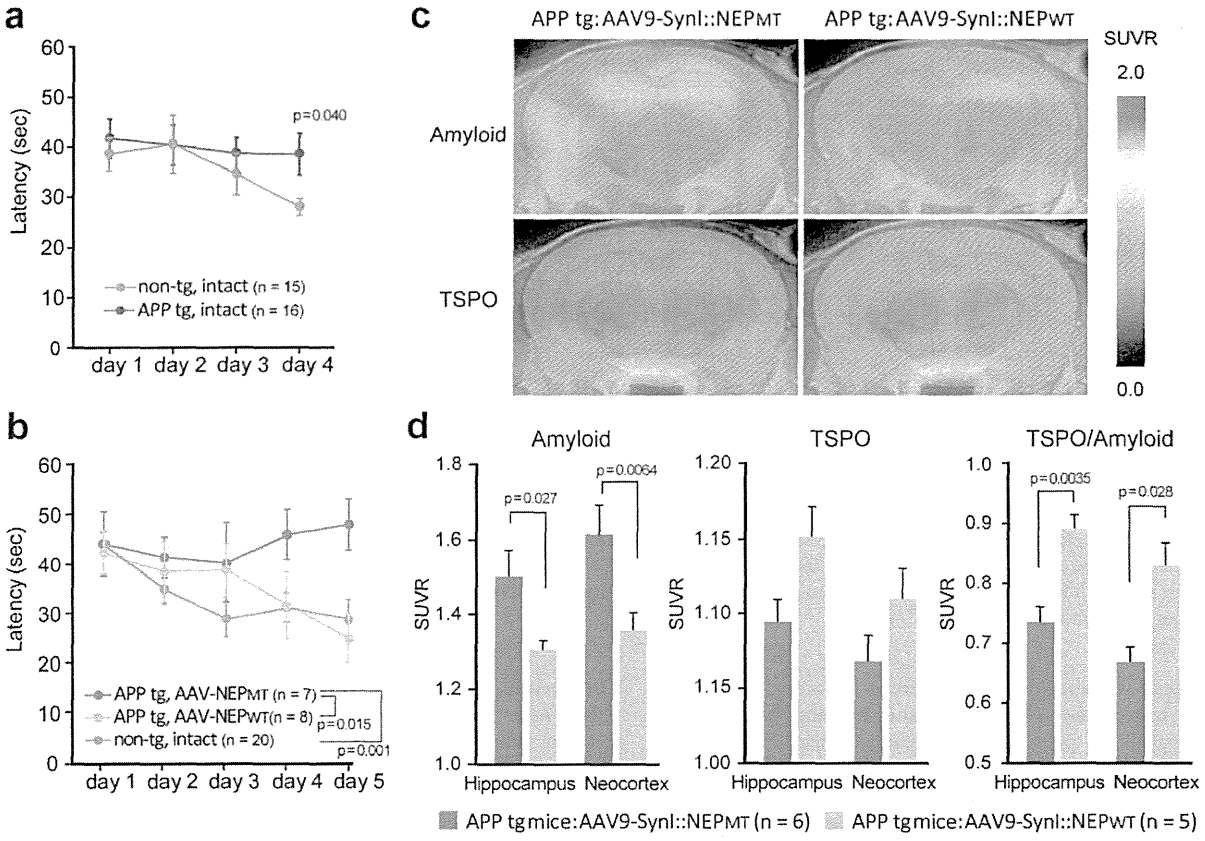

Figure 4 | NEP gene transfer ameliorated impaired spatial learning and memory function, amyloid burden, and modified glial activation in aged APP tg mice. Reference memory was examined using a water maze task. Escape latency in each block to the hidden platform during a 60-sec session was measured. (a) Impaired reference memory function of APP tg mice at the ages of 15 months was detected, and they were divided into two groups, followed by administration of AAV9-SynI::NEP_{MT} or AAV9-SynI::NEP_{WT} (1.5×10^{12} genome copies). Data represent mean ± s.e.m. (b) Five months after the gene transfer, their memory functions were re-examined. Two-way ANOVA showed a significant main effect of neprilysin gene transfer ($F_{(2,160)} = 6.287$; $p < 0.05$). *Post hoc* analysis revealed that the escape latency of rAAV9-SynI::NEP_{MT}-injected APP tg mice was significantly different from the other groups ($p < 0.05$). Data represent mean ± s.e.m. (c) Plaque deposition and glial activation in living brains of APP tg mice 5 months after injection of AAV9-SynI::NEP_{MT} and AAV9-SynI::NEP_{WT} assessed by PET with radioligands for amyloid ([^{11}C]PIB) (top) and TSPO ([^{11}C]Ac5216) (bottom). Amyloid and TSPO images are derived from the same individuals. Data represent mean ± s.e.m. (d) The levels were estimated as SUVRs. TSPO upregulation relative to amyloid abundance was also determined by calculating the quotient of the SUVRs for [^{11}C]Ac5216 and [^{11}C]PIB (right). The main effect of the treatment was significant on amyloid load ($F_{(1,12)} = 9.17$, $p < 0.05$) and TSPO-to-amyloid ratio ($F_{(1,10)} = 16.4$, $p < 0.01$) but insignificant on TSPO level ($F_{(1,10)} = 4.2$, $p > 0.05$) by repeated-measures 2-way ANOVA. The p values show significant differences between rAAV9-SynI::NEP_{WT} and rAAV-SynI::NEP_{WT}. Data represent mean ± s.e.m.

we showed that intracardiac administration of AAV9 can deliver neprilysin gene into broad areas of the adult mouse brain, and results in a marked and widespread reduction of Aβ levels. Although the mechanism by which AAV9 penetrates the blood-brain barrier (BBB) remains unknown, tyrosine mutation of the adeno-associated viral capsid protein may contribute to the enhanced expression levels of transgenes delivered by AAV[21].

It is noteworthy that a relatively small increase of neprilysin activity in the brain was sufficient to yield a significant reduction of Aβ, with subsequent alleviation of abnormal spatial learning and memory function. The exogenous neprilysin was abundantly present in late and early endosomes of neurons throughout the brain, including the neocortex and hippocampal formation. This localization appears to provide a rationale for the effective degradation of Aβ, as discussed below. It is considered that Aβ is generated in late endosomes, then is secreted from presynaptic terminals of neurons by neuronal activity-dependent exocytosis[22], and is temporally concentrated and may be oligomerized/aggregated. In addition, Walsh et al. reported that Aβ oligomerization occurred after generation of the peptide within specific intracellular vesicles including recycling endosomes[23], which could be modulated by Aβ *per se*[24], and the oligomers are subsequently secreted from the cell. This observation is supported by the fact that the mildly acidic environment (pH 5 ~ 6) in endosomes appears to promote Aβ oligomerization/aggregation[25]. After this event, Aβ or Aβ oligomers impair neuronal transmission via binding to *N*-methyl-D-aspartic acid (NMDA) or acetylcholine α7 nicotinic (α7nACh) receptors and prion protein[17–19]. Aβ/Aβ oligomers are diffused in the synaptic clefts after secretion, but they maintain a higher concentration in endosomal membrane, and may also be tethered in part at the plasma membrane. Although neprilysin is a neutral endopeptidase, its pH optimum is around 6.0[26], so neprilysin could degrade Aβ oligomers efficiently under the mildly acidic conditions in endosomes.

We succeeded in excluding membrane-associated Aβ oligomers from the brain by means of neprilysin gene transfer, ameliorating the impairment of spatial learning and memory function, although the contribution of a reduction in the total amount of amyloid deposition cannot be neglected. However, mounting evidence suggests that Aβ oligomers are highly neurotoxic[17–19] and may be more directly responsible for the pathological and symptomatic changes in AD,