により優性遺伝性のLewy小体を伴うパーキンソン病を生じる[9]．現在までにpark13まで知られており更に増え続けている．なお，park2は我が国で記載されていた常染色体劣性遺伝性の若年性パーキンソニズムであり，原因遺伝子も我が国で発見されparkinと命名された[10]．従来，特発性パーキンソン病の定義としてLewy小体は必須とする意見が強かったが，park2にはLewy小体はみられず，現在はLewy小体の有無にかかわらずparkの名称が用いられている．このこともある意味ではパーキンソン病の概念の大きな変化といえる．一般に，神経変性疾患の中で遺伝性の症例は10％未満であり残りは孤発性である．しかしながら，症候はもちろん病理変化など必ず共通点(最終共通経路)があり，遺伝性病型の発症機序の解明が孤発性症例の発症機序の解明や治療法の開発に役立つと期待される．実際，α-synucleinは孤発性パーキンソン病のhallmarkであるLewy小体の主要な構成成分であることがすぐに明らかにされた．

現在，分子遺伝学的研究の中心は，Mendelの遺伝を示す単一遺伝子の変異の探索から，孤発性パーキンソン病など多因子疾患における疾患感受性遺伝子の分析に移行しており，長足の進歩を遂げているDNA解析技術を背景に全ゲノムを対象とした網羅的解析が主流となりつつある．パーキンソン病ではα-synuclein遺伝子の多型やGaucher病の遺伝子変異が危険因子であることが明らかになっている．

3. 治療法の変遷—薬物療法

パーキンソン病の薬物治療は，19世紀末のベラドンナ・アルカロイドの使用に始まり，スコポラミンを経て1949年に抗コリン薬の有効性が報告された．抗コリン薬は特に振戦に有効で，認知症を増悪させうるという副作用はあるものの今日でも使用されている．最も劇的なデビューは何といってもレボドパである．1960年にEhringer，Hornykiewiczが脳炎後パーキンソニズムとパーキンソン病患者脳の線条体でドパミンの低下の報告を受け，翌1961年にはBirkmayer，Hornykiewiczはドパミンの前駆物質であるレボドパを患者に静注し全く動けなかった患者が歩き出すのを目撃し[11]，Barbeauらもレボドパの経口投与を行いその有効性を報告した．このとき既にモノアミン酸化酵素阻害薬の効果が確認されており，間もなくドーパ脱炭酸酵素阻害薬の併用も研究され始めており，現在ではドーパ脱炭酸酵素阻害薬との合剤がほとんどである．レボドパは夢の薬として全世界に普及したが，すぐさま症候の変動やジスキネジアの出現などの副作用が判明し，その対策として1974年には最初のドパミンアゴニストであるブロモクリプチンが使用され，現在では更にペルゴリド，カベルゴリン，タリペキソール，プラミペキソール，ロピニロールと多数が使用可能である．やはり胸膜炎，弁膜症といった副作用も知られるようになり，その使用には注意が肝心である．

この間，抗インフルエンザ薬のアマンタジン，ノルアドレナリン前駆物質のドプス，COMT阻害薬のエンタカポンなどが開発され，近年では抗てんかん薬のゾニサミドにも抗パーキンソン病効果があることが知られ臨床試験が終了したところである．まだ神経細胞死を治す本質的治療薬がない現状では，対症治療薬であっても様々な機序によるものの開発は重要なことと思われる．

4. 治療法の変遷—機能外科学

パーキンソン病の外科的治療は，1930年のPollock，Davisによる後根切除による筋固縮の軽減に始まり，錐体路切断や運動皮質切除などが行われたが，1940年Meyersは初めて基底核を標的とした破壊術で振戦と筋固縮が改善するとした．我が国では1952年に楢林が片側性パーキンソニズム患者の対側淡蒼球に対する定位脳手術を行い振戦と筋固縮の消失を達成した．その後，楢林らは視床を，米国のSpiegelらは淡蒼球を中心に多くの手術を行ったが，前述のレボドパの普及により外科的治療はいったんは急速に衰退していった．しかし，この間も我が国では楢林らを中心に症例を選び定位脳手術の治療や研究が脈々と続けられていた．著者は幸

いにもそのなかで色々教えて頂くことのできた数少ない神経内科医の一人ではないかと感謝する次第である．その後，1990年代になりLaitinenらにより薬物治療で難治性の症例に淡蒼球の定位脳手術が復活し[12]，我が国でもメディアなどにも取り上げられ急速に全国に広まった．更に標的として視床下核も加わり，破壊術のみならず脳深部刺激治療(deep brain stimulation: DBS)が可能となった．DBSにより両側同時かつ任意に治療を行うことができるようになるとともに，多くの臨床経験が積まれ近年は安定した治療法として定着している．更に，脚橋核など新たな標的に対する治療研究も進んでいる．

5. 将来への展望

以上述べてきたように，現在も様々な研究が日夜行われており，発症機序の解明，治療法の開発などパーキンソン病の克服に向けて飛躍的に発展するよう大きな期待を抱いている．実は，まだ触れていないがこれから発展してほしいものとして，細胞移植治療と遺伝子治療をあげたい．パーキンソン病では現在話題の神経幹細胞，胚性幹細胞あるいはiPS細胞などが話題になるずっと前から，胎児の中脳黒質細胞，副腎髄質細胞あるいは交感神経節細胞などの移植治療が試みられていた．有効との報告もあるが，十分な合意は得られておらず，少なくとも先進国ではあまり行われていないと思われる．一方，成人脳内にも神経幹細胞が存在することが明らかにされ，これらの内在性幹細胞をドパミン細胞に分化，増殖させたり，外から移植することで治療しようという研究が進められている．これらはまさに再生医療であるが，広い意味では機能再建を目指すリハビリテーションにも通じる．今回は，触れることができなかったがPET，SPECT，NIRSなど脳機能を視覚化する機能画像検査も大きな進歩を遂げており，神経科学の進歩と合わせて，より科学的で有効なリハビリテーションの開発が進むと期待される．

また，変性する細胞体は黒質にあり，ドパミン欠乏の標的である線条体は障害されていないという幸運なる特徴を活かして，線条体にドパミン産生系酵素の遺伝子を導入する遺伝子治療の臨床試験が，我が国でも行われ有効性が報告されている．いずれも1日も早いパーキンソン病の克服を目指す一人として，これらの先進治療研究に大いに期待したい．

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Age at onset influences on wide-ranged clinical features of sporadic amyotrophic lateral sclerosis

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ABSTRACT

Purpose: To profile the detailed clinical features of sporadic amyotrophic lateral sclerosis (ALS) on large-scale samples in Japan.
Methods: We assessed the clinical features of sporadic ALS patients in Japan, based on the nationwide registration system of the Ministry of Health, Labor and Welfare of Japan. We described 3428 new cases registered cases between 2003 and 2006 to analyze initial symptoms and related clinical features, 4202 cases registered in the single year of 2005 to describe the cross-sectional overview of the ALS patients, and a total of 2128 cases with tracheostomy positive pressure ventilation (TPPV) from all of the registration data from 2003 to 2006 to describe the features of ALS patients with TPPV.
Results: The patients with an older age at onset progressed more rapidly to the TPPV stage than those with a younger age at onset. The subpopulation of patients with long-standing TPPV showed ophthalmoplegia, while its appearance rate was less in the patients with an older age at onset than in those with a younger age at onset. Furthermore, age at onset strongly influenced the frequency of initial symptoms: dysarthria, dysphagia, neck weakness and respiratory disturbance were more frequent in patients with an older age at onset, while upper or lower limb weakness was observed more frequently in patients with a younger age at onset. In addition, those initial symptoms were still the most prominent at the follow-up stage, suggesting that the initial symptoms determine the major clinical features even in advanced illness.
Conclusions: Our present study demonstrated that symptomatic features of ALS are strongly influenced by the age at onset by the large scale of samples.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is one of the most devastating neurodegenerative diseases affecting upper and lower motor neurons preferentially, and shows progressive muscle wasting of the limb, bulbar and respiratory musculatures. Almost half of ALS patients expire within three years of onset, primarily due to respiratory failure [1–6]. Approximately 5–10% of ALS patients show a familial trait, while more than 90% of the patients are sporadic, and the causal mechanism of the motor neuron degeneration is largely unknown. Although many clinical trials of potential therapeutic agents for the treatment of sporadic ALS have been performed [7], effective therapeutics against motor neuron degeneration in ALS except for riluzole [8,9] have not been developed. The clinical features of ALS have been established for the most part. However, many aspects of symptomatic manifestations such as the influence of age at onset on clinical features, the frequency of rare symptoms and many other symptomatic details have not been well characterized, particularly

Abbreviations: ALS, amyotrophic lateral sclerosis; TPPV, tracheostomy positive pressure ventilation.

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based on a nationwide scale sample. In Japan, the proportion of the ALS patients with TPPV is relatively higher than in other countries [10,11]. Rare symptoms such as ophthalmoplegia are more frequently seen in those who receive TPPV to prolong survival [12,13], so the clinical profile of ALS patients in Japan might have unique features. Data concerning the clinical features are important to establish an early diagnosis, treatment plan, and prognostic estimation, as well as to design clinical trials.

The aim of this study was to profile the detailed clinical features of sporadic ALS on large-scale samples in Japan.

2. Research design and methods

A nationwide registration of patients with intractable diseases including ALS has been conducted by the Ministry of Health, Labor and Welfare of Japan since 1974. When a patient is diagnosed as having ALS, the patient can apply for registration in this system, and receive financial support from the state for medical expenses incurred for the treatment of ALS, independent of the disease severity. In 2003, a data collection system was developed for research use of this registration system. Concurrently with that, the registration form for ALS was revised substantially. Since 2003, the annual renewal of registration of each patient has been conducted. The data from registration forms were input to the database in each prefectural office and consolidated in the Ministry of Welfare, Health and Labor of Japan. In the revised registration form, the overview of the clinical state is to be indicated, including the severity, neurological symptoms, activities of daily living and conditions of tube feeding or non-invasive positive pressure ventilation (NIPPV) and TPPV of ALS patients in Japan on a nationwide scale. Using the data accumulated from 2003 to 2006, we analyzed the clinical features of sporadic ALS patients in Japan. Clinical profiles of sporadic ataxias in Japan were previously described using this registration system [14].

The inclusion criteria of the registration system for ALS are: 1) adult onset, steady progressive course; 2) the presence of clinical or electrophysiological evidence of lower motor neuron (LMN) degeneration in at least two topographical anatomic regions (brainstem, cervical, thoracic or lumbosacral region), together with clinical evidence of upper motor neuron (UMN) degeneration in at least one region; and 3) the absence of electrophysiological and pathological evidence of other disease processes that might explain the signs of LMN and/or UMN degeneration, and neuroimaging evidence of other disease processes that might explain the observed clinical and electrophysiological signs. Therefore the patients registered in this system satisfy definite, probable or possible ALS based on the revised El Escorial Criteria [15] for the diagnosis of ALS.

The data collection system was developed in 32 of 47 prefectures in Japan. In proportion to the total population, 63% of total registered patients in Japan were integrated into the computerized database. The data were comprised of initial registration form and renewal registration form. When a patient was diagnosed as ALS, the initial registration form was used to apply for the system, and the renewal registration form was used in the following year. However, the information on the patients initially registered before 2003 was comprised of data from only renewal registration.

After 2003, 3694 ALS patients were newly registered in the system. Records were eliminated from the analysis if information was missing for age at onset and age at registration. Ninety-four patients were also excluded who had a family history of motor neuron disease or an abnormality of genes related to neurodegenerative disease such as the SOD1 mutation. The inclusion age range was above 20 years at onset. After these data clearing, the data from a total of 3428 patients were available. In order to analyze the age at onset, initial symptoms and related clinical features, we used this data set.

In a single year, 2005, 4546 ALS patients were registered using the initial registration form or renewal registration form. The number included those initially registered before 2003. To describe the cross-sectional overview of the medical and social conditions of ALS patients in Japan, we used this data set. After the data described above were excluded, the data from 4202 patients were used.

From 2003 to 2006, 2440 ALS patients with TPPV were registered at least once, mostly using the renewal registration form. The number included those initially registered before 2003. To describe the conditions of ALS patients with TPPV, we analyzed this data set. After the data cleaning, the data from 2128 patients with TPPV were used.

All of the patients provided written informed consent for the research use of the data, and the anonymity of the data was strictly secured. We implemented the guidelines for research use of the data from the nationwide registration system of intractable diseases and the ethics guidelines for clinical studies endorsed by the Japanese government. The research project was approved by the Ministry of Health, Labor and Welfare, Japan, and by the ethics committee of Nagoya University Graduate School of Medicine.

2.1. Assessment of clinical features

Age at onset was considered as the time of the patient's initial awareness of weakness. As for the initial symptoms, six symptoms including dysarthria, dysphasia, respiratory disturbance, weakness of neck, weakness of upper extremities and weakness of lower extremities were noted. In most cases, one symptom was assessed as an initial symptom, however, two or more symptoms may be recorded. The activities of daily living and clinical symptoms were assessed by 6 items from the 12 items of ALSFRS-R (Speech, Swallowing, Handwriting, Dressing and Hygiene, Walking and Dyspnea). The Japanese version of ALSFRS-R was validated previously for ALS, showing that the assessment values are highly equivalent among well-trained neurologists, general physicians and nurses, and that intra-rater assessment values are also highly equivalent [16]. Intra-rater and inter-rater reliability of each item of the Japanese version of ALSFRS-R were also validated. The presence of oculomotor disturbance was assessed through a bedside neurological examination.

2.2. Data analysis

All variables were summarized using descriptive statistics, including mean, standard deviation (S.D.), and percentages. Correlations

Table 1
Clinical features of patients newly registered from 2003 to 2006 (n=3428)

Age at onset (years, mean±S.D.)	65.4±10.7
Male/female (%)	57.8/42.2
Duration from disease onset to registration (years, mean±S.D.)	1.7±2.2
Symptoms at registration (%)	
Dysarthria	64.2
Dysphagia	57.8
Weakness of neck	70.0
Respiratory distress	34.2
Weakness of upper extremities	86.6
Weakness of lower extremities	76.2
Initial symptoms (%)	
Dysarthria	36.3
Dysphagia	21.1
Weakness of neck	7.1
Respiratory disturbance	6.3
Weakness of upper extremities	48.1
Proximal dominant	26.1
Distal dominant	50.8
Diffuse	23.0
Weakness of lower extremities	34.1
Proximal dominant	19.7
Distal dominant	42.6
Diffuse	37.8

Table 2
Cross-sectional living conditions of patients registered in 2005 (n=4202)

Living condition	Frequency (%)
At work or school	6.7
Household work	6.5
Under home care	58.2[a]
In hospital	27.5[a]
In nursing-care facility	2.4

[a] 1.2% of patients overlap.

between age at onset and duration from disease onset to invasive procedures were analyzed using Pearson's correlation coefficient, and the cumulative incident curves of two age groups were assessed by the log-rank test. Difference of frequencies of symptoms between two age groups was assessed by the chi-square test. p-values <0.05 were considered to be statistically significant. Calculations were performed using the statistical software package SPSS 15.0J for Windows (SPSS Japan Inc., Tokyo Japan).

3. Results

3.1. Clinical features of sporadic ALS patients

The mean age at onset was 65.4±10.7 years, the male to female ratio was 1.37:1, and the mean duration from disease onset to registration was 1.5±1.4 years. The initial symptom was dysarthria in 36.3%, dysphagia in 21.1%, weakness of neck in 7.1%, respiratory disturbance in 6.3%, weakness of the upper extremities in 48.1%, weakness of lower extremities in 34.1%, when allowing overlapping descriptions (Table 1). When we analyzed these demographic clinical features between male and female patient groups, age at onset was slightly higher in the female patients. The proportion of the patients with bulbar symptom onset was higher in the female patients, whereas, the proportion of the patients with weakness of upper extremities was higher in the male patients (Supplemental Table 1).

The cross-sectional state of living conditions of ALS patients in Japan in 2005 is shown in Table 2. The proportion of the patients at work or school was 6.7%, 6.5% engaged in household work, 58.2% under home care, 27.5% in hospital and 2.4% in a nursing-care facility. The state of nutrition and respiratory support is shown in Table 3. The frequency of patients with a gastrostomy tube was 28.7%, and 7.8% were using a nasogastric tube. NIPPV was used by 7.2% of the patients, and 29.3% were under TPPV. The clinical profiles of the patients with TPPV were shown in Table 4. Mean duration from introduction of TPPV was 3.7 years, and 42.2% of the patients with TPPV were living under home care.

3.2. Age at onset influences progression of disease assessed by duration from onset to introduction of TPPV

The mean interval between the onset of disease and the introduction of TPPV was 3.0 years. Intervals from the disease onset to the introduction of TPPV became shorter as the age at onset advanced (Fig. 1A). There was a significant correlation between the

Table 3
Nutritional and respiratory support of patients registered in 2005 (n=4202)

Nutritional and respiratory support	Frequency (%)
Tube feeding	
Gastrostomy tube	28.7
Nasogastric tube	7.8
NIPPV [a]	
Intermittent use	2.0
All-night use	2.6
All-day use	2.6
TPPV [b]	29.3

[a] Non-invasive positive pressure ventilation.
[b] Tracheostomy positive pressure ventilation.

Table 4
Clinical profiles of patients with TPPV (n=2128)

Male/female (%)	59.9/40.1
Age at onset (years, mean±SD)	59.8±11.7
Duration of disease (years, mean±SD)	6.7±5.0
Duration from disease onset to introduction of TPPV	3.0±3.2
Duration from TPPV introduction	3.7±3.5
Living conditions	
Under home care (%)	42.2[a]
In hospital (%)	57.4[a]
In nursing-care facility (%)	2.1

[a] 1.8% of patients overlap.

age at onset and the interval from disease onset to introduction of tube feeding or TPPV, when analyzed using Pearson's correlation coefficient (r=−0.39 p<0.001). Since 65 years was the mean age of onset, we assessed the cumulative frequency of TPPV in subgroups of patients with an age at onset of 65 years or more and less than 65 years, showing that the duration from onset to introduction of TPPV was significantly shorter in patients with an onset age of 65 years or older (p<0.001) (Fig. 1B). The age at onset influences the progression from onset to the advanced stage assessed by the introduction of TPPV.

3.3. Appearance of ophthalmoplegia under TPPV influenced by age at onset

In the patients with long-standing TPPV, rare symptoms such as ophthalmoplegia were frequently observed. Ophthalmoplegia, which is particularly well assessed by bedside examination, was seen in only

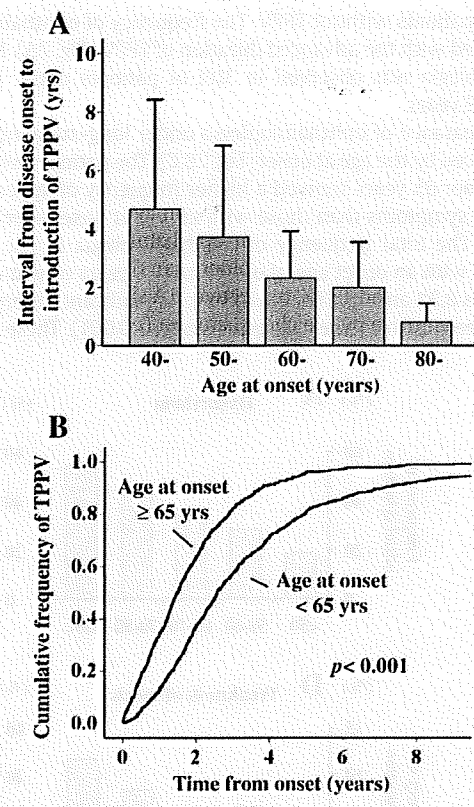

Fig. 1. Relationship between age at onset and introduction of tube feeding and TPPV. Interval from disease onset to introduction of TPPV (A) is shown. An older age at onset strongly correlates to shorter intervals from onset to TPPV. Cumulative frequencies of patients with TPPV in the patient population with an onset age older or younger than 65 years are shown (B). Cumulative curves for patients with an onset age of 65 years or more show significantly shorter intervals between disease onset and introduction of TPPV than those with an onset age of under 65 years of age, suggesting that age at onset markedly influences the time from onset to introduction of TPPV. n=2128.

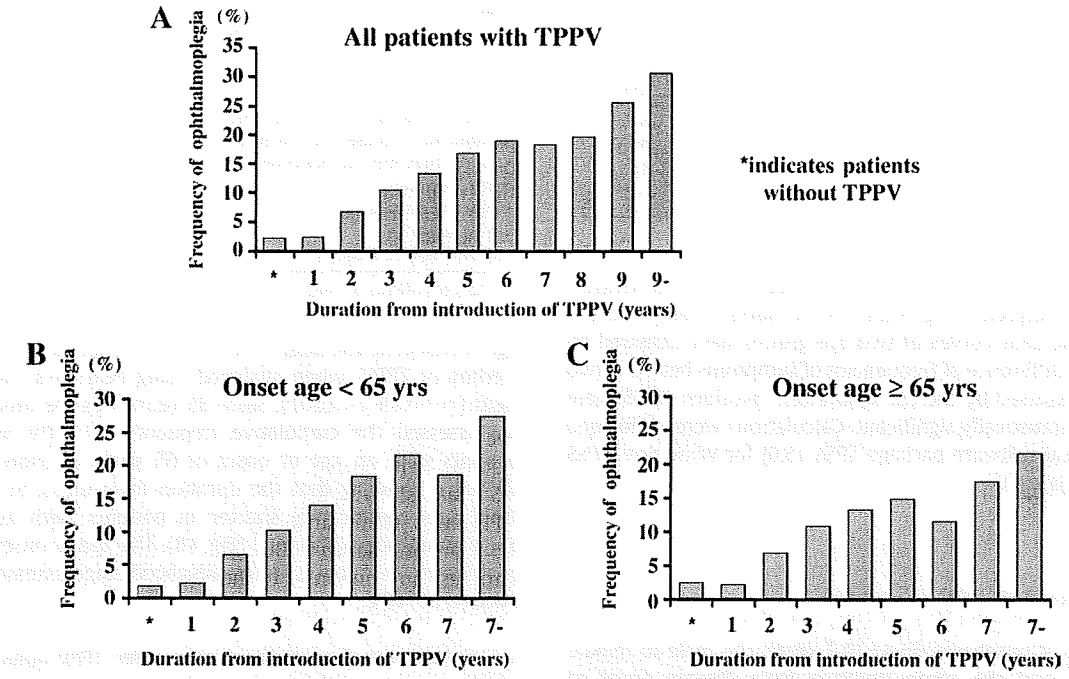

Fig. 2. Frequency of ophthalmoplegia in patients under TPPV, in terms of duration of TPPV and the influence of onset age on its appearance. Ophthalmoplegia rarely occurs in patients without TPPV (*), while its occurrence gradually increases with advanced duration of TPPV (A). Following 9 years of TPPV, almost 30% of patients show ophthalmoplegia. Frequencies of ophthalmoplegia in the patient population with onset age older or younger than 65 years are shown in B and C. Ophthalmoplegia is less frequent in patients with an age at onset of 65 years or older (C). The total frequency of ophthalmoplegia in the patients with onset age older than 65 years or younger than 65 years is 8.3% and 15.1%, respectively. A significant difference exists between them by the chi-square test ($p<0.001$). $n=2128$.

2.0% of the patients without TPPV. The frequency of ophthalmoplegia was increased with the advanced duration of TPPV (Fig. 2A). However, ophthalmoplegia was observed in 30% of patients under TPPV for more than 9 years.

The appearance of ophthalmoplegia under long-standing TPPV is also influenced by the age at onset (Fig. 2B,C). The patients with an age at onset under 65 years showed a higher frequency of appearance of oculomotor symptoms than those with an age at onset over 65 years (Fig. 2B,C). The total frequency of ophthalmoplegia in the patients under TPPV with an onset age of older than 65 years or younger than 65 years was 8.3% and 15.1%, respectively. A significant difference was found between them by the chi-square test ($p<0.001$). These observations suggest that a younger age at onset advances the appearance of ophthalmoplegia compared to patients with an older age at onset. The average time from onset to introduction of TPPV was, however, 1.86± 1.70 years in the patients with an onset age over 65 years, and 3.60± 3.72 years in those with an onset age of younger than 65. This difference influenced the appearance rate of ophthalmoplegia.

3.4. Age at onset influences the frequency of initial symptoms

We analyzed the relationships between the age at onset and the initial symptoms. Dysarthria and dysphagia as the initial symptoms were markedly increased in patients with an advanced age at onset

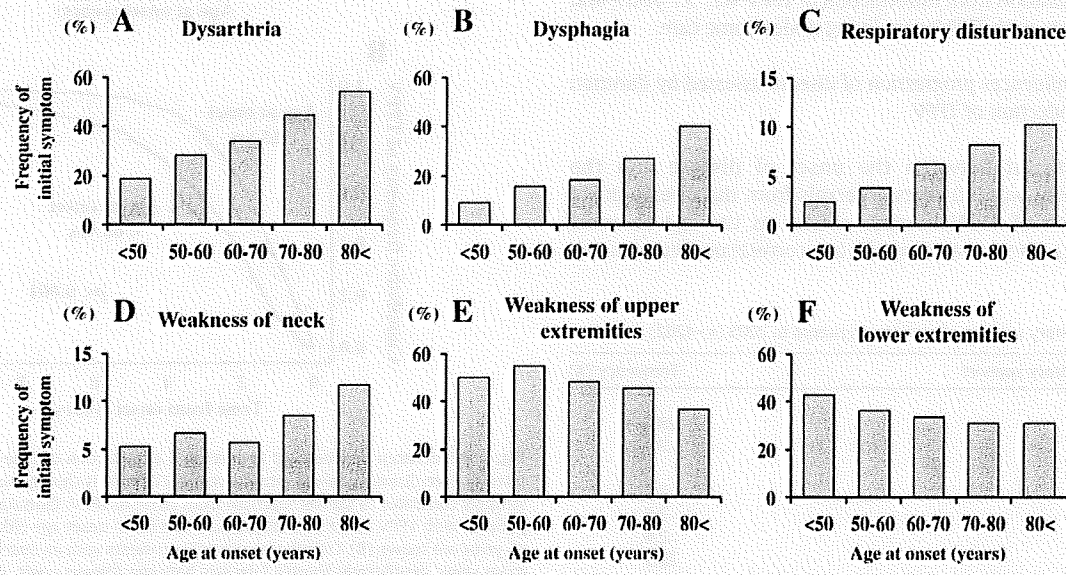

Fig. 3. Age at onset and frequency of initial symptoms. Dysarthria (A), dysphagia (B), respiratory disturbance (C) and weakness of neck (D) are increased in frequency as an initial symptom as the age at onset increases. In contrast, weakness of the upper extremities (E) or lower extremities (F) decreased as the onset age increases. $n=3428$.

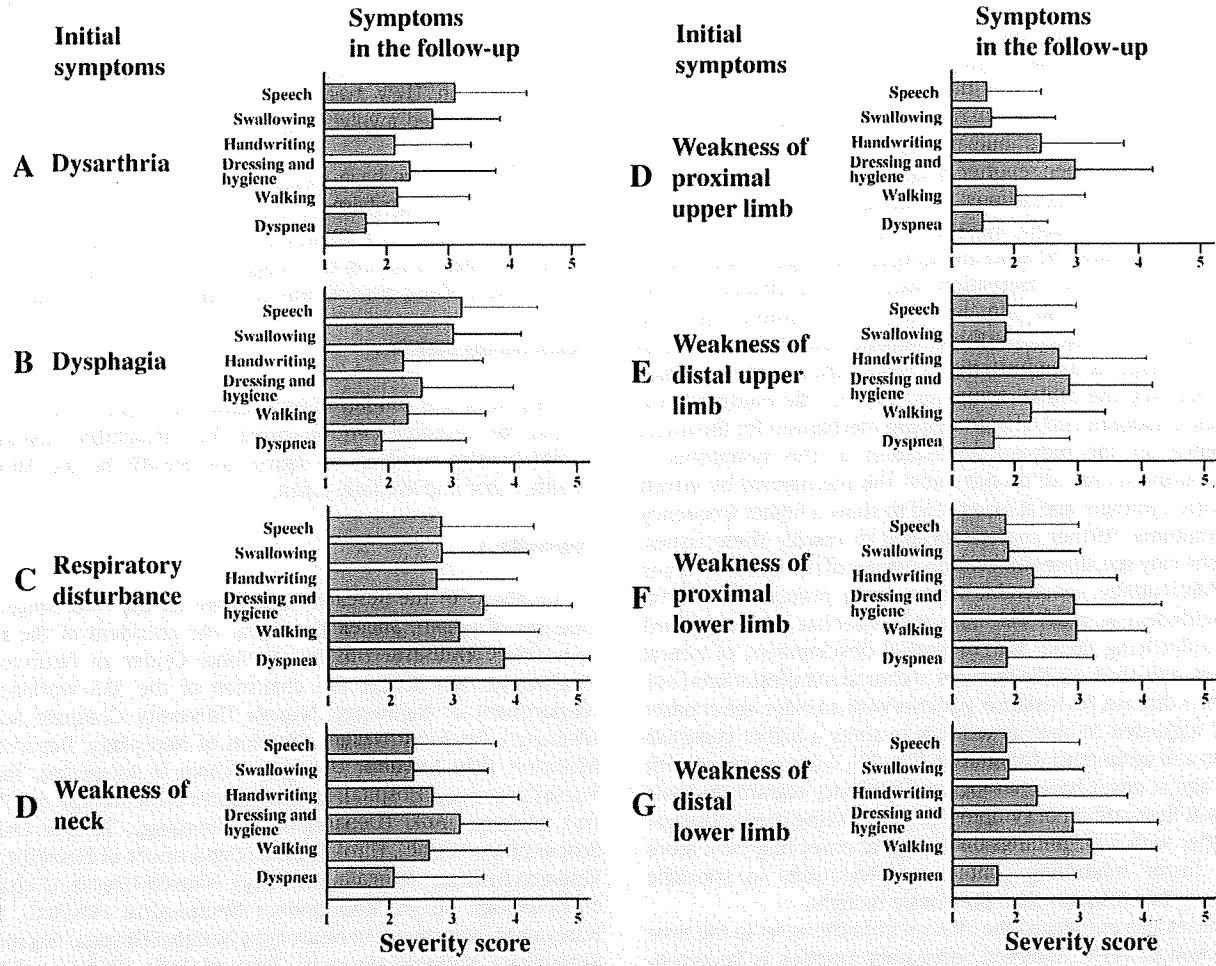

Fig. 4. Relationship between initial symptoms and symptoms at the follow-up stage. Severity scores of Speech, Swallowing, Handwriting, Dressing and Hygiene, Walking and Dyspnea are shown as subscales of ALSFRS-R. The score of "5" represents the most severe state, and "1" represents the absence of the symptom. Initial symptoms remain the most prominent or related symptoms even in the follow-up stage for 1.7±2.2 years from onset, suggesting that initial symptoms significantly determine the prominent features of symptoms throughout the disease course. $n=3428$.

(Fig. 3A,B). On the other hand, weakness in the upper or lower limbs as an initial symptom was seen more frequently in patients with a younger age at onset, and these frequencies gradually decreased with increasing age at onset. As for the respiratory disturbance and dropping head due to weakness of the neck muscles, the frequencies increased gradually with increasing age at onset. When we divided the patients between those with an onset age of older than 65 years and those younger than 65 years and analyzed the data with the chi-square test, the differences in frequencies of dysarthria, dysphagia, respiratory disturbance, weakness of upper extremities and weakness of lower extremities as initial symptoms were also significant between those groups ($p<0.001$, $p<0.001$, $p<0.001$, $p=0.001$, $p=0.019$, respectively). The difference in the frequency of neck weakness was not significant ($p=0.07$), although the tendency was apparent, and may be due to the small number of patients with neck weakness as an initial symptom. These observations suggest that age at onset is a determining factor of the features of the initial symptoms. Correlations between age at onset and the frequency of initial symptoms were similarly observed in the male and female patient groups (Supple. Fig. 1).

3.5. Initial symptoms determine major clinical features in follow-up stage

We examined the relationship between the initial symptoms and the symptoms assessed by 6 items of ALSFRS-R at examination at 1.7± 2.2 years after the onset (Fig. 4). At the follow-up stage, the patients who showed a bulbar symptom as an initial symptom showed speech or swallowing disturbance as a major symptom in the follow-up stage. Patients that showed respiratory disturbance as an initial symptom also showed dyspnea as the most prominent disturbance; patients with weakness of distal upper limb muscles showed the most prominent disturbance in handwriting and dressing; patients with weakness of proximal upper limbs showed prominent disturbance in dressing and hygiene; and patients with weakness of lower limbs, either proximal or distal, all showed a prominent disturbance in walking. These observations strongly suggested that the initial symptoms remained the most prominent or related symptoms even in the follow-up stage, and support the view that the initial symptoms determine the clinical features of the individual patient even in the follow-up stage. A similar tendency was observed in the male and female patient groups (Supple. Fig. 2).

4. Discussion

The results of the present study demonstrate the characteristic clinical profiles of Japanese sporadic ALS patients. A very high rate of Japanese ALS patients (29.3%) were under TPPV compared to patients in North America or Europe [10,11,17,18] which are 2.1–5.4%, respectively. The frequency of patients showing rare symptoms such as ophthalmoplegia increased with disease progression, particularly under long-standing TPPV.

A striking observation in the present study is that the age at onset greatly influences the wide-ranging clinical features, including the initial symptoms, progression to the endstage assessed by introduction of TPPV, and the frequency of rare symptom in the long-standing course. A higher incidence of bulbar involvement in patients with an older age at onset has been reported in some previous studies [19–23]. We extended these observations in that almost all of the initial symptoms, such as dysphagia, dysarthria, upper or lower limb weakness, respiratory failure and head dropping are strongly influenced by the age at onset. This observation was also confirmed in the subpopulation of male and female patients. In addition, since the initial symptoms also determine the prominent clinical phenotypes in the follow-up stage as demonstrated in this study, age at onset may influence not only the initial symptoms, but also the entire clinical phenotypes of sporadic ALS. The underlying mechanism for the onset age influence on the initial manifestation of the symptoms is unknown. Furthermore, we do not know the mechanism by which patients with a younger age at onset tend to show a higher frequency of rare symptoms. Further study is needed to resolve these issues, although one may speculate that subpopulations of the motor neurons may be differentially vulnerable to the aging process. In several sporadic neurodegenerative diseases, age at onset has been suggested to be an influencing factor for the spatial development of neural involvement, and, thus, for the features of clinical manifestations [24]. In Parkinson's disease, for instance, patients with an older age at onset have been suggested to have a tendency to show a higher cognitive dysfunction and autonomic dysfunction [25–27], whereas, those with a younger age at onset have an increased tendency toward dystonia and a diurnal fluctuation of symptoms [28,29]. Taking these observations together with our findings on ALS, age at onset may be a more important factor modifying clinical manifestations in sporadic neurodegenerative diseases than previously thought.

Age at onset also influenced the interval from the onset to the time of introduction of TPPV. Reserved respiratory function is known to decrease with advancing age [19]. Therefore, the short interval between the onset and the introduction of TPPV may be explained by the smaller reserved respiratory capacity in elderly patients. Indeed, serial examinations of the respiratory function in elderly patients start at a lower vital capacity and reach a critical point more quickly than younger patients [19,30]. It is congruent with the fact shown in the previous reports [1,3,5,6,22], that younger ALS patients survive longer than older patients.

Therefore, in taking into account the age at onset, initial symptoms, occurrence of rare symptoms and progression, the age at onset greatly affects the clinical profiles of sporadic ALS patients. In addition, the onset age-related initial symptoms are important to estimate the patient's prognosis as well as the design of clinical trials [31].

A high proportion of ALS patients in Japan are under TPPV compared to patients in other countries, possibly for social, cultural and economic reasons [13,17,18]. The presence of a subgroup of patients extending involvement to other systems beyond motor neurons, such as oculomotor, autonomic, sensory and higher functional systems, has been described in Japanese ALS patients under long-term TPPV treatment [32–36]. Pathologically, these patients show an extensive involvement of the tegmentum of the brainstem, substantia nigra, Clarke's dorsal nuclei and spinocerebellar tract, and frequent involvement of the thalamus and globus pallidus. Our present observations have confirmed these reports on sporadic Japanese ALS patients, particularly those with long-standing TPPV, and demonstrated that these subpopulations with a rare extension of involvements include almost 30% of the patients with 9 years or more under TPPV, particularly those assessed for oculomotor system involvement. However, further studies are needed to determine whether all the patients would eventually show an extended involvement beyond the motor system or whether these patients with an extended form are restricted to a given subpopulation. This is an important issue to determine the natural history of sporadic ALS. Since European and American ALS patients are not generally maintained on TPPV treatment for a longer period as Japanese patients, extended involvement is very rarely observed in Europe or North America.

In summary, we have presented the clinical profiles of sporadic Japanese ALS patients based on a large-scale sample. As demonstrated, age at onset may be a remarkable factor influencing wide-ranging clinical profiles including the progression and prognosis. We should take account of this observation in cohort studies or clinical trials.

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Appendix A

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Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jns.2008.09.024.

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ORIGINAL ARTICLE

Association of HTRA1 Mutations and Familial Ischemic Cerebral Small-Vessel Disease

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ABSTRACT

BACKGROUND

The genetic cause of cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL), which is characterized by ischemic, nonhypertensive, cerebral small-vessel disease with associated alopecia and spondylosis, is unclear.

METHODS

In five families with CARASIL, we carried out linkage analysis, fine mapping of the region implicated in the disease, and sequence analysis of a candidate gene. We also conducted functional analysis of wild-type and mutant gene products and measured the signaling by members of the transforming growth factor β (TGF-β) family and gene and protein expression in the small arteries in the cerebrum of two patients with CARASIL.

RESULTS

We found linkage of the disease to the 2.4-Mb region on chromosome 10q, which contains the HtrA serine protease 1 (HTRA1) gene. HTRA1 is a serine protease that represses signaling by TGF-β family members. Sequence analysis revealed two nonsense mutations and two missense mutations in HTRA1. The missense mutations and one of the nonsense mutations resulted in protein products that had comparatively low levels of protease activity and did not repress signaling by the TGF-β family. The other nonsense mutation resulted in the loss of HTRA1 protein by nonsense-mediated decay of messenger RNA. Immunohistochemical analysis of the cerebral small arteries in affected persons showed increased expression of the extra domain–A region of fibronectin and versican in the thickened tunica intima and of TGF-β1 in the tunica media.

CONCLUSIONS

CARASIL is associated with mutations in the HTRA1 gene. Our findings indicate a link between repressed inhibition of signaling by the TGF-β family and ischemic cerebral small-vessel disease, alopecia, and spondylosis.

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Hypertension is a well-known risk factor for nonhereditary cerebral small-vessel disease.[1] Genetic causes have been identified for hereditary cerebral small-vessel diseases — cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy,[2] autosomal dominant retinal vasculopathy with cerebral leukodystrophy,[3] brain small-vessel disease with hemorrhage,[4] and familial cerebral amyloid angiopathy.[5] Although arteriopathy in these cerebral small-vessel diseases is well documented, little is known about its genetic basis.

Cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL) is characterized by nonhypertensive cerebral small-vessel arteriopathy with subcortical infarcts, alopecia, and spondylosis, with an onset in early adulthood.[6-8] On neuropathological examination, arteriosclerosis associated with intimal thickening and dense collagen fibers, loss of vascular smooth-muscle cells, and hyaline degeneration of the tunica media has been observed in cerebral small arteries.[7-9] These pathological findings resemble those seen in patients with nonhereditary ischemic cerebral small-vessel disease.[7-11] We conducted a study to determine whether mutations in *HTRA1*, a gene encoding HtrA serine protease 1, cause CARASIL.

METHODS

SUBJECTS AND GENETIC ANALYSIS

We enrolled a total of six probands from six consanguineous families of Japanese ancestral origin and some of their family members. The first five families were included in a linkage analysis, and one member of the fifth family and one of the sixth family underwent neuropathological examination. Ancestry was reported by the participant or family members.

We isolated genomic DNA from 11 subjects from five of the families with CARASIL: 5 probands, 3 unaffected siblings, and 3 parents. We performed a genomewide linkage analysis using 763 microsatellite markers (Applied Biosystems). Pairwise lod scores were calculated with the MLINK program of the LINKAGE software package (version 5.2) and the FASTLINK package (version 4.1).[12,13] We established five new microsatellite markers — *M1236*, *M1238*, *M1241*, *M1260*, and *M1264* — on the basis of simple-repeat information from the University of California, Santa Cruz, Human Genome Browser. Primer sequences of these markers are summarized in the Supplementary Appendix (available with the full text of this article at NEJM.org). We designed primer pairs for amplification of the nine coding exons of *HTRA1*.

We isolated genomic DNA from all participants, including healthy persons of Japanese ancestral origin, as determined by means of self-report. These control subjects were between 74 and 90 years of age and had no signs of dementia, as defined by the Mini–Mental State Examination. We obtained fibroblast specimens from four controls and from Subject II-2 in Family 1.

We obtained written informed consent from the affected persons and their family members and written informed consent from the controls. The institutional review board of Niigata University approved this study.

ASSAY OF HTRA1 PROTEASE ACTIVITY

To express HTRA1 in *Escherichia coli* as fusions with glutathione *S*-transferase, we subcloned wild-type or mutant *HTRA1* complementary DNA (cDNA), lacking codons 1 through 140, into the vector pGEX 6P-3 (GE Healthcare). The N-terminus of HTRA1 is toxic to *E. coli*. Amino acid substitution of the serine protease motif S328A, which abolishes the protease activity in HTRA1, was used as a negative control.[14] Glutathione *S*-transferase fusion proteins were overexpressed and purified. Protease activity, measured as fluorescein isothiocyanate–labeled substrate β-casein, was evaluated with the use of a QuantiCleave Fluorescent Protease Assay Kit (Pierce) and recombinant glutathione *S*-transferase–HTRA1. To eliminate the possibility that a deletion of the N-terminus in HTRA1 affects protease activity, we also performed the identical protease assay using the serum-free medium containing cells stably expressing full-length wild-type or mutant HTRA1 tagged with a green fluorescent protein at the C-terminus. Green fluorescent protein–tagged HTRA1 proteins were detected by means of anti–green fluorescent protein antibody (Medical and Biological Laboratories, Nagoya, Japan).

To assay the formation of a stable complex with α_1-antitrypsin, we transiently expressed α_1-antitrypsin and either wild-type or mutated *HTRA1* cDNA with a simian virus 5 peptide (V5) tag at the C-terminus in the human embryonic kidney cell line HEK293. These cells were grown in serum-free medium, and samples of the resul-

tant conditioned medium were then immunoblotted with anti–V5 antibody.[14]

EXPRESSION OF *HTRA1* AND *NOG*

Total RNA was isolated from specimens of whole blood or cultured skin fibroblasts, and cDNA was synthesized with the use of the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). We assayed the expression of *HTRA1* messenger RNA (mRNA) in whole-blood samples by using gene-specific primers for *HTRA1*. To assay *HTRA1* mRNA levels in cultured skin fibroblasts in relation to the expression of glyceraldehyde-3-phosphate dehydrogenase, we performed a real-time quantitative reverse-transcriptase polymerase-chain-reaction (RT-PCR) assay by using specific TaqMan probes and primer sets (Applied Biosystems). We assayed mRNA levels of the noggin gene (*NOG*) in cultured skin fibroblasts in relation to the levels of β-actin by using real-time quantitative RT-PCR and SYBR Green (Applied Biosystems) (for details, see the Methods section of the Supplementary Appendix).

ASSAY OF SIGNALING BY TGF-β FAMILY PROTEINS

We used a site-directed mutagenesis system (GeneTailor, Invitrogen) to synthesize cDNA encoding mutant HTRA1 and cDNA encoding a constitutively active TGF-β1 proprotein (pro–TGF-β1 containing the activating amino acid mutations C223S and C225S).[15] We then individually subcloned this cDNA into the vector pcDNA DEST-40 (Invitrogen). Constitutively active TGF-β1 was synthesized from pro–TGF-β1 containing the activating mutations C223S and C225S. We isolated cDNA from the SMAD family member 2 gene (*SMAD2*), obtained from a library of human whole-brain cDNA (Clontech), and subcloned it into the pcDNA DEST-40 vector. Luciferase assays were performed as previously described.[16,17] Mouse C2C12 myoblasts (mesenchymal precursor cells, obtained from the American Type Culture Collection) were cotransfected with HTRA1-expression vectors, the pRL-TK renilla luciferase expression plasmid, and the following constructs: the $(\text{Smad binding element})_4$–firefly luciferase vector (TGF-β–responsive reporter vector) and vectors containing *SMAD2*, the SMAD family member 4 gene (*SMAD4*), and *TGFB1* (encoding pro–TGF-β1 with the two point mutations C223S and C225S)[15]; the pGL3-Id985WT–firefly luciferase vector (bone morphogenetic protein [BMP]–responsive reporter vector)[17] and vectors containing the SMAD family member 1 gene (*SMAD1*), *SMAD4*, and *BMP-4* (encoding pro–BMP-4); and the pGL3-Id985WT–firefly luciferase vector[17] and vectors containing *SMAD1*, *SMAD4*,

Table 1. Clinical Characteristics of the Six Probands with CARASIL.*

Characteristic	Proband and Family No.					
	II-2, Family 1	II-3, Family 2	II-1, Family 6	II-7, Family 3	II-3, Family 4	II-3, Family 5
Consanguinity of family	Yes	Yes	Yes	Yes	Yes	Yes
Mutation (nucleotide and amino acid)	1108C→T R370X	904C→T R302X	904C→T R302X	889G→A V297M	889G→A V297M	754G→A A252T
Sex	Female	Male	Female	Female	Male	Female
Age at time of study (yr)	44	28	46	50	33	48
Age at onset (yr)	18	16	14	16	14	Teens
Initial symptom	Alopecia	Alopecia	Alopecia	Alopecia	Alopecia	Lumbago
Leukoaraiosis on brain MRI	Yes	Yes	Yes	Yes	Yes	Yes
Alopecia, age at onset (yr)	18	16	14	16	14	—
Spondylosis, age at onset (yr)	21	21	29	39	33	Teens
Dementia, age at onset (yr)	35	37	29	50	33	—
Acute stroke, age at onset (yr)	—	31	—	—	—	38
Gait disturbance, age at onset (yr)	35	26	32	31	29	38
Pseudobulbar palsy, age at onset (yr)	35	26	32	50	33	38
Pyramidal sign, age at onset (yr)	35	27	32	50	29	48
Hypertension	No	No	No	No	No	No

* None of the patients had hypertension, any cancer, abnormalities in the retinal artery, or macular degeneration. CARASIL denotes cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy, and MRI magnetic resonance imaging.

and *BMP-2* (encoding pro–BMP-2).[18] Cell extracts were assayed for luciferase activity with the use of the Dual-Luciferase Reporter Assay System (Promega). The luciferase activity was corrected for transfection efficiency by dividing it by the pRL-TK renilla luciferase activity. Every sample was transfected in triplicate, and every experiment was repeated three times.

PHOSPHORYLATION OF SMAD PROTEINS

HEK293 cells were cotransfected with vectors containing *HTRA1* and the following constructs: vectors containing *SMAD2*, *SMAD4*, and *TGFB1* (encoding pro–TGF-β1 with two point mutations [C223S and C225S]); vectors containing *SMAD1*, *SMAD4*, and *BMP-4*; and vectors containing *SMAD1*, *SMAD4*, and *BMP-2*.[15,18] The cells were lysed in radioimmunoprecipitation assay buffer containing phosphatase inhibitor. We performed Western blotting to detect SMAD1, phosphorylated SMAD1, SMAD2, and phosphorylated SMAD2, using the corresponding anti-SMAD antibodies (Cell Signaling): anti-SMAD1, anti–phospho-SMAD1/5/8, anti-SMAD 2/3, and anti–phospho-SMAD2 antibodies.

IMMUNOHISTOCHEMICAL STUDIES AND IN SITU HYBRIDIZATION

We carried out immunoperoxidase staining on formalin-fixed, paraffin-embedded brain specimens obtained from two patients with CARASIL and from four controls (a 40-year-old woman with amyotrophic lateral sclerosis, an 84-year-old woman, a 62-year-old man with a stroke, and a 36-year-old woman with schizophrenia).[8,9] The primary antibodies used were those against TGF-β1 (1:50, Santa Cruz), against versican (1:100, Seikagaku), and against the extra domain–A region of fibronectin (1:100, Abcam). The negative control was prepared with the use of nonimmune IgG as the primary antibody. We used cDNA encoding the extra domain–A region of fibronectin (spanning nucleotides 5404 through 5704 of fibronectin isoform 1 [region NM_212482.1]) as a template for digoxigenin-labeled antisense and sense-complementary RNA probes. The sense probe was used as a negative control. We carried out in situ hybridization on the paraffin-embedded sections by using the probes. After the sections had been washed and blocked, they were incubated with alkaline phosphatase–conjugated anti-digoxigenin antibodies, stained with 4-nitroblue tetrazolium chloride–5-bromo-4-chloro-3-indolyl phosphate solution (Roche), and counterstained with fast red.

RESULTS

SUBJECTS

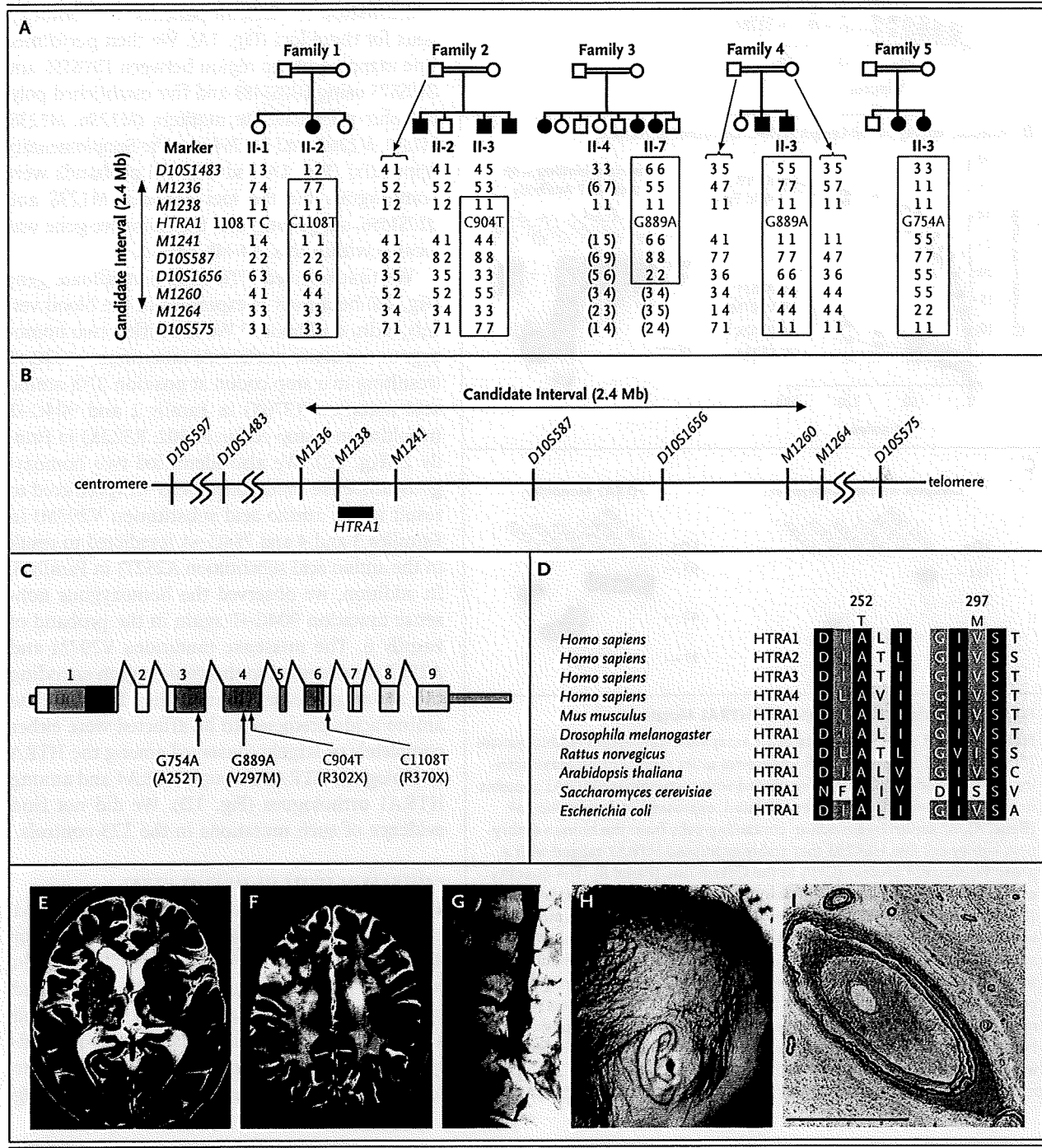
Clinical characteristics of the six probands with CARASIL are listed in Table 1, and the pedigrees are shown in Figure 1A. The patients in Families

Figure 1 (facing page). *HTRA1* Mutations in Families with Cerebral Autosomal Recessive Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CARASIL).

Panel A shows the pedigrees of families with CARASIL. Squares denote men; circles, women; solid symbols, affected family members; open symbols, unaffected members; and double horizontal lines, consanguineous marriage. Pairs of alleles of selected family members are listed for several microsatellite markers; the alleles are identified with the use of identification numbers 1 through 8. Genotypes whose haplotypic phases are unknown are shown in parentheses. Regions of homozygosity are delineated in boxes. Panel B shows the physical map of the candidate region for CARASIL on chromosome 10q. In Panels A and B, the microsatellite markers are listed in order from the centromere to the q-arm terminus; the candidate gene and the nucleotide mutations therein, carried by the probands, are indicated in red. Panel C shows the distribution of mutations in *HTRA1*, which consists of nine exons (squares): those encoding the insulin-like growth factor–binding protein domain (green), the Kazal-type serine protease inhibitor domain (red), the trypsin-like serine protease domain (blue), and the PDZ domain (yellow), as well as untranslated regions (gray). Nucleotide (and corresponding amino acid) mutations are listed — missense in black and nonsense in red. Panel D shows the conservation of mutated HTRA1 amino acid residues in patients with CARASIL and in nonhuman species. Conserved residues are shaded (black, 100% conserved; dark gray, 80% conserved; gray, 60% conserved). The T at position 252 and the M at position 297 are highlighted in red; mutations at these positions are either completely or largely conserved. Sequences were obtained from GenBank. The results of magnetic resonance imaging (MRI) in patients with CARASIL are shown in Panels E, F, and G. Panels E and F show T_2-weighted images of the brain of Subject II-7, Family 3 (repetition time, 5000 msec; echo time, 150 msec; section thickness, 5 mm), revealing an ischemic region in the basal ganglia and white matter. T_1-weighted lumbar MRI (repetition time, 519 msec; echo time, 19 msec; section thickness, 5 mm) revealed spondylotic changes of the lumbar spine in Subject II-3, Family 2 (Panel G). Diffuse hair loss in the temporal and parietal areas of the head was observed in Subject II-2, Family 1 (Panel H) at 48 years of age. Cerebral small arteries in the arachnoid mater from Subject II-1, Family 6, show marked intimal thickening, narrowing of the lumen, hyalinosis, and splitting of the internal elastic membrane (Panel I, elastic van Gieson stain; scale bar, 1 mm).

1 through 5 were enrolled initially, and the causative gene for CARASIL was identified. We then enrolled an additional subject with pathologically confirmed CARASIL, in a sixth family, to perform immunohistochemical analysis. On neuropathological examination of this additional subject, arteriosclerosis associated with intimal thickening and dense collagen fibers were observed in cerebral small arteries (Fig. 1I).

The probands from the first five families had diffuse white-matter lesions on magnetic resonance imaging (MRI), autosomal recessive inheritance, an onset of initial symptoms between the second and fifth decade, and spondylosis or alopecia (Table 1 and Fig. 1E through 1H).[6-8] Although the affected persons in Family 5 did not have alopecia and cognitive impairment, we enrolled them because they had diffuse white-



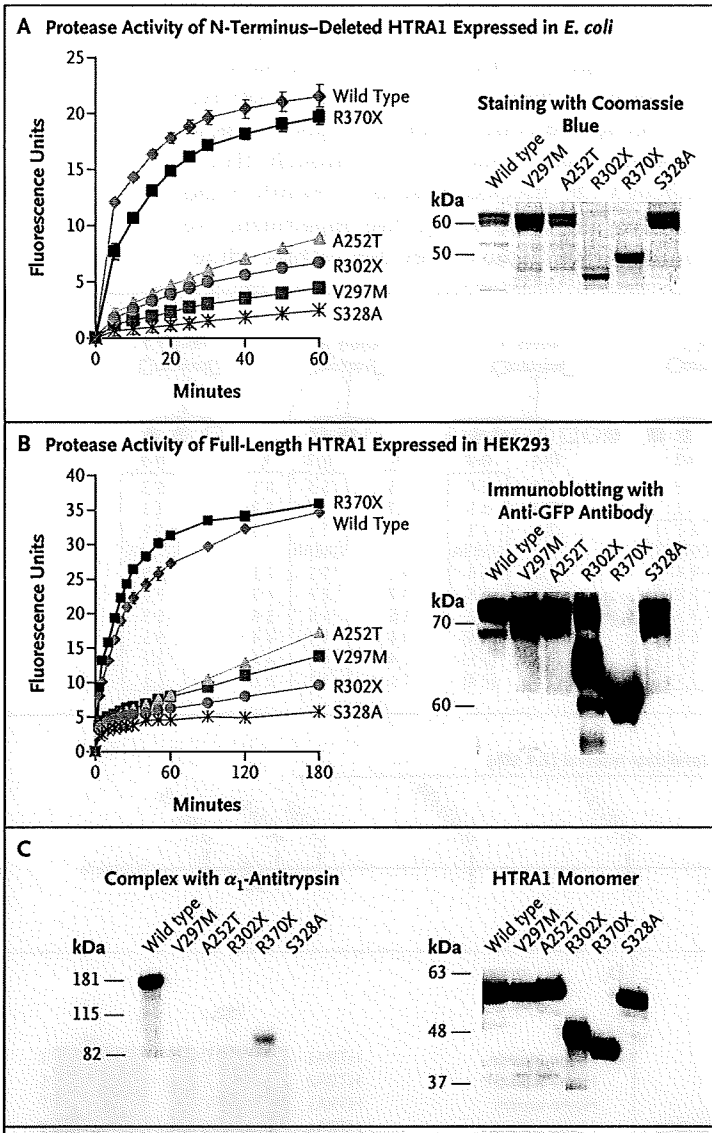


Figure 2. Functional Consequences of HTRA1 Mutations.

Panels A and B show the results of an assay with fluorescein isothiocyanate (FITC)–labeled β-casein. The fluorescence units represent protease activity. FITC-labeled β-casein was incubated with recombinant HTRA1 proteins in which the N-terminus has been deleted, expressed in *Escherichia coli* (Panel A), or serum-free medium containing cells from the human embryonic kidney cell line HEK293 that stably expressed HTRA1 tagged with a green fluorescent protein (GFP) at the C-terminus (Panel B). The quantity of HTRA1 protein was measured through staining with Coomassie blue (Panel A); the quality of the samples was ensured through immunoblotting with an anti-GFP antibody (Panel B). In Panels A and B, the I bars represent standard errors. Amino acid substitution of the serine protease motif S328A, which abolishes the protease activity in HTRA1, was used as a negative control.[14] Panel C (left) shows the formation of covalent complexes (high-molecular-weight products) between α_1-antitrypsin and wild-type or R370X HTRA1. The HTRA1 protein level was measured through immunoblotting with an anti–simian virus 5 antibody (right).

matter lesions on MRI and spondylosis, and one (a sibling of the proband in this family) had pathological findings identical to those in the patients with CARASIL.[8]

GENETIC ANALYSIS

From genomewide linkage analysis of the five families enrolled, we obtained maximal cumulative pairwise lod scores of 3.97 and 3.59 at *D10S587* and *D10S1656* (θ=0.0); all patients were homozygous for these loci (Fig. 1A). We then performed fine mapping of the region between *D10S597* and *D10S575* using *D10S1483* and five established polymorphic microsatellite markers (*M1236*, *M1238*, *M1241*, *M1260*, and *M1264*; see the Supplementary Appendix) (Fig. 1A and 1B). All probands were homozygous for the loci between *M1238* and *D10S1656*, suggesting that the causative gene was located within this 2.4-Mb region.

We first selected *HTRA1* as a candidate gene (Fig. 1B) because it is expressed in the blood vessels, skin, and bone.[19] We identified two homozygous nonsense nucleotide mutations: 1108C→T (resulting in a stop codon at position 370; amino acid mutation R370X) in Family 1 and 904C→T (resulting in a stop codon at 302; R302X) in Family 2 (Fig. 1C). We also identified two homozygous missense mutations: 889G→A (predicted to result in the amino acid substitution V297M) in Families 3 and 4 and 754G→A (predicted to result in the amino acid substitution A252T) in Family 5. In addition, we observed the homozygous nonsense mutation 904C→T again in the proband of Family 6. The missense mutations V297M and A252T were located in the genic region encoding the serine protease domain (Fig. 1C), and the amino acids predicted to be affected were either completely or largely conserved among the HTRA homologues HTRA1 through HTRA4 and among HTRA1 orthologues (Fig. 1D). We did not find evidence of such mutations in the 125 controls.

PROTEASE ACTIVITY OF MUTANT HTRA1

The level of protease activity in the mutant HTRA1 encoded by cDNA containing V297M, A252T, or R302X was 21% to 50% of the activity level in wild-type HTRA1. In contrast, HTRA1 encoded by a construct containing the R370X mutation had a protease activity level similar to that in wild-type HTRA1 (Fig. 2A and 2B). HTRA1 attacks the reactive center loop of α_1-antitrypsin, instigating

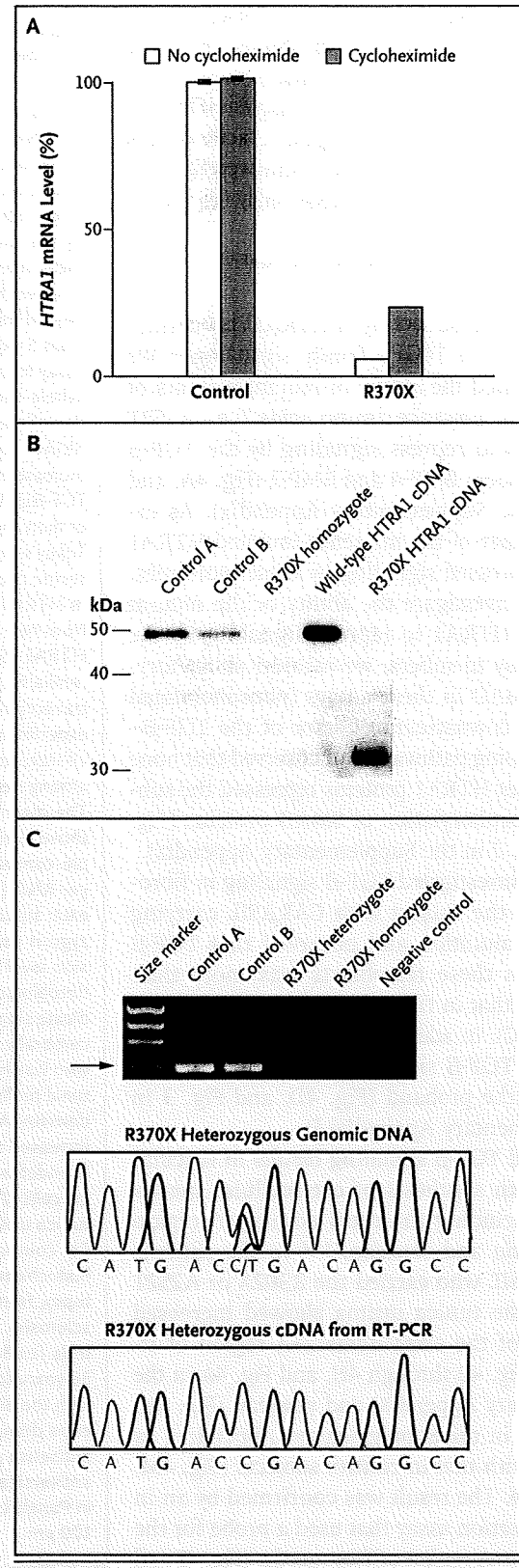


Figure 3. Nonsense-Mediated Decay of Mutant *HTRA1* Messenger RNA.

Panel A shows *HTRA1* messenger RNA (mRNA) levels in cultured skin fibroblasts from Subject II-2, Family 1, who is a homozygous carrier of R370X HTRA1, as a percentage of the average level of *HTRA1* mRNA in cells among four controls, with or without treatment with cycloheximide (100 μg per milliliter), an inhibitor of nonsense-mediated decay, for 4 hours. The I bars represent the standard errors. Panel B shows results of Western blotting of cultured skin fibroblasts obtained from Subject II-2, Family 1, the homozygous carrier of R370X, and two controls (A and B) with the use of an anti-HTRA1 antibody (MAB2916, R&D Systems). Wild-type and R370X complementary DNA (cDNA) was also assayed, for reference. Panel C shows the results of a reverse-transcriptase–polymerase-chain-reaction (RT-PCR) assay with the use of primers specific to the *HTRA1* sequence. We found PCR amplicons of the expected size (600 bp, arrow) with cDNA prepared from peripheral blood of Subject II-1, Family 1, an unaffected heterozygous carrier of R370X, but not with cDNA from Subject II-2, Family 1, the R370X homozygote. The negative control was a sample obtained from the heterozygous Subject II-1 that did not undergo reverse transcription. The electropherograms show wild-type and mutant (1108C→T) products derived from the genomic DNA of leukocytes from the heterozygous Subject II-1, whereas a wild-type allele only was detected in the RT-PCR products derived from the RNA of the leukocytes from the same subject.

the serine protease activity of α_1-antitrypsin, which thereby mediates the formation of a covalent complex between the two molecules.[14] We did not observe the formation of a stable complex between α_1-antitrypsin and mutant HTRA1 encoded by cDNA containing V297M, A252T, or R302X. In contrast, wild-type HTRA1 and HTRA1 encoded by cDNA containing R370X did form stable complexes with α_1-antitrypsin (Fig. 2C).

NONSENSE-MEDIATED DECAY

If a premature stop codon is located at least 50 to 55 nucleotides upstream of the exon–exon junction close to the 3′ end, mRNA may become degraded through nonsense-mediated decay.[20] Because the location of R370X fulfills this criterion for decay (Fig. 1C), we determined whether R370X-containing *HTRA1* mRNA is degraded by means of nonsense-mediated decay. The level of *HTRA1* mRNA expression in fibroblasts from the patient with the R370X mutation was 6.0% of that in fibroblasts from control subjects, and treatment with cycloheximide, an inhibitor of nonsense-mediated decay, increased this level by a factor of

four (Fig. 3A). We did not detect HTRA1 protein in the culture medium of fibroblasts from the patient carrying the R370X mutation (Subject II-2, Family 1) (Fig. 3B). Furthermore, analysis of *HTRA1* in leukocytes from a heterozygous carrier of this mutation (Subject II-1, Family 1) showed the presence of wild-type *HTRA1* mRNA only (Fig. 3C).

MUTANT HTRA1 AND SIGNALING BY TGF-β HOMOLOGUES

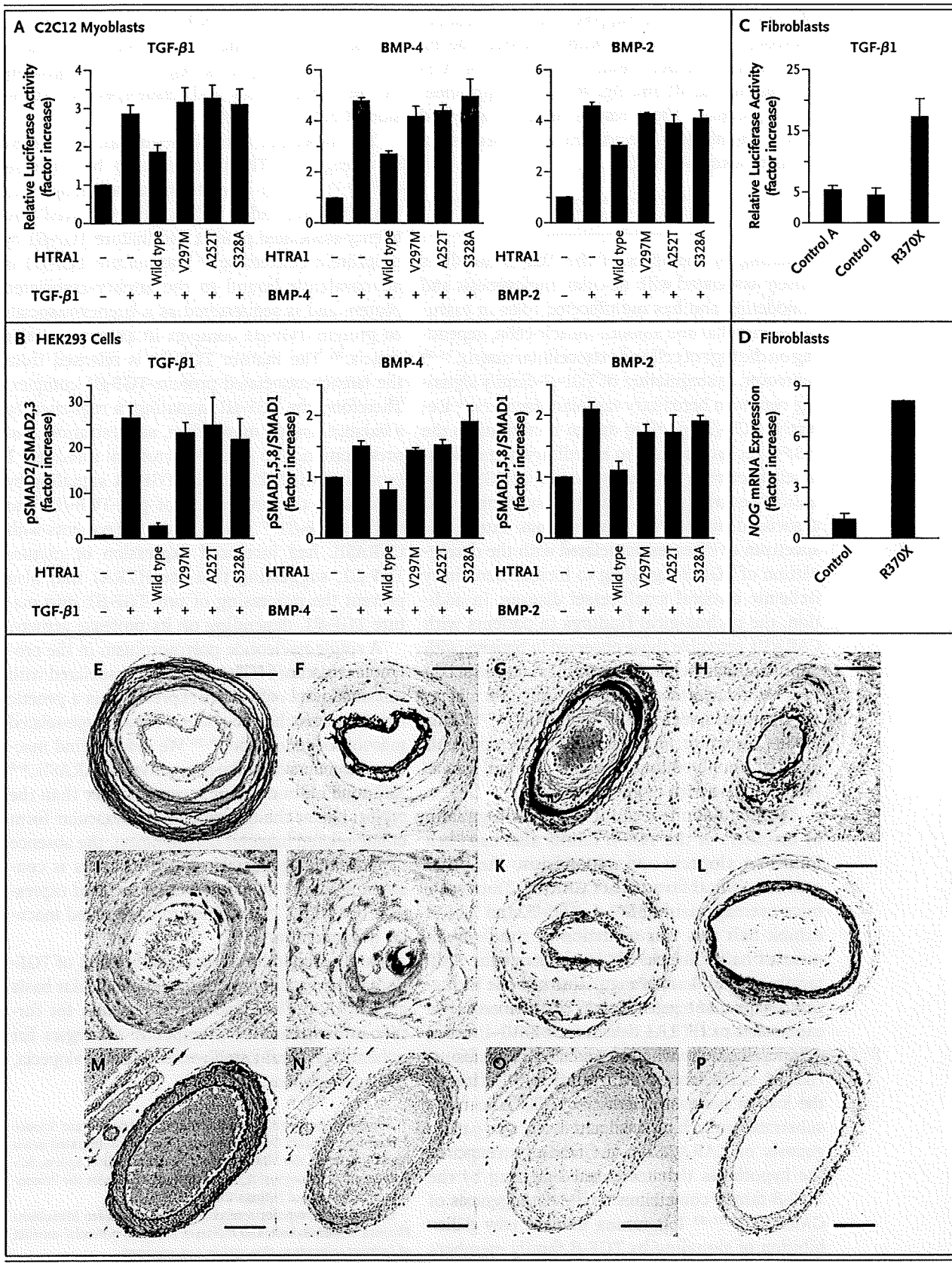
The serine protease activity of HTRA1 is necessary for inhibition of TGF-β family signaling.[16] We therefore tested the ability of mutant variants of HTRA1 with missense amino acids (i.e., A252T and V297M) to repress signaling by the TGF-β family members BMP-4 and BMP-2 (Fig. 4A, and Fig. 1 in the Supplementary Appendix). As expected, neither of the missense-mutated HTRA1 proteins repressed signaling by these molecules. To further investigate the ability of the mutant variants of HTRA1 to repress signaling by the TGF-β family members, we assayed phosphorylation of SMAD in these assays (phosphorylated SMAD is a downstream effector of the TGF-β–family signaling pathway), and observed that none of the mutant HTRA1 proteins repressed the subsequent phosphorylation of SMAD proteins (Fig. 4B, and Fig. 2 in the Supplementary Appendix).

We next investigated TGF-β signaling in fibroblasts from the subject with CARASIL carrying the R370X mutation and observed that TGF-β signaling in these fibroblasts was more than three times that in fibroblasts from control subjects (Fig. 4C). In addition, *NOG* mRNA, which is induced by TGF-β signaling in fibroblasts, was elevated in the proband (Fig. 4D, and Fig. 3 in the Supplementary Appendix).[21]

Increased TGF-β signaling results in vascular fibrosis, with synthesis of extracellular matrix proteins, including the extra domain–A region of fibronectin and versican.[22-24] In the patients with CARASIL who carried the R302X or A252T mutation, the tunica intima showed increased expression of the extra domain–A region of fibronectin (Fig. 4E through 4H, and Fig. 4A in the Supplementary Appendix) and versican (Fig. 4K, and Fig. 4B in the Supplementary Appendix), as compared with that in control subjects (Fig. 4M, 4N, and 4O). The result was confirmed by an in situ hybridization assay that used a probe for the extra domain–A region of fibronectin (Fig. 4I and

Figure 4 (facing page). Effect of Mutations on Transcription by Members of the Transforming Growth Factor β (TGF-β) Family.

Panel A shows the results of cotransfection of mouse C2C12 myoblasts with the pRL-TK renilla luciferase expression plasmid, the wild-type or a mutated HTRA1 expression plasmid, and other vector constructs designed to assay transcription by TGF-β1, bone morphogenetic protein (BMP-4 or BMP-2). Data are the means (from three independent experiments) of firefly luciferase activity, divided by the renilla luciferase activity, shown as the factor increase over the mean level among controls (negative for HTRA1 and TGF-β1). Panel B shows the results of cotransfection of the human embryonic kidney cell line HEK293 with the wild-type or a mutated HTRA1–simian virus 5 expression vector and other vector constructs designed to assay transcription by pro–TGF-β1, pro–BMP-4, or pro–BMP-2. Whole-cell lysates were immunoblotted. The data are the mean ratios of the expression levels of phosphorylated SMAD (pSMAD) and SMAD, among four independent experiments, shown as the factor increase over the mean ratio among controls (negative for HTRA1 and TGF-β1). The mean values for wild-type HTRA1 were significantly lower than those of the mutant HTRA1 ($P<0.05$ for all comparisons, by Tukey's multiple-comparison test). Amino acid substitution of the serine protease motif S328A, which abolishes the protease activity in HTRA1, was used as a negative control.[14] In Panel C, fibroblasts from two controls (A and B) and a homozygous carrier of R370X HTRA1 (Subject II-2, Family 1) were cotransfected with the pRL-TK renilla luciferase expression plasmid and vectors designed to assay transcription by TGF-β. Data are the means (from three independent experiments) of firefly luciferase activity, divided by the renilla luciferase activity, shown as the factor increase over the mean level among negative controls containing a reporter vector only (not shown). The mean values for the R370X cells are significantly higher than those in either control ($P<0.05$ for both comparisons, by Tukey's multiple-comparison test). Panel D shows noggin gene (*NOG*) messenger RNA (mRNA) levels (among three independent experiments for each subject) in fibroblasts from four controls and from the homozygous carrier of R370X HTRA1 (Subject II-2, Family 1). The expression level for Subject II-2 is shown as a ratio of the mean level in fibroblasts from four controls. In Panels A through D, the T bars indicate standard errors. Panels E through L show images of small cerebral arteries, obtained on autopsy, from Subject II-1, Family 6, who was homozygous for the R302X mutation. There was marked intimal proliferation (Panels E and G), increased expression of an extra domain–A region of fibronectin in the tunica intima (Panels F and H), increased mRNA expression of an extra domain–A region of fibronectin in endothelial cells (Panels I and J) and subendothelial smooth-muscle cells (Panel I), and increased expression of a versican in the tunica intima (Panel K) and TGF-β1 in the tunica media (Panel L). For comparison, Panels M, N, and P show images from immunohistochemical analysis of small cerebral arteries, obtained on autopsy, from a control (a 40-year-old woman with amyotrophic lateral sclerosis). The same results were obtained for three additional controls (an 84-year-old woman, a 62-year-old man with a stroke, and a 36-year-old woman with schizophrenia). Staining was performed with the elastic van Gieson stain in Panels E, G, and M; the anti–extra domain–A fibronectin antibody IST-9 in Panels F, H, and N; antisense probe for extra domain–A fibronectin in Panels I and J; antiversican antibody in Panels K and O; and anti–TGF-β1 antibody in Panels L and P. The scale bars in Panels E through P indicate 100 μm.



4J, and Fig. 5 in the Supplementary Appendix). Moreover, in the patients with CARASIL, the tunica media exhibited elevated expression of TGF-β1 (Fig. 4L and 4P, and Fig. 4C in the Supplementary Appendix). These results indicate increased TGF-β signaling in the cerebral small arteries in patients with CARASIL.

DISCUSSION

Signaling by members of the TGF-β family is closely associated with vascular angiogenesis and remodeling and has multifaceted roles in vascular endothelial and smooth-muscle cells, depending on the type of cell and extracellular matrix.[25,26] Moreover, dysregulation of TGF-β–family signaling results in hereditary vascular disorders.[26] Defective TGF-β signaling due to mutations in the TGF-β receptors leads to hereditary hemorrhagic telangiectasia, whereas activation of TGF-β signaling contributes to Marfan's syndrome and associated disorders.[26] Our findings extend the spectrum of diseases associated with the dysregulation of TGF-β signaling to include hereditary ischemic cerebral small-vessel disease. In addition, the pathological findings in patients with CARASIL resemble those observed in patients with nonhereditary ischemic cerebral small-vessel disease with hypertension, suggesting that hypertension may increase TGF-β signaling.[7-11,27] Thus, TGF-β signaling might underlie the molecular basis of nonhereditary ischemic cerebral small-vessel disease with hypertension.

Dysregulation of the inhibition of signaling by members of the TGF-β family also has been linked to alopecia and spondylosis, the other cardinal clinical features of CARASIL. Transgenic mice overexpressing BMP-4, BMP-2, and TGF-β exhibit hair loss or retardation of the development of hair follicles.[28,29] Members of the BMP family are well-known regulators of bone formation, repair, and regeneration.[30] Furthermore, overexpression of HTRA1 decreases BMP-2–induced mineralization, whereas reduced expression of HTRA1 accelerates mineralization.[31] Although the loss of protease activity by HTRA1 on other substrates may be associated with the pathogenesis of CARASIL, our findings strengthen the hypothesis that increased signaling by the TGF-β family contributes to the pathogenesis of CARASIL.[14,31-33] It remains unclear why disinhibition of signaling by TGF-β family members caused by mutant HTRA1 results in narrowly restricted clinical phenotypes. Possible explanations are tissue-specific regulation of signaling by the TGF-β family and tissue-specific expression of HTRA1.[14,19,33,34]

The molecular basis for regulation of TGF-β1 signaling by HTRA1 remains to be elucidated.[16,35,36] TGF-β1 is synthesized as a proprotein (pro–TGF-β1) and is subsequently cleaved into latency-associated protein and mature TGF-β1 by proprotein convertase.[26] The mature TGF-β1 is noncovalently bound to the latency-associated protein and is sequestered as a latency-associated protein–TGF-β1 complex in an extracellular matrix.[26] The mature TGF-β1 is released from the latency-associated protein–TGF-β1 complex. Therefore, the TGF-β1 signaling is regulated by a balance among maturation, sequestration, and presentation. The elastin microfibril interfacer 1 protein (EMILIN1) inhibits TGF-β1 signaling by preventing the processing of pro–TGF-β1 into mature TGF-β1.[37] In our study, the patients with CARASIL had increased expression of mature TGF-β1, suggesting that the HTRA1 may also prevent the processing of pro–TGF-β1 into mature TGF-β1, depending on its protease activity.

A single-nucleotide polymorphism in the promoter region of *HTRA1*, which is associated with elevated levels of HTRA1 expression, is a genetic risk factor for the neovascular form of age-related macular degeneration.[38,39] We did not find macular degeneration in the persons with CARASIL.[6-8] Although all our patients were younger than the typical age at the onset of the neovascular form of age-related macular degeneration, the absence of macular degeneration in the patients is consistent with the hypothesis that increased expression of HTRA1 contributes to age-related macular degeneration.[6-8,38]

Our results indicate that disinhibition of TGF-β–family signaling underlies the molecular basis of CARASIL. They also provide a basis for further investigation of therapeutic strategies for ischemic cerebral small-vessel disease, alopecia, and spondylosis.

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