である.

わが国で最も多いのが *PRNP* コドン 180 のバリン（V）からイソロイシン（I）への変異による遺伝性 CJD（V180I 変異遺伝性 CJD）で，本邦では gPrD 全体の 40％に認めている．平均発症年齢は 76.5 歳と高齢で，初発症状は記銘力障害以外に，失語や失行などの高次脳機能障害で発症する例が多く，緩徐に進行するため，MRI を撮影しないでアルツハイマー病（Alzheimer disease）と誤診されていることがある．全経過の平均は約 1.9 年であるが，無動性無言になってから数年にわたる場合もある．ほぼ全例が孤発性の発症様式であるため，sCJD との鑑別も重要である．脳波で PSD を認めるのはわずかに 10％程度であるが，ほぼ全例に脳 MRI の拡散強調画像で後頭葉と中心溝前後を除いたほぼ全域に大脳皮質のリボン状の高信号と基底核領域の高信号を認める（**2**-B）．髄液検査では 14-3-3 蛋白の陽性率は約 70％で，QUIC 法による異常プリオン蛋白の陽性率は約 40％と低い[9].

GSS で最も頻度が多いのが *PRNP* コドン 102 のプロリン（P）からロイシン（L）への変異による GSS（P102L 変異 GSS：GSS[102]）で，gPrD 全体の中では約 16％を占める．発症年齢は 40〜60 歳代で，平均約 53.7 歳である．特定の地域に偏って発症する傾向があることが知られている．浸透率は高く，約 85％に認知症の家族歴を認める．約 90％が小脳症状で発症し，歩行障害を主訴とする．その後に認知症を伴って両者が緩徐に進行する．平均罹病期間は 4.5 年で，末期には寝たきりから無動性無言となる．比較的急速に認知症が進行し，CJD 様の経過を呈する型が，同一家系内でも存在する．脳波上約 23％に周期性同期性放電（PSD）を認める．髄液検査では約 25％で 14-3-3 蛋白の上昇を認める．QUIC 法による異常プリオン蛋白陽性率は約 88％である[9].脳 MRI 拡散強調画像や FLAIR 画像では変化を認めることが少ない．

プリオン蛋白遺伝子コドン 200 のグルタミン（E）からリジン（K）への変異による遺伝性 CJD（E200K 変異 CJD）はわが国では 2 番目，欧米では最も頻度が高い．浸透率はほぼ 100％とされているが，本邦では家族歴が確認されている例は約半数である．発症平均年齢は 58.6 歳で，症状は上述の古典型 sCJD に類似している．特定の地域に多発していることが知られている．

その他，M232R 変異 CJD は古典型 sCJD と同様の臨床経過，検査所見を呈する急速進行例が多いが，急速進行型と緩徐進行型が存在することが知られており，緩徐進行型では脳波上 PSD が出現しない例がほとんどである．同一家系内の発症例は報告されていない[9].

獲得性（感染性）プリオン病

欧米で用いられている environmentally-acquired prion diseases の日本語訳として，獲得性プリオン病と呼ばれている．クールー，医原性 CJD，変異型 CJD（variant CJD：vCJD）の 3 種類に大別される．本邦では 2014 年 2 月の時点で，2004 年に報告された変異型 CJD 1 例以外はすべて硬膜移植による CJD（dura mater graft associated CJD：dCJD）である．

■クールー

パプアニューギニア（Papua New Guinea）の東部高地のオカパ（Okapa）地域のフォーレ（Fore）族（集落）のカニバリズム（食人）が原因で感染が蔓延したが，1959 年からカニバリズムの禁止が徹底されていった結果，1959 年以降に生まれた子どもからはクールーの発症は報告されていない．最近では，潜伏期間が最長 50 年ほどにもなることが指摘されている[10].

■医原性 CJD

医原性 CJD の感染経路としては，移植（硬膜移植，角膜移植），医療器具（脳外科手術器具，深部脳電極），血液（vCJD における輸血，ヒト下垂体製剤）などが報告されている．

硬膜移植による CJD（硬膜 CJD，dCJD）

脳外科手術時のヒト由来乾燥硬膜の移植により CJD が感染した例で，アルカリ処理をしていないドイツ製のヒト死体由来の乾燥硬膜（商品名 Lyodura®）を使用していたことが証明されている．これまでに本邦で調査された 144 例の

Lyodura®

ドイツのBブラウン社では, Lyodura® のガンマ線滅菌処理による製造を 1987 年に変更し, 水酸化ナトリウム処理を加え, 1996 年に製造中止となったが, 日本では厚生省（当時）が 1973 年に Lyodura® の輸入を承認したのち, 1997 年にヒト乾燥硬膜製品の使用を禁止するまで何の措置も取らなかった. その間に手術を受けた患者の中には Lyodura® が使用された例があったため, 1987 年以降に手術を受けた患者でも dCJD を発症している. 2013 年の Hamaguchi らの報告では, 全世界で報告されている 195 例の乾燥脳硬膜による CJD のうち驚くべきことに, 142 例が日本での発症例である[16]. プラーク型 dCJD は孤発性 CJD（sCJD）のうち, 主に VV2 型, あるいは MV2 型の症例から移植された硬膜が原因であることが実験的に確認されている[17].

うち使用硬膜が確認されている症例は, すべて Lyodura® である. 潜伏期間は 1〜30 年（平均 12 年）で, 発症年齢は 50 歳代が多く, sCJD と比べると若い. dCJD 患者では, 硬膜移植を受けた年が 1975〜1993 年までと, 幅広く確認されている. 初発症状は小脳性運動失調が多く, 眼球運動障害, 視覚異常の出現頻度が高い傾向がある. 2/3 の症例は古典型 sCJD と大差なく, PSD やミオクローヌスが出現し, 罹病期間は約 1.6 年である. 残り 1/3 は, プラーク型と呼ばれる病理組織変化を呈し, 緩徐進行性で発症 1 年後にも簡単な応答が可能である. プラーク型ではミオクローヌスや PSD はみられないか, みられても出現が遅い[11].

■変異型 CJD（vCJD）

BSE（bovine spongiform encephalopathy：ウシ海綿状脳症）罹患ウシ由来の食品の経口摂取によってウシからヒトに伝播したと考えられている. 1994 年からイギリスを中心に発生しており, 2014 年 7 月現在, 累積患者数は 226 人確認されている（http://www.cjd.ed.ac.uk/documents/worldfigs.pdf）. vCJD の全例でプリオン蛋白遺伝子コドン 129 多型は MM 型であるが, MV 型で潜伏感染が知られている（保因者）.

発症年齢は 12〜74 歳であるが, 平均 29 歳と若年である. 初期には抑うつ, 焦燥, 不安, 自閉, 無関心, 不眠, 強迫観念, 錯乱, 興奮, 異常な情動, 性格変化, 異常行動, 記憶障害などの精神症状が中心である. 進行すると認知症が徐々に顕著となり, 全例に失調症状を認め, 顔・四肢の痛み, 異常感覚, 感覚障害も高頻度に認められる. ミオクローヌスははっきりとておらず出現期間, 頻度ともに少ない. 経過緩徐進行性で罹病期間は平均 1.5 年である. 波では通常 PSD を認めず, 髄液検査では約数で 14-3-3 蛋白が陽性となる. 脳 MRI では散強調画像や FLAIR 画像で視床枕に高信号域が認められる（視床枕徴候：pulvinar sign）（C）. 視床内側も同時に高信号領域を呈するとがある（ホッケー杖徴候：hockey stick sign 大脳皮質のリボン状の高信号領域は認められい.

vCJD は輸血などの血液を介した感染の危性が指摘されている[12,13]. 発症前の vCJD 症が献血した血液を輸血した 67 人中 3 人が vC を発症し, 1 人が輸血から 5 年後に偶然腹部動脈瘤破裂で死亡した際に, 剖検で脾臓と頸リンパ節に異常プリオン蛋白が検出された.

■感染予防

プリオン病は発症後のみならず潜伏期間においても患者に対して使用した器具や, 患者か提供された臓器などを介して[14], 伝播する可能性が指摘されている[12]. プリオン病患者に使用した手術器具に対して, 現在推奨されている消毒・滅菌方法は, ①焼却可能な器具, 用はすべて焼却, ②器具に付着した血液・組織をできる限り取り除いた後, 3％ SDS 溶液に

3～4 分間 100℃煮沸し，手作業またはウォッシャーディスインフェクターによる洗浄後にプレバキューム方式のオートクレーブで 134℃ 10 分処置，③軟性内視鏡などの加圧・加熱処理ができない手術器具に関しては適切な洗浄剤による十分な洗浄後に過酸化水素低温ガスプラズマ滅菌による洗浄・不活化処理，④病理標本に関しては 90％蟻酸で 1 時間処理することとされている（http://prion.umin.jp/guideline/cjd_2008all.pdf）.

（三條伸夫，水澤英洋）

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Further reading

厚生労働科学研究費補助金難治性疾患克服研究事業「プリオン病及び遅発性ウイルス感染症に関する調査研究班」（編）．プリオン病と遅発性ウイルス感染症．東京：金原出版；2010.
厚労省研究班によるプリオン病および遅発性ウイルスに関する参考書

Nozaki I, et al. Prospective 10-year surveillance of human prion diseases in Japan. *Brain* 2010；133 (10)：3043-3057.
1999 年から開始された本邦のプリオン病に関するサーベイランスの 10 年間の総集編

橋本順（編）．特集／知っておきたい認知症の臨床と画像．臨床放射線 2010；55（10 月増刊号）.
プリオン病を含む各種認知症の画像上のポイントが掲載されている

厚生労働科学研究費補助金・難治性疾患克服研究事業「プリオン病及び遅発性ウイルス感染症に関する調査研究班」（編）．プリオン病感染予防ガイドライン（2008 年版）
http://prion.umin.jp/guideline/cjd_2008all.pdf
プリオン病に関する感染予防法マニュアルで，完全版と要約版がある

Original Article

Descriptive Epidemiology of Prion Disease in Japan: 1999–2012

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ABSTRACT

Background: Epidemiologic features of prion diseases in Japan, in particular morbidity and mortality, have not been clarified.

Methods: Since 1999, the Research Committee has been conducting surveillance of prion diseases, and the surveillance data were used to assess incident cases of prion diseases. For the observation of fatal cases, vital statistics were used.

Results: Both incidence and mortality rates of prion diseases increased during the 2000s in Japan. However, this increase was observed only in relatively old age groups.

Conclusions: The increased number of patients among old age groups might be due to increased recognition of the diseases. If so, the number of cases should plateau in the near future.

Key words: prion diseases; Creutzfeldt-Jakob syndrome; incidence; mortality; secular trends

INTRODUCTION

In 1996, when the paper indicating the relationship between bovine spongiform encephalopathy (BSE) and the human variant Creutzfeldt-Jakob disease (CJD) was published,[1] full-scale epidemiologic research for prion diseases, such as CJD, Gerstmann-Sträusslar-Scheinker disease (GSS), and fatal familial insomnia (FFI), started in Japan.[2] Since 1999, a nationwide surveillance system for prion diseases has been implemented, and patients with prion diseases have been registered.[3] The epidemiologic features of prion diseases in Japan are summarized as follows[2,3]: (1) the annual incidence rate is about 1 case per 1 million population, which is similar to the worldwide standard; (2) the incidence rate is highest among those aged in their 60s and 70s; and (3) CJD associated with cadaveric dura mater transplantation is more prevalent in Japan than in other countries.

Over the past decade, the number of patients with prion diseases has increased in Japan. While the reason for this increase is unclear, epidemiologists must consider that increased recognition of the disease may lead to a subsequent increase in the number of patients. In other words, whether prevalence has truly increased or whether some other factors have made the number merely seem increased should be clarified. Detailed observation of the epidemiologic features of prion diseases might shed light on this apparent increase in prevalence.

We conducted this descriptive epidemiologic research with two purposes: to clarify the recent epidemiologic features of prion diseases in Japan and to obtain some hints about the cause of increasing incidence of the diseases in Japan.

METHODS

We used two data sets in this study. One was the registry data of prion diseases in Japan obtained through the surveillance system conducted by the Surveillance Committee, which is financially supported by the Ministry of Health, Labour and Welfare of the Japanese government.[3] The system was first implemented in April 1999. There are several routes to obtain information about the existence of potential patients with prion diseases: a mandatory reporting system from

physicians, the public-aid-for-treatment system, and clinical examinations.

First, since 1999, prion diseases have been designated as reportable diseases by the Prevention of Infectious Diseases and Medical Care for Infectious Patients Act (Act No. 114 of 1998). When a physician diagnoses a patient as having a prion disease, he or she must report the fact to a local public health center.

Second, prion diseases are designated as diseases qualifying for public aid. Patients with one of the designated diseases receive treatment from a hospital with public aid, and he or she is not required to pay any fee. The aid is based on a claim made by the patient or his or her family to a public health center, so information on the patient is obtained when making the claim.

Lastly, the Surveillance Committee conducts human prion protaineous gene analyses at Tohoku University and cerebrospinal fluid analyses (14-3-3 protein and total tau protein) at Nagasaki University. Physicians who suspect that a patient has a prion disease or who want to certify the diagnosis of a prion disease may send blood or cerebrospinal fluid to these universities with a patient's informed consent. The cost is covered by research funds from the national government, and the patient and physician are not responsible for paying any fees.

When the Surveillance Committee obtains information about a potential patient through one of these three routes, one of the committee members, who is a neurologist or a psychiatrist familiar with prion diseases, obtains detailed data about the patient by meeting the patient if possible or using hospital records. Based on the obtained data, the Committee members discuss whether or not the patient has a prion disease, and patients recognized to have a prion disease are registered anonymously.

In the present study, we used only data of registered patients with disease onset from 1999 through 2012. We observed epidemiologic features of prion diseases in Japan calculated standardized morbidity rates by prefecture and secular trends of age-specific incidence rates. As shown in Figure 4, the 2012 data are still incomplete, so patients diagnosed in this year were excluded in observation of time and place.

We also used data on vital statistics in Japan from 1999 through 2012 (http://www.e-stat.go.jp/SG1/estat/GL08020101.do?_toGL08020101_&tstatCode=000001028897&requestSender=dsearch). Since 1999, the statistics presented the numbers of fatal cases with prion diseases (ICD-10th; A81.0 [Creutzfeldt-Jakob disease] + A81.8 [Other atypical virus infections of central nervous system]) by prefecture as an infectious disease as well as the fatal numbers by age and sex. Therefore, we calculated standardized mortality ratios by prefecture in addition to age-specific mortality rates in Japan. For calculating 95% confidence intervals (CIs) of standardized morbidity and mortality ratios, we used the table presented by Schoenberg.[4]

In addition to these analyses, standardized morbidity and mortality ratios—the former of which were based on the surveillance data and the latter of which were based on the vital statistics—were compared with the number of neurologists authorized by the Societas Neurologica Japonoca (http://www.kktcs.co.jp/jsn-senmon/secure/senmon.aspx) per population by prefecture.

The age-specific population for incidence and mortality rates by calendar year was that used in vital statistics, while that for calculation of standardized morbidity and mortality ratios by prefecture was the census population in 2005.

RESULTS

There were 2026 incident cases of prion diseases (854 males and 1172 females) from 1999 through 2012 and 2334 fatal cases (1035 males and 1299 females) from 1999 through 2012 according to vital statistics in Japan. The average annual incidence rate was 1.09 cases per 1 million population (0.95 for males and 1.22 for females, calculated from 1999 through 2011) and average annual mortality rates were 1.32 per 1 million population (1.20 for males and 1.44 for females).

The results below are described according to the three major characteristic headings of descriptive epidemiology: persons, place, and time.[5]

Persons

Table 1 shows the characteristics of 2026 incident patients with prion diseases in Japan during the 14 years from 1999 through 2012. The number of patients by age class was largest in the group aged 70–79 years, followed by those aged 60–69 years. Of the 2026 patients with prion diseases, 77% was sporadic CJD patients.

Place

The standardized morbidity and mortality ratios for each prefecture are shown in Table 2. The standardized morbidity ratios ranged from 0.28 (Shiga Prefecture) to 2.15 (Saga Prefecture), while the ratios for mortality ranged from 0.24 (Tottori Prefecture) to 1.90 (Yamanashi Prefecture). As shown in Figures 1 and 2, no geographical clustering of prevalent prefectures was observed. The correlation coefficient between the morbidity and mortality ratios was 0.53 (95% CI 0.29 to 0.71; Figure 3). We assessed the relationship between the morbidity and mortality of prion diseases and the number of neurologists per population because there is a possibility that shortage of neurological medical services introduces misdiagnosis of prion diseases. However, the correlation coefficient between the standardized morbidity ratio and the number of neurologists was −0.12 (95% CI −0.39 to 0.17), while that for the standardized mortality ratio was −0.09 (95% CI −0.37 to 0.20). The coefficients for the relationship between standardized morbidity and mortality ratios and the number of neurologists per population aged ≥65 years were

Table 1. Demographic characteristics of patients with prion diseases in Japan, 1999–2012

	Whole patients	Sporadic CJD[a]	Variant CJD	CJD with dura mater transplantaion	Familiar prion diseases			Unclassified CJD[c]
					Familiar CJD[b]	GGS	FFI	
Total sample	2026 (100)	1550 (77)	1	83 (4)	298 (15)	84 (4)	4	6
Sex								
Male	854 (42)	636 (41)	1	35 (42)	136 (46)	41 (49)	3	2
Female	1172 (58)	914 (59)		48 (58)	162 (54)	43 (51)	1	4
Total	2026 (100)	1550 (100)	1	83 (100)	298 (100)	84 (100)	4	6
Age at onset (years)								
10–19	3			2 (2)	1 (0)			
20–29	8 (0)			5 (6)	1 (0)	2 (2)		
30–39	29 (1)	12 (1)		7 (8)	1 (0)	9 (11)		
40–49	69 (3)	40 (3)	1	5 (6)	10 (3)	11 (13)	1	1
50–59	304 (15)	212 (14)		20 (24)	30 (10)	40 (48)	2	
60–69	613 (30)	498 (32)		25 (30)	70 (23)	18 (21)	1	1
70–79	738 (36)	590 (38)		17 (20)	123 (41)	4 (5)		4
80–89	245 (12)	186 (12)		2 (2)	57 (19)			
90–99	14	9			5			
Unknown	3	3						
Total	2026 (100)	1550 (100)	1	83 (100)	298 (100)	84 (100)	4	6
Mean	67.9	68.7		57.9	70.7	53.8	54.5	
SD	11.1	9.8		16.2	11.4	10.7	6.4	
Oldest age	94	94		85	93	75	61	
Youngest age	15	30		15	15	22	46	

CJD, Creutzfeldt-Jakob disease; FFI, fatal familial insomnia; GSS, Gerstmann-Sträusler-Scheinker disease; SD, standard deviation.
Percentages are shown in parentheses. Percentages may not add up to exactly 100% because of rounding.
[a]Including CJD without prion protein gene analyses.
[b]Including patients without prion protein gene variation but with family histories of CJD.
[c]Those whose diagnosis has been confirmed as CJD, but whose type of CJD has not been surveyed.

−0.17 (95% CI: −0.44 to 0.12) and −0.00 (95% CI: −0.29 to 0.29), respectively.

Time

Figure 4 shows the annual incidence and mortality cases by year. Because the cases of prion diseases in recent years have not all been discussed by the Committee yet, the numbers of new cases in recent years (specifically 2012) were small. Figures 5 and 6 show the age-specific incidence and mortality rates of prion diseases by year. Although the rates increased among older subjects, those among younger subjects did not increase.

DISCUSSION

In the present study, we described the epidemiologic features of prion diseases in Japan using two data sources—the prion disease surveillance system and vital statistics—from three descriptive epidemiologic viewpoints: persons, place, and time.

The prion disease surveillance system in Japan intends to collect information when a person receives a prion disease. The vital statistics comprise the data when the patient dies. According to the natural history of the disease, which is that patients with prion diseases die within a few years of disease onset, the numbers of incident patients and deceased ones

should be similar, although they were not identical because of the time lag between onset and death. We recognized 2026 incident cases and 2334 fatal cases in this study for the observed 14 years. There are several possible explanations for the difference between the two figures (308 cases). First, as mentioned before, a time lag exists between onset and death. Second, the surveillance data were incomplete for recent calendar years (Figure 4). In addition, discrepancies in diagnoses may exist between the two databases. Cases in the surveillance system should be true prion diseases because their diagnoses were based on the discussion of the Surveillance Committee, which consists of neurologists, psychiatrists, and neuropathologists and uses clinical findings and medical records gathered by the committee members, gene analyses, and pathological findings including results of western blot analyses. On the other hand, the vital statistic data consists of death certificate data for which the recorded cause underlying death was a prion disease. Because all physicians are able to create death certificates, some of these might be cases described by physicians who were not experts in prion diseases. These points might be the reasons for the gap between the numbers of incident and fatal cases. Nonetheless, we consider the validity of the two datasets used in this study to be quite high and believe that our findings accurately reflect the true epidemiologic features of prion diseases in Japan.

Table 2. Morbidity, mortality, and other variables concerning prion diseases in Japan, by prefecture

	Population[a] (thousands)	Number of newly diagnosed patients	Number of newly diagnosed patients (1999–2011)[b]	Crude incidence rate (per million population/year)	Standardized morbidity ratio (95% CI)	Number of fatal patients (1999–2012)	Crude mortality rate (per million population/year)	Standardized mortality ratio (95% CI)
Total	127768	2026	1814	1.09		2334	1.30	
Hokkaido	5628	138	124	1.69	1.47 (1.23–1.76)	106	1.35	0.97 (0.80–1.18)
Aomori	1437	19	16	0.86	0.72 (0.41–1.16)	20	0.99	0.69 (0.42–1.07)
Iwate	1385	19	19	1.06	0.85 (0.51–1.35)	22	1.13	0.75 (0.47–1.14)
Miyagi	2360	44	43	1.40	1.31 (0.95–1.77)	43	1.30	1.01 (0.73–1.37)
Akita	1146	28	28	1.88	1.40 (0.93–2.03)	32	2.00	1.22 (0.84–1.72)
Yamagata	1216	20	20	1.26	1.00 (0.61–1.54)	18	1.06	0.69 (0.41–1.09)
Fukushima	2091	38	35	1.29	1.10 (0.77–1.53)	36	1.23	0.87 (0.61–1.20)
Ibaraki	2975	18	15	0.39	0.36 (0.20–0.59)	35	0.84	0.66 (0.46–0.91)
Tochigi	2017	16	10	0.38	0.36 (0.17–0.66)	40	1.42	1.11 (0.79–1.51)
Gunma	2024	41	39	1.48	1.33 (0.95–1.82)	36	1.27	0.95 (0.67–1.32)
Saitama	7054	67	60	0.65	0.66 (0.50–0.85)	119	1.20	1.04 (0.86–1.24)
Chiba	6056	97	85	1.08	1.05 (0.84–1.30)	100	1.18	0.97 (0.79–1.19)
Tokyo	12577	166	149	0.91	0.90 (0.76–1.06)	228	1.29	1.08 (0.94–1.23)
Kanagawa	8792	154	134	1.17	1.19 (1.00–1.42)	153	1.24	1.07 (0.91–1.26)
Niigata	2431	51	49	1.55	1.28 (0.94–1.68)	53	1.56	1.06 (0.79–1.39)
Toyama	1112	12	10	0.69	0.57 (0.27–1.05)	18	1.16	0.79 (0.47–1.25)
Ishikawa	1174	38	35	2.29	2.05 (1.43–2.84)	22	1.34	1.00 (0.63–1.51)
Fukui	822	7	6	0.56	0.48 (0.18–1.05)	7	0.61	0.43 (0.17–0.89)
Yamanashi	885	26	23	2.00	1.77 (1.12–2.65)	32	2.58	1.90 (1.30–2.68)
Nagano	2196	21	19	0.67	0.55 (0.33–0.86)	47	1.53	1.04 (0.77–1.39)
Gifu	2107	38	32	1.17	1.04 (0.71–1.46)	38	1.29	0.95 (0.68–1.31)
Shizuoka	3792	71	67	1.36	1.22 (0.95–1.55)	86	1.62	1.21 (0.97–1.51)
Aichi	7255	104	94	1.00	1.01 (0.82–1.23)	121	1.19	1.02 (0.85–1.22)
Mie	1867	35	34	1.40	1.23 (0.85–1.72)	41	1.57	1.15 (0.82–1.56)
Shiga	1380	6	5	0.28	0.28 (0.09–0.65)	24	1.24	1.04 (0.67–1.55)
Kyoto	2648	16	14	0.41	0.37 (0.20–0.63)	40	1.08	0.83 (0.59–1.13)
Osaka	8817	109	101	0.88	0.84 (0.68–1.02)	145	1.17	0.94 (0.80–1.11)
Hyogo	5591	61	54	0.74	0.68 (0.51–0.89)	112	1.43	1.10 (0.91–1.33)
Nara	1421	8	7	0.38	0.34 (0.14–0.70)	22	1.11	0.84 (0.53–1.27)
Wakayama	1036	6	6	0.45	0.36 (0.13–0.78)	14	0.97	0.65 (0.35–1.09)
Tottori	607	6	4	0.51	0.42 (0.11–1.07)	3	0.35	0.24 (0.05–0.70)
Shimane	742	23	21	2.18	1.66 (1.02–2.53)	28	2.69	1.68 (1.12–2.43)
Okayama	1957	32	30	1.18	1.01 (0.68–1.45)	28	1.02	0.73 (0.49–1.06)
Hiroshima	2877	46	41	1.10	0.98 (0.70–1.33)	51	1.27	0.95 (0.71–1.24)
Yamaguchi	1493	35	32	1.65	1.29 (0.88–1.82)	42	2.01	1.30 (0.94–1.76)
Tokushima	810	16	13	1.23	1.00 (0.53–1.70)	9	0.79	0.53 (0.26–1.01)
Kagawa	1012	16	14	1.06	0.89 (0.48–1.49)	20	1.41	0.98 (0.60–1.51)
Ehime	1468	26	25	1.31	1.07 (0.69–1.58)	28	1.36	0.92 (0.61–1.33)
Kochi	796	28	28	2.70	2.10 (1.39–3.04)	23	2.06	1.32 (0.83–1.97)
Fukuoka	5050	120	111	1.69	1.57 (1.30–1.90)	93	1.32	1.03 (0.83–1.26)
Saga	866	29	28	2.49	2.15 (1.43–3.12)	19	1.57	1.12 (0.68–1.75)
Nagasaki	1479	27	25	1.30	1.08 (0.70–1.60)	34	1.64	1.13 (0.78–1.59)
Kumamoto	1842	20	17	0.71	0.60 (0.35–0.95)	42	1.63	1.13 (0.81–1.52)
Oita	1210	27	26	1.65	1.34 (0.87–1.96)	26	1.54	1.02 (0.67–1.51)
Miyazaki	1153	14	13	0.87	0.72 (0.38–1.23)	22	1.36	0.94 (0.59–1.42)
Kagoshima	1753	32	29	1.27	1.04 (0.70–1.50)	39	1.59	1.07 (0.76–1.46)
Okinawa	1362	25	24	1.36	1.55 (1.00–2.32)	16	0.84	0.81 (0.47–1.32)
Unknown		30				1		

CI, confidence interval.
[a]Data from 2005 Census.
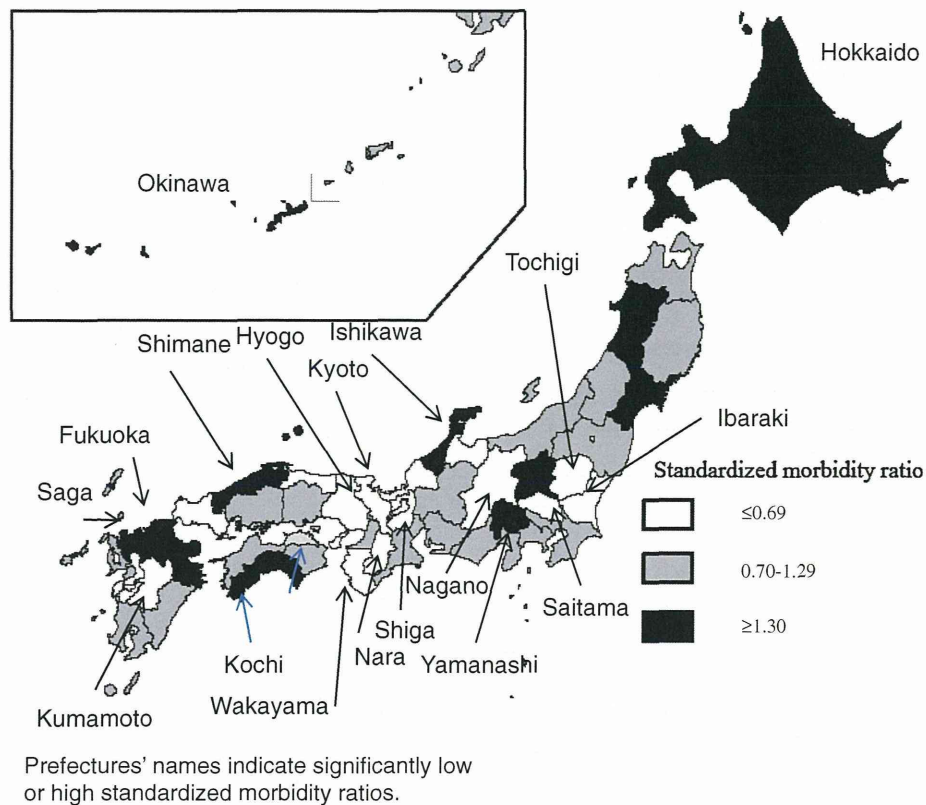[b]Patients whose address was unknown were excluded.

Figure 1. Standardized morbidity ratios of prion diseases in Japan by prefecture, 1999–2011
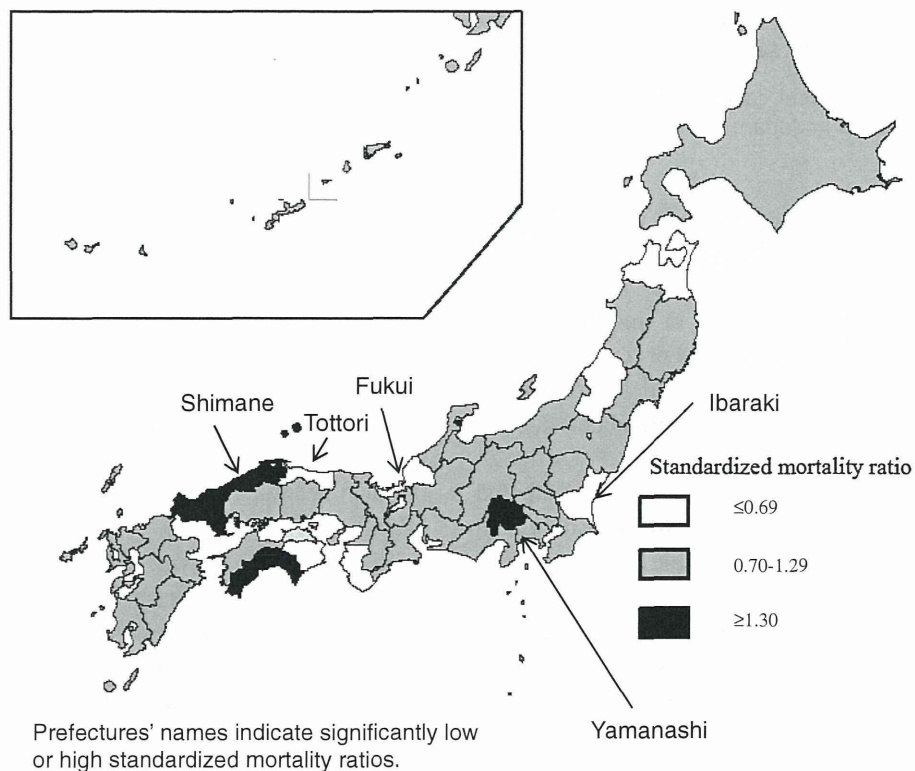
Figure 2. Standardized mortality ratios of prion diseases in Japan by prefecture, 1999–2012

Different epidemiologic aspects from those of prion diseases in European countries and North America[6,7] were observed in Japan in the present study. The proportion of patients with acquired CJD among all prion disease patients was high. Many of them developed CJD following cadaveric dura mater transplantation.[8–12] Currently, we have data of 147 such cases, and detailed epidemiologic features will be presented in another article. On the other hand, we observed
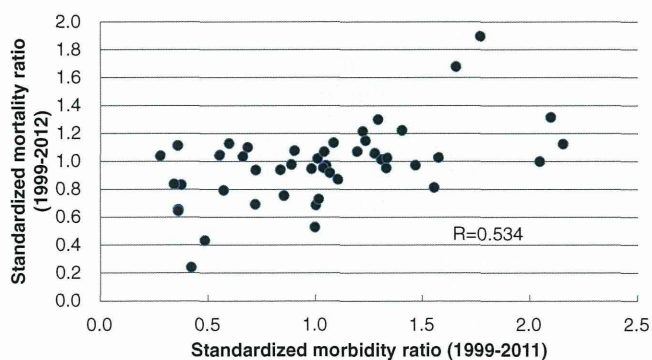
Figure 3. Relationship between standardized morbidity ratios and standardized mortality ratios of prion diseases by prefecture
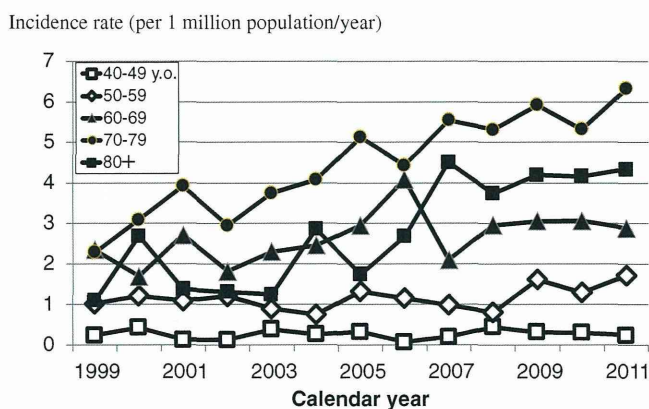
Figure 5. Annual incidence rates of prion diseases in Japan by age
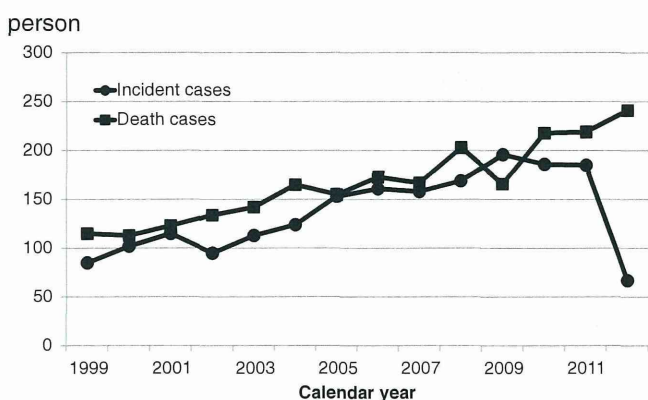
Figure 4. Numbers of incident cases of and deaths from prion diseases in Japan, 1999–2012
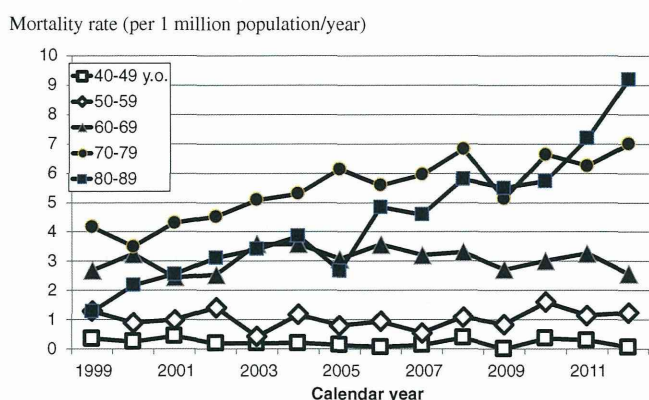
Figure 6. Annual mortality rates of prion diseases in Japan by age

only a single case of acquired prion diseases other than dura-related CJD, which was a case of variant CJD in 2005.[13,14]

Although there was no geographical clustering of prefectures with high incidence rates or mortality rates, some prefectures presented high rates. For example, both incidence and mortality rates were high in Yamanashi prefecture. There have been several articles about familial clustering of prion diseases in this prefecture.[15–19] Of the 23 patients reported in Yamanashi, 57% (13 cases) had familial CJD, although only 15% of prion disease patients had familial CJD in Japan as a whole, as shown in Table 1.

The numbers of incident and fatal patients increased in Japan during the last decade, as shown in Figure 4, although no chronological changes in the number of patients with prion diseases were observed in European countries.[20] The increase in number of fatal cases was reflected in the increase in number of incident cases in our study. As shown in Figures 5 and 6, the increases in numbers of incident and fatal cases were also reflected in the increased number of cases among the elderly. This phenomenon might be due to the substantial network of gene and spinal fluid analytic systems in Japan.

The Surveillance Committee has publicized the system not only to neurologists but also to general physicians. A patient with rapidly progressive dementia dying before diagnosis might be diagnosed with a prion disease through these gene and/or cerebrospinal fluid analyses. If a physician uses these systems, the Surveillance Committee is automatically notified of the existence of a potential patient, starts getting information about the patient, and discusses whether or not the patient has a prion disease. Further dissemination of information about the analytic system might increase the rate of identification of such patients, particularly among older patients.

The number of gene analyses conducted at Tohoku University increased from 132 cases in 1999 to 273 in 2012. Given that relatively young patients (ie, in their 40s or 50s) with rapidly progressing dementia are rare, such patients are typically referred to specialists in dementia (including prion diseases), so any issues with recognition have been negligible. If knowledge of the gene and spinal fluid analyses system is propagated to all physicians in this country, the issue of recognition should be diminished even further, and the chronological increase of the incidence rate should plateau. However, despite this expected plateau in the near future, the number of patients may still increase because of the growing number of old people in Japan.

Selection bias and information bias may have affected this study. The Surveillance Committee has made an effort to obtain information for all patients with prion diseases in Japan, but the database is not complete. Similar selection bias may be present in the vital statistics data as well; those with prion diseases whose deaths were attributed to conditions other than prion diseases would not be counted as prion disease deaths. Information bias may also exist on the vital statistics data; we were unable to clarify the validity of the diagnosis on death certificates, whereas diagnoses obtained through the surveillance system were validated by the Committee members, including neurologists and neuropathologists.

In conclusion, we showed here the epidemiologic features of prion diseases in Japan. Increased recognition of prion diseases may account for the observation of chronological increases in the number of patients with prion diseases, and the increasing trend of numbers of patients might soon plateau.

ONLINE ONLY MATERIAL ——————

Abstract in Japanese.

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Relationship and factor structure in multisystem neurodegeneration in Parkinson's disease

Objectives – Parkinson's disease (PD) is a multisystem neurodegenerative disease. We aimed to identify the relationship and factor structure among its different features. *Materials & methods* – Motor, olfactory and cognitive function, and cardiac sympathetic denervation were evaluated in 125 patients with PD using the Unified Parkinson's Disease Rating Scale (UPDRS) part III score, odor stick identification test for the Japanese (OSIT-J), Mini-Mental State Examination (MMSE), and [123I] meta-iodobenzylguanidine (MIBG) cardiac scintigraphy (heart-to-mediastinum (H/M) ratio). Pearson's correlation and multiple regression analysis were used to evaluate the association among the four measures with age, gender, and disease duration as the covariates. Exploratory factor analysis was used to identify the underlying factor structure among the measures and covariates. *Results* – Pearson's correlation and multiple regression analysis showed correlations between OSIT-J score and MIBG H/M ratio, OSIT-J and MMSE scores, UPDRS part III score and MIBG H/M ratio, UPDRS part III score and disease duration, and MMSE score and age. Factor analysis identified three factors: (i) age and MMSE score; (ii) MIBG H/M ratio and OSIT-J score; and (iii) UPDRS part III score and disease duration. *Conclusions* – Our results suggest that aging, PD-related pathogenesis, and disease duration underlie the multisystem neurodegeneration present in PD. Moreover, age and disease duration are the major risk factors for cognitive impairment and motor symptoms, respectively. Olfactory impairment and cardiac sympathetic denervation are strongly associated in PD.

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Introduction

PD is a multisystem neurodegenerative disease. The presence of Lewy bodies is the pathological hallmark of PD; they progressively accumulate in neurons and neurites of multiple neurotransmitter systems (1, 2). Although some studies reported a relationship among different features of patients with PD (3–9), there are no previous studies which systematically explored such relationships.

We focused on motor, cognitive, and olfactory functions, and cardiac sympathetic denervation in patients with PD. As neurodegeneration is a multifactorial process, confounding factors should be considered as covariates to assess relationships. PD commonly develops in an older population and age is known to be a risk factor (10, 11). Male gender is also a risk factor for developing PD (10, 11). PD is a neurodegenerative disorder and its features progress over time. Thus, we considered age, gender and disease duration as covariates.

We hypothesized that some of the clinical and laboratory measures and covariates are correlated, reflecting the underlying pathophysiology. We used Pearson's correlation and multiple regression analysis to prove the relationships. We also used exploratory factor analysis, which

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is based on the assumption that there are unobserved latent variables accounting for the correlations among observed variables, to identify the underlying factor structure in the relationships.

This study aimed to identify the relationships and factor structure in multisystem neurodegeneration in PD by using clinical and laboratory measures and covariates for a large cohort of patients.

Materials and methods

Patients

We performed a retrospective chart review for 176 patients with PD who visited Kanto Central Hospital, a community hospital in Tokyo, Japan from 2008 to 2012. All examinations were performed for clinical purposes. All data were extracted from the hospital database.

Inclusion criteria were subjects who had an established diagnosis of PD according to the clinical diagnostic criteria of the United Kingdom Parkinson's Disease Society Brain Bank (12) and responded symptomatically to dopaminergic therapy.

Exclusion criteria were subjects with moderate or severe dementia, which was defined as having a Clinical Dementia Rating (CDR) ≥ 2 because they could not cooperate appropriately with the testing. Subjects who had severe diabetes mellitus or a past medical history of ischemic heart disease and/or thoracic surgery were also excluded. Subjects who were not evaluated for motor, olfactory, and cognitive function and cardiac sympathetic denervation within a 6-month interval were also ineligible.

Patients were evaluated with general and neurological examinations and the Mini-Mental State Examination (MMSE). CDR was scored with a semi-structured interview for patients and caregivers by neurologists (T.H., S.O., and R.A.). Olfactory function and cardiac sympathetic denervation were assessed using the odor stick identification test for the Japanese (OSIT-J) and ^{123}I-MIBG cardiac scintigraphy, respectively. Levodopa equivalent dose (LED) was calculated according to a previous study (13). Patients were classified by Hoehn-Yahr (HY) stage, and evaluated using the Unified Parkinson's Disease Rating Scale (UPDRS) by neurologists.

Based on the inclusion and exclusion criteria, 125 patients were included in the analysis, and 51 patients were excluded because their data were not evaluated within a 6-month interval.

This study was approved by the Institutional Review Board of Kanto Central Hospital, Japan.

As this is a retrospective observational cohort study and extracted data were de-identified, written informed consent was waived.

Olfactory assessment

Olfactory function was assessed by OSIT-J (Daiichi Yakuhin Sangyo Co., Ltd., Tokyo, Japan). OSIT-J was described in detail in a previous study (4). In brief, we tested 12 different odorants familiar to the Japanese population: India ink, wood, perfume, menthol, Japanese orange, curry, gas for cooking, rose, Japanese cypress (hinoki), condensed milk, socks smelling of sweat, and roasted garlic. A subject was given six alternatives: four odor names, including one correct name, "not detected," and "unknown." The total number of correct answers for the 12 odorants constituted the OSIT-J score.

^{123}I-MIBG cardiac scintigraphy

Subjects were given an intravenous injection of 111 MBq ^{123}I-MIBG (FUJIFILM RI Pharma Co., Ltd., Tokyo, Japan). Chest images were obtained after 15 min for the early phase, and 3 h for the late phase, using a gamma camera (e.cam Signature, SIEMENS, Munich and Berlin, Germany). The relative organ uptake of ^{123}I-MIBG was determined by region-of-interest (ROI) analysis in the anterior planar view. The ratio of the average pixel count in the heart (H) to that in the mediastinum (M) (H/M ratio), and the washout ratio were calculated. The MIBG late phase H/M ratio (MIBG H/M ratio) was used as a marker of cardiac sympathetic denervation.

Statistical analysis

The assumption of normality was evaluated based on the residuals using the Shapiro–Wilk test. Box–Cox transformation was applied to the variables which did not meet the assumption. Thus, an inverse transformation was used for the MIBG H/M ratio and square-root transformation for the UPDRS part III score.

In the simple linear correlation analysis, Pearson's correlation coefficient determined the linear correlation between two continuous variables. Point biserial correlation coefficient determined associations between gender (categorical variable) and the other (continuous) variables.

In the multiple linear regression analysis, stepwise regression analysis was applied to explore the association among the seven clinical and laboratory measures and covariates. Here, MMSE,

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UPDRS part III, OSIT-J score, and MIBG H/M ratio were treated as dependent and independent variables, while age, gender, and disease duration were the covariate variables. The significance level of 0.1 was used for model selection. Multi-co-linearity among independent variables was evaluated using the variance inflation factor (VIF). $P < 0.05$ was reported as statistically significant.

To identify the underlying factor structure, exploratory factor analysis was applied for the six clinical and laboratory measures and covariates. Principal component analysis was used to extract factors, followed by Varimax rotation and Kaiser Normalization. The number of factors was determined by interpretability. The absolute factor loading value of ≥0.60 was defined as a variable's large contribution to a factor. Absolute loading value <0.45, but ≥0.25 was defined as the intermediate contribution.

Statistical analysis was performed with the Scientific Package for Social Sciences version 20 (SPSS 20) (Armonk, NY, USA) and Statistical Analysis Software (SAS) (Cary, NC, USA).

Results

Patients' clinical and laboratory data are described in Table 1.

Pearson's correlation coefficients between measures and covariates are shown in Table 2. Gender was associated with OSIT-J score (mean 4.2 for men and 5.4 for women) and MMSE score (mean 25.9 for men and 27.4 for women).

Table 1 Demographic and clinical data of 125 patients with Parkinson's disease

Characteristics	Mean	SD
Age	72.9	8.4
Gender: M/F	57/68	–
Disease duration (months)	49.3	44.3
Hoehn-Yahr stage	1.8	0.4
Levodopa equivalent dose	183.5	256.2
MMSE score	26.7	2.9
OSIT-J score [8.2 (2.4)]	4.8	2.8
MIBG early phase H/M ratio [2.32 (0.33)]	1.81	0.43
MIBG late phase H/M ratio [2.35 (0.36)]	1.61	0.47
MIBG washout rate (%) [18.4 (9.0)]	29.0	8.9
UPDRS part I score	2.0	1.7
UPDRS part II score	10.0	5.4
UPDRS part III score	22.0	11.9
UPDRS part IV score	1.6	2.0

SD, standard deviation; UPDRS, Unified Parkinson's Disease Rating Scale; MMSE, Mini-Mental State Examination; OSIT-J, the odor stick identification test for the Japanese; MIBG, [123I] meta-iodobenzylguanidine; H/M, heart/mediastinum.
Normal range of OSIT-J score, MIBG H/M ratio and MIBG washout rate are described by [mean (SD)].

Table 2 Pearson's (or point biserial) correlation coefficients

	OSIT-J score	MIBG late phase H/M ratio[a]	MMSE score	UPDRS part III score[b]
Pearson's correlation coefficient				
OSIT-J score	–	0.407***	0.336***	−0.300***
MIBG late phase H/M ratio[a]	0.407***	–	NS	−0.334***
MMSE score	0.336***	NS	–	−0.279**
UPDRS part III score[b]	−0.300***	−0.334***	−0.279**	–
Age	−0.259**	NS	−0.432***	0.336***
Disease duration	NS	−0.263**	NS	0.361***
Point biserial correlation coefficient				
Gender	−0.223*	NS	−0.264**	NS

n, number; OSIT-J, the odor stick identification test for the Japanese; MIBG, [123I] meta-iodobenzylguanidine; H/M, heart/mediastinum; MMSE, Mini-Mental State Examination; UPDRS, Unified Parkinson's Disease Rating Scale.
[a]Inverse transformed.
[b]Square-root transformed.
NS: $P > 0.05$.
*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$.

The results of multiple regression analyses are summarized in Table 3. All variables included in the final models had VIF <2. Scatter plots for clinical and laboratory measures and covariates which were correlated in the multiple regression analysis are shown in Fig. 1.

Factor analysis was applied for the six clinical and laboratory measures and covariates, OSIT-J, MMSE, UPDRS part III score, MIBG H/M ratio, age, and disease duration. For these variables, Kaiser's MSA (measures of sampling adequacy) values were >0.62 (>0.5 is acceptable for factor analysis). The factor loadings are listed in Table 4. Factor analysis extracted three factors, which accounted for 62.6% of the total variance, from the six variables. For factor 1, MMSE score and age had high loadings while the OSIT-J score and UPDRS part III score had intermediate loadings. For factor 2, the MIBG H/M ratio and OSIT-J score had high loadings while UPDRS part III score had intermediate loading. For factor 3, UPDRS part III score and disease duration had high loadings while the MIBG H/M ratio had intermediate loading.

Discussion

To our knowledge, this is the first study to identify multiple relationships among motor, olfactory, and cognitive function and cardiac sympathetic denervation using Pearson's correlation and multiple regression analyses. We also identified three underlying factors in the relationships using factor analysis.

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Table 3 Multiple regression analysis

Independent variable	Dependent variable			
	OSIT-J score	MIBG late phase H/M ratio[a]	MMSE score	UPDRS part III score[b]
	Squared type II semipartial correlation			
OSIT-J score		0.103***	0.035*	–
MIBG late phase H/M ratio[a]	0.142***		–	0.061**
MMSE score	0.089***	–		–
UPDRS part III score[b]	–	0.050**	–	
Age	–	–	0.126***	0.085***
Gender	–	–	0.036*	–
Disease duration	–	–	–	0.060**
R^2 for the model including all significant variables	0.255	0.215	0.276	0.276

n, number; OSIT-J, the odor stick identification test for the Japanese; MIBG, [^{123}I] *meta*-iodobenzylguanidine; H/M, heart/mediastinum; MMSE, Mini-Mental State Examination; UPDRS, Unified Parkinson's Disease Rating Scale.
[a]Inverse transformed.
[b]Square-root transformed.
'–' = Dropped from the model with $P > 0.1$.
*$P < 0.05$.
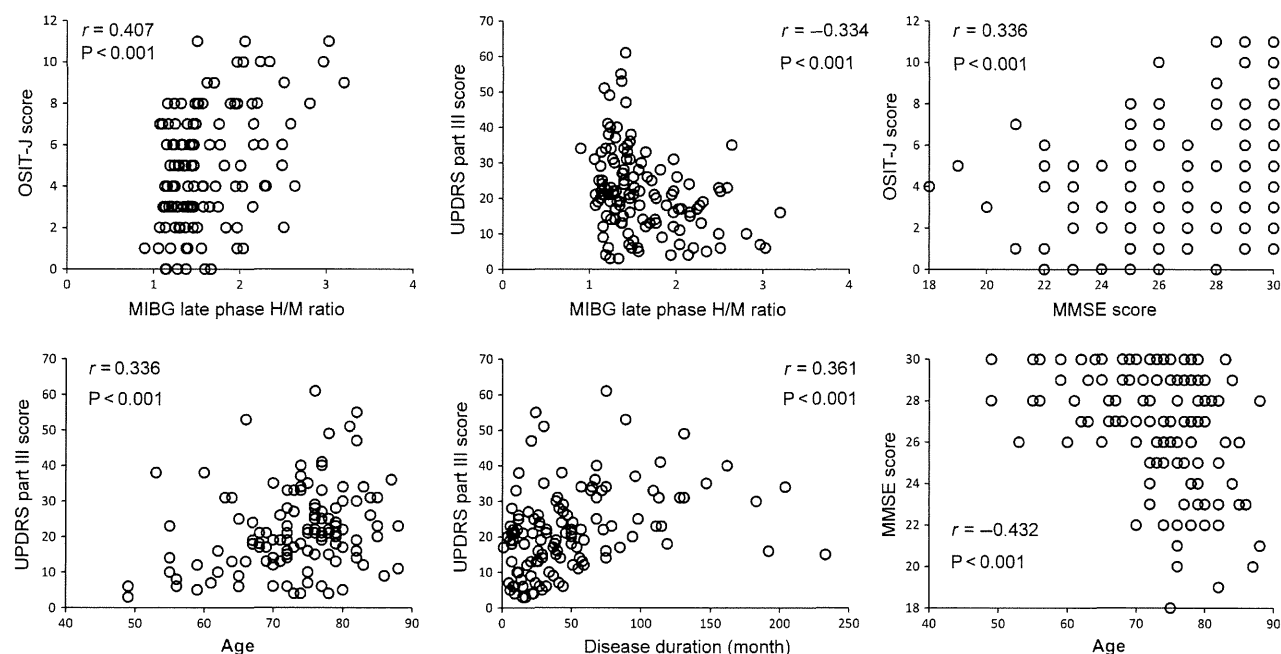**$P < 0.01$.
***$P < 0.001$.

Figure 1. Scatter plots for clinical and laboratory measures and covariates which were correlated in a multiple regression analysis. OSIT-J, the Odor Stick Identification Test for the Japanese; MIBG, [123I] metaiodobenzylguanidine; H/M, heart/mediastinum; UPDRS, Unified Parkinson's Disease Rating Scale; MMSE, Mini-Mental State Examination. Pearson's correlation coefficient (r) and p value are calculated by Pearson correlation; MIBG late phase H/M ratio and UPDRS part III score are inverse transformed and square-root transformed, respectively. 90x48mm (300 x 300 DPI).

For factor 1, age and MMSE score had high loading while OSIT-J score and UPDRS part III score had intermediate loading. In the multiple regression analysis, age was correlated with MMSE, OSIT-J, and UPDRS part III score. Thus, we consider that factor 1 represents the aging effect on the clinical features of patients with PD. In other words, aging is the risk factor

for cognitive function followed by smell and motor function. This finding is consistent with previous studies indicating that advanced age is a risk factor for developing PD (10, 11) and dementia in patients with PD (14, 15).

For factor 2, the MIBG H/M ratio and OSIT-J score had high loading, and UPDRS part III score had intermediate loading. The OSIT-J score

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Table 4 Factor analysis of clinical and laboratory measures and covariates

Variable interpretation of factor	Factor 1 aging	Factor 2 PD-related pathogenesis	Factor 3 disease duration	Final communality estimate
Age	**0.826**	0.032	0.198	0.72
MMSE score	**−0.805**	0.194	0.008	0.69
MIBG late phase H/M ratio[a]	0.119	**0.828**	−0.299	0.79
OSIT-J score	−0.358	**0.790**	0.003	0.75
Disease duration	−0.013	−0.068	**0.893**	0.80
UPDRS part III score[b]	0.369	−0.277	**0.649**	0.63
Variance explained by each factor	1.61	1.43	1.35	4.38
	22.3%	20.4%	19.2%	62.6%

MMSE, Mini-Mental State Examination; MIBG, [123I] *meta*-iodobenzylguanidine; H/M, heart/mediastinum; OSIT-J, the odor stick identification test for the Japanese; UPDRS, Unified Parkinson's Disease Rating Scale.
The high loading (larger than 0.60 by absolute value) in each factor is shown in bold and intermediate loading (larger than 0.25 and <0.60 by absolute value) is underlined.
[a]Inverse transformed.
[b]Square-root transformed.

and MIBG H/M ratio were correlated in the multiple regression analysis. While the exact pathophysiology of olfactory impairment remains to be elucidated, the broad olfactory-related areas, including the olfactory bulb, primary olfactory cortex, amygdala, hippocampus and orbitofrontal cortex, are susceptible to Lewy pathology. These structures are thought to be the neural substrates for olfactory impairment (16–19). Cardiac sympathetic nerves are also susceptible to Lewy pathology in PD. It is known that a decreased MIBG H/M ratio indicates the presence of degenerated noradrenergic cardiac sympathetic fibers due to Lewy body pathology (20). Both olfactory bulb and cardiac sympathetic nerves are supposed to have Lewy pathology in the very early phase of PD, even preceding the onset of motor symptoms (19–21). Thus, we consider that factor 2 represents the effect of PD-related pathogenesis, particularly resulting in Lewy pathology in the olfactory-related areas and cardiac sympathetic nerves in the very early stage of PD. On the other hand, while motor and cognitive functions are also supposed to be related to Lewy pathology, their loadings were intermediate (−0.277) and low (0.194) in factor 2, respectively. Although the reason for this is not clear, it might be because motor and cognitive functions are also strongly influenced by disease duration (factor 3) and age (factor 1), respectively, resulting in relatively lighter loading in factor 2.

For factor 3, the UPDRS part III score and disease duration had high loading and the MIBG H/M ratio had intermediate loading. Disease duration was correlated with motor function in multiple regression analyses and with the MIBG H/M ratio in only the Pearson's correlation analysis. Thus, we consider that factor 3 represents the effect of disease duration for motor function followed by the MIBG H/M ratio.

Taken together, we determined that aging, PD-related pathogenesis, and disease duration underlie the multisystem neurodegeneration in PD. It is interesting to note that motor symptoms and non-motor features (cognitive and olfactory impairment and cardiac sympathetic denervation) were loaded in separate factors, suggesting that a somewhat different pathophysiology accelerates the motor symptoms and non-motor features of PD. Our results suggest that age is the major risk factor for cognitive impairment in PD (factor 1). To date, various pathogenetic mechanisms (2, 22), for example cortical Lewy bodies (23, 24), Alzheimer's disease-type pathologies (25) and white matter damage (26) were suggested as neural correlates of cognitive impairment in PD. It is also known that multiple neurotransmitter systems, for example acetylcholine, dopamine, serotonin, and noradrenaline, could be targeted in PD, and their degeneration may underlie the different phenotypes of cognitive deficits (27, 28). We speculate that aging may interact with the PD-related pathogenesis in the cognitive-related areas, accelerating the degeneration of certain neurotransmitter systems, resulting in cognitive deficits. On the other hand, our study also showed that disease duration is the main risk factor for motor symptoms (factor 3). This is consistent with the natural history of PD as a progressive neurodegenerative disease, that is motor symptoms progress throughout the course of the disease. Furthermore, our results showed a strong association between olfactory impairment and cardiac sympathetic denervation (factor 2). A similar association was also reported in several previous studies (3–5). While ^{123}I-MIBG cardiac scintigraphy specifically evaluates noradrenergic nerves, olfactory impairment is supposed to reflect impaired multiple neurotransmitter systems such as acetylcholine, serotonin, dopamine, and noradrenaline (29). We speculate that the two systems share a common pathogenesis which might be affected even during the very early phase of PD. For example, they may share the catecholamine (i.e., dopamine and noradrenaline) systems, which might have similar biochemical processes such as aldehyde formation (30), resulting in Lewy pathology and underlying their strong association. The exact pathological and

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biochemical correlates should be investigated in future studies.

In the multiple regression analysis, OSIT-J and MMSE scores were also correlated. To date, this relationship is somewhat controversial. Some studies did not find the relationship (4, 5), whereas Baba et al. (6) reported a weak correlation between OSIT-J score and MMSE score and Bohnen et al. (31) found a relationship between odor identification score and cognitive measures of episodic verbal learning. We enrolled various patients with PD ranging from those who were cognitively normal to those who had mild dementia, despite the fact that most previous studies evaluated only PD patients without dementia. Thus, we found the association because we had a greater number of subjects with wide-ranging clinical variability. On the other hand, the neural basis of this correlation is still obscure. Olfactory perception is known to require semantic processing (32). Hudry et al. (33) pointed out that olfactory impairment in patients with PD is partly due to deficiencies in perceptual and semantic olfactory processes. Moreover, it was also reported that olfactory impairment is related to cholinergic denervation of the limbic archicortex (31); severe hyposmia predicts future development of dementia in these patients (34). Thus, olfactory and cognitive function may share the same anatomy, impairment in the same neurotransmitter systems and/or the same functional systems such as semantic processing. Further study is needed to identify the neural correlates of this association.

In a multiple regression analysis, UPDRS part III score and the MIBG H/M ratio were negatively correlated. Kim et al. (7) reported that the MIBG H/M ratio is related to severity of midline motor symptoms in patients with PD, but not to HY stage nor UPDRS part III score. Spiegel et al. (8) reported that the MIBG H/M ratio correlated with the severity of hypokinesia and rigidity, but not with the severity of a resting or postural tremor in patients with PD. While we used only the total score of UPDRS part III score, certain components of the motor symptoms might specifically correlate with the MIBG H/M ratio, supporting the correlation that we found. Future study is needed to clarify the pathophysiology of this association.

Braak et al. (19) proposed the progression patterns of Lewy pathology in PD. In summary, Lewy pathology is found in the anterior olfactory structures in stages 1–2, progresses to the substantia nigra and other nuclei of the basal mid- and forebrain, and motor and cognitive symptoms manifest in stages 3–4 (23). Lewy pathology

further extends to the neocortex, and motor, and cognitive function worsens in stages 5–6. As discussed above, OSIT-J and MMSE score reflect the synergistic cortical functions of olfactory- and cognitive-related areas. Thus, the association between OSIT-J and MMSE score may suggest parallel neurodegeneration due to Lewy pathology in the olfactory- and cognitive-related areas during stages 3–6. On the other hand, pathology in the cardiac sympathetic nerves is not defined in Braak staging (19). Based on the association between MIBG H/M ratio and OSIT-J score or HY/UPDRS part 3 score, we speculate that cardiac sympathetic nerves also degenerate in parallel to Lewy pathology in brain. Thus, the MIBG H/M ratio could be used as a biomarker of accumulated Lewy pathology in the brains of patients with PD. The correspondence between Braak staging and olfactory impairment/cardiac sympathetic denervation should be investigated in a future study.

Some limitations of the present study should be addressed. First, this is both cross-sectional and retrospective in design and did not allow us to observe features longitudinally. A longitudinal study is needed to prove the associated degeneration directly. Second, this study did not include data on several key non-motor symptoms such as psychiatric symptoms, hallucination, mood disorders, and sleep problems. A future prospective study assessing the various features of PD is needed to thoroughly investigate the relationships. Third, this study design does not identify the underlying pathogenesis behind the observed multisystem degeneration. Various levels of future studies, such as genetic, molecular, receptor (35), neurotransmitter and biochemical process (30), are needed to elucidate this point.

In conclusion, this study suggests that three parallel processes, aging, PD-related pathogenesis and disease duration underlie the multisystem neurodegeneration in PD. Our study also revealed that age and disease duration are the main risk factors for cognitive impairment and motor function, respectively, while cardiac sympathetic denervation and olfactory impairment are strongly associated. The data taken together support the current idea of PD as a progressive neurodegenerative disease. Age plays a significant role in the disease process and interacts with PD-related pathogenesis to accelerate PD symptoms (15).

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Chimeric Antisense Oligonucleotide Conjugated to α-Tocopherol

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We developed an efficient system for delivering short interfering RNA (siRNA) to the liver by using α-tocopherol conjugation. The α-tocopherol–conjugated siRNA was effective and safe for RNA interference–mediated gene silencing *in vivo*. In contrast, when the 13-mer LNA (locked nucleic acid)-DNA gapmer antisense oligonucleotide (ASO) was directly conjugated with α-tocopherol it showed markedly reduced silencing activity in mouse liver. Here, therefore, we tried to extend the 5′-end of the ASO sequence by using 5′-α-tocopherol–conjugated 4- to 7-mers of unlocked nucleic acid (UNA) as a "second wing." Intravenous injection of mice with this α-tocopherol–conjugated chimeric ASO achieved more potent silencing than ASO alone in the liver, suggesting increased delivery of the ASO to the liver. Within the cells, the UNA wing was cleaved or degraded and α-tocopherol was released from the 13-mer gapmer ASO, resulting in activation of the gapmer. The α-tocopherol–conjugated chimeric ASO showed high efficacy, with hepatic tropism, and was effective and safe for gene silencing *in vivo*. We have thus identified a new, effective LNA-DNA gapmer structure in which drug delivery system (DDS) molecules are bound to ASO with UNA sequences.

Introduction

Antisense oligonucleotides (ASOs) and small interfering RNA (siRNA) are both recognized therapeutic agents for the silencing of specific genes at the posttranscriptional level.[1] Chemical modifications, particularly the use of locked nucleic acids (LNAs),[2–4] 2′-*O*-methoxyethyl (2′-*O*-MOE),[5,6] and constrained ethyl BNA (cEt),[7,8] markedly improve ASO binding affinity for the target mRNA, resulting in increased steric block efficiency. Currently, the mainstream of the ASO is gapmer ASOs.[1] Gapmer oligonucleotides, which contain two to five chemically modified nucleotides (LNA, 2′-*O*-MOE RNA, or cEt) as "wings" at each terminus flanking a central 5- to 10-base "gap" of DNA, enable cleavage of the target mRNA by RNase H, which recognizes DNA/RNA heteroduplexes.[9,10]

Recently, the FDA approved Kynamro (mipomersen sodium, Isis Pharmaceuticals, Carlsbad, CA) as a treatment for familial hypercholesterolemia.[11,12] Kynamro, a DNA 10-mer with 2′-*O*-MOE-modified-5-mers at both ends, targets *Apolipoprotein B* (*ApoB*). It has a strong target gene–silencing effect and greatly reduces serum low-density lipoprotein (LDL)-cholesterol in patients with familial hypercholesteremia. Since the approval of Kynamro, the higher binding affinity of LNAs has prompted the development of far shorter ASOs, which have been shown recently to increase the gene silencing effect, probably because of their increased intracellular availability.[13] Despite this progress in the design of new chemical modifications of oligonucleotides, methods that improve the potency of oligonucleotide drugs in animals are still highly desirable. The inadequate delivery and poor cellular uptake of oligonucleotides, coupled with their inability to efficiently access the target mRNA during intracellular trafficking,[14] are major impediments to *in vivo* silencing.[15]

The development of effective delivery systems for oligonucleotides is essential for their clinical application. Previously, we hypothesized that the best *in vivo* carrier for siRNA would be a molecule that the target cells need but cannot synthesize. Vitamins meet these requirements, and the least toxic, fat-soluble vitamin (even at high doses) is vitamin E.[16] Therefore, we directly conjugated α-tocopherol, a natural isomer of vitamin E, to siRNA and obtained a substantial reduction in the expression of an endogenous gene in mouse liver and brain.[17,18] In this study, we tried to use α-tocopherol (Toc) conjugation as a delivery system for ASO.

Results
Design of Toc-ASO targeting mouse *ApoB* mRNA
We used the 13-mer LNA/DNA gapmer that targets mouse *ApoB* mRNA (NM_009693) and has been described previously.[13] Toc was conjugated to several lengths of gapmers or chimeric ASOs. The structures of the α-tocopherol-bound ASO (Toc-ASOs) are shown in **Figure 1**. For example, the 20-mer Toc-ASO is an α-tocopherol–conjugated chimeric 20-mer, with a 7-mer "second wing" (**Figure 1**) of artificial nucleotides extending from the 5′-end of the original 13-mer ASO. The 7-mer second wing was composed of phosphodiester-bound unlocked nucleic acid (UNA) (Toc-20-mer ASO) or phosphorothioate-bound UNA (Toc-20-mer ASO PS). To estimate the effect of the artificial modification second wing, we

13-mer ASO

Toc-13-mer ASO

Toc-13-mer ASO PEG

Toc-17-mer ASO

Toc-20-mer ASO PS

Toc-20-mer ASO

Second Wing Gap Wing
wing

● Locked nucleic acid (LNA)
● DNA
● Chemically modified RNA
∧ Phosphodiester bond
∧ Phosphorothioate bond
■ α-tocopherol
— Polyethylene glycol (PEG) linker

Figure 1 Design of several types of Toc-ASOs.

Table 1 Melting temperatures (T_m) of ASOs targeting mouse Apolipoprotein B (ApoB) mRNA

ASO	T_m (°C)
13-mer ASO	58.9
Toc-13-mer ASO	58.0
14-mer ASO	58.1
Toc-14-mer ASO	57.9
17-mer ASO	57.8
Toc-17-mer ASO	56.5
Toc-17-mer ASO F	71.4
Toc-17-mer ASO OMe	69.9
20-mer ASO	58.1
Toc-20-mer ASO	57.4
Toc-20-mer ASO PS	55.8
Toc-23-mer ASO	56.8

synthesized α-tocopherol-bound 17-mer ASO with second wing consisting of phosphodiester-bound 2′-Fluoro modified RNA (Toc-17-mer ASO F) and phosphodiester-bound 2′-O-methyl RNA (Toc-17-mer ASO OMe).

To estimate the length effect of the second wing, we designed several lengths of Toc-chimeric ASOs that contained phosphodiester-bound UNA, namely Toc-14-mer ASO, Toc-17-mer ASO, and Toc-23-mer ASO. To estimate α-tocopherol conjugation effect, we designed α-tocopherol–unconjugated ASOs with phosphodiester-bound UNA second wing: 14-mer ASO, 17-mer ASO, and 20-mer ASO.

The UV melting temperatures (T_m) of various Toc-ASOs are shown in **Table 1**. All of the Toc-ASOs had approximately the same T_m value, with the exception of Toc-17-mer ASO OMe and Toc-17-mer ASO F.

Efficacy of the Toc-ASOs

First, we made a nucleic acid Toc-13-mer ASO, in which the α-tocopherol was directly conjugated to the 13-mer ASO by a phosphodiester bond. Mice were injected with 0.75 mg/kg ASO and examined 3 days later. Quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was performed using total RNA extracted from liver homogenates. We found that the Toc-13-mer ASO had no gene silencing effect (**Figure 2a**). Because conjugation of α-tocopherol interfered with the 13-mer ASO's gene silencing effect, we introduced a spacer between the 13-mer ASO and α-tocopherol. Because Toc-13-mer ASO PEG (α-tocopherol–conjugated to the 13-mer ASO via hexaethylene glycol) also had no effect, we then inserted additional nucleotides as a linker for spacing. Although Toc-20-mer ASO PS had no gene silencing effect

(**Figure 2a**), Toc-17-mer and Toc-20-mer ASOs reduced target gene expression, especially Toc-17-mer ASO had significantly greater effect than that of the parent 13-mer ASO (**Figure 2a**).

Length effect of the second wing

Toc-13-mer (no second wing sequences) and Toc-14-mer ASO had no obvious effect, but Toc-17-mer and Toc-20-mer ASOs decreased the target gene expression. Importantly, these silencing effects were more potent than that of the 13-mer ASO (**Figure 2b**). To verify the advantage of α-tocopherol conjugation, the gene silencing effects of several length of ASOs with α-tocopherol conjugation or without α-tocopherol conjugation were evaluated. The ASOs without α-tocopherol did not have target gene silencing effect (**Figure 2b**). The knockdown effect was specific for the target molecule, as evidenced by the findings that the negative control of Toc-17-mer or Toc-20-mer ASOs targeting an unrelated gene did not affect the ApoB mRNA level (**Figure 2a,b**), and that ApoB targeting Toc-ASOs did not change the levels of the other endogenous mRNAs in the liver—for example, glyceraldehyde-3-phosphate dehydrogenase (Gapdh), transthyretin (Ttr), superoxide dismutase 1 (Sod1), and hypoxanthine guanine phosphoribosyltransferase (Hprt) (**Figure 2c**).

Chemical modification of the second wing

The target gene silencing effects of Toc-17-mer ASO F and Toc-17-mer ASO OMe was markedly reduced in comparison with Toc-17-mer ASO which had UNA second wing (**Figure 2a**).

To evaluate the difference of mechanisms between effective Toc-ASO and noneffective one, northern blot analysis was performed on mouse liver at 72 hours after 0.75 mg/kg injection of Toc-ASOs. Toc-17-mer ASO OMe produced only one band corresponding to full length of Toc-17-mer ASO OMe itself, and the Toc-17-mer ASO produced a band corresponding to 13-mer ASO, which indicated that the 13-mer ASO was cleaved from Toc-17-mer ASO in vivo (**Figure 2d**). Additionally, Toc-17-mer ASO F produced two bands: the cleaved 13-mer and the full length of Toc-17-mer
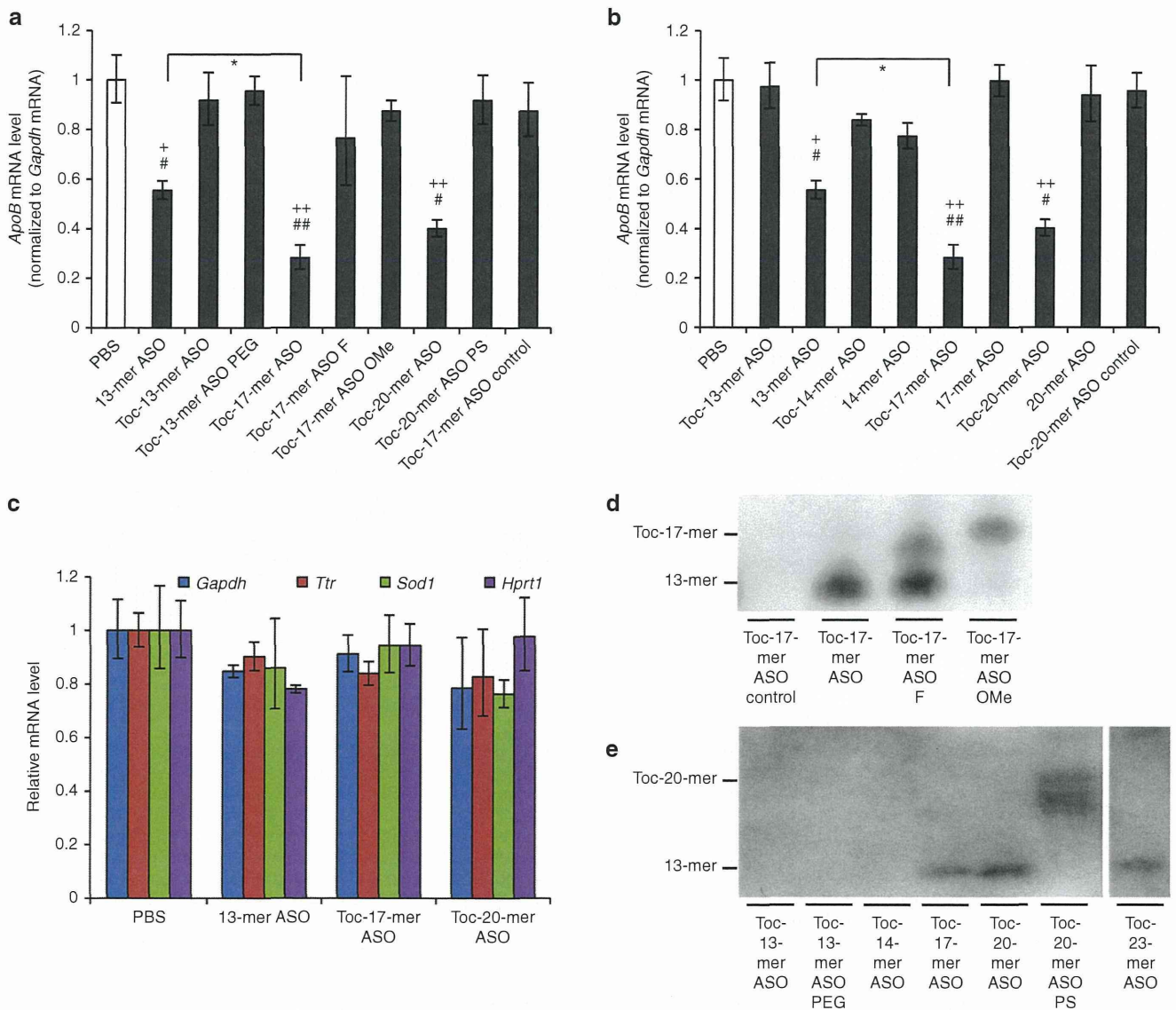
Figure 2 Gene-silencing effect of intravenous injection of Toc-ASO. (**a**) Quantitative RT-PCR analyses of *Apolipoprotein B* (*ApoB*) mRNA levels relative to *gapdh* mRNA levels in the liver 3 days after injection of 0.75 mg/kg α-tocopherol–conjugated ASOs. The data shown are relative to those from mice that received PBS alone and are presented as mean values ± SEM ($n = 3$, $^{+}P < 0.05$, $^{++}P < 0.01$ versus PBS, $^{#}P < 0.05$, $^{##}P < 0.01$, versus Toc-17-mer ASO control, and $^{*}P < 0.05$ Toc-17-mer ASO versus 13-mer ASO). (**b**) Quantitative RT-PCR analyses of *ApoB* mRNA levels (normalized to *gapdh* mRNA levels) in the liver 3 days after injection of 0.75 mg/kg ASOs bound to α-tocopherol by UNA second wings of various lengths or ASO without α-tocopherol. The data shown are relative to those from mice that received PBS alone and are presented as mean values ± SEM ($n = 3$, $^{+}P < 0.05$, $^{++}P < 0.01$ versus PBS, $^{#}P < 0.05$, $^{##}P < 0.01$ versus Toc-20-mer ASO control, and $^{*}P < 0.05$ between each groups). (**c**) Quantitative RT-PCR analyses of endogenous mRNAs (*Gapdh*, *Ttr*, *Sod1*, and *Hprt*) in the liver 3 days after injection of 0.75 mg/kg ASO, Toc-ASOs, or PBS alone. Data are relative to the total input RNA and are expressed as mean values ± SEM ($n = 3$). (**d**) Northern blot analysis to detect 13-mer gapmer sequences of Toc-ASO in the liver 3 days after injection of mice with 0.75 mg/kg Toc-ASO. (**e**) Northern blot analysis to detect 13-mer gapmer sequences of Toc-ASOs in the liver 3 days after injection of mice with 0.75 mg/kg Toc-ASOs of various lengths.

ASO F, it suggested that Toc-17-mer ASO F was thought to be less likely to be cleaved than Toc-17-mer ASO. In the liver samples from the 0.75 mg/kg Toc-ASO–injected mice on 72 hours after injection, the 13-mer band was clearly detected when mice were injected with the Toc-17-mer, Toc-20-mer, and Toc-23-mer ASOs (**Figure 2e**). On the other hand, samples from Toc-ASO–injected mouse liver in which Toc-ASOs had no silencing effect did not produce a 13-mer band (**Figure 2e**).

Dose dependency and time course of the Toc-ASOs effect

We derived dose-response curves from our quantitative RT-PCR results and then calculated the median effective dose (ED_{50})—that is, the dose of ASO that produced a 50% reduction in the target gene expression. We administered 0.75, 1.5, and 3 mg/kg of ASOs to mice and then sampled their livers (**Figure 3a**). We observed a dose dependent gene silencing effect in both 13-mer ASO and Toc-17-mer ASO-injected