Review

Difficulty in determining the association of a single nucleotide polymorphism in the ZNF512B gene with the risk and prognosis of amyotrophic lateral sclerosis

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Abstract: The advent of next-generation sequencing technology is expected to accelerate the identification of novel genes, and this technology will likely supersede Sanger sequencing. Thus, genome-wide association studies (GWASs) are performed more routinely in an effort to identify disease-susceptibility genes for sporadic amyotrophic lateral sclerosis (ALS). Previously, a Japanese team conducted a large-scale GWAS with 1,305 Japanese ALS patients and discovered a new single nucleotide polymorphism (SNP) rs2275294 associated with susceptibility to sporadic ALS (sALS) in the *ZNF512B* gene on chromosome 20q13.33. Ju *et al.* recently performed a case-control study to examine the possible association of rs2275294 with the risk of sALS. Their results, however, indicated that the SNP in *ZNF512B* is not associated with sALS susceptibility in the Chinese population. A precise diagnosis of neurodegenerative diseases, especially ALS, is highly challenging. For GWASs and other clinical research studies that require a large sample size, if true ALS patients are not selected initially, then all subsequent research is futile. Here, I evaluate the factors that are likely responsible for the inconsistent results obtained by GWASs and propose the development of a new classification system and diagnostic criteria for ALS as the first step towards conducting better clinical studies on ALS. I have attempted to explain the reasons for the inconsistent association between rs2275294 and ALS progression by listing the gene–gene/gene–environment interactions, age of onset, sample size, odds ratio, and inappropriate ALS diagnosis criteria for stratifying this heterogeneous disease in this review.

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Key words： amyotrophic lateral sclerosis (ALS), single nucleotide polymorphism (SNP), genome-wide association studies (GWASs), *ZNF512B*, criteria for diagnosis

Introduction

Amyotrophic lateral sclerosis (ALS), a delegate neurological malady, is an adult-onset neurodegenerative disease in which motor neurons specifically deteriorate, leading to death or the necessity of counterfeit ventilation at 3–4 years after onset. Currently, the number of patients in Japan has been assessed to be approximately 8,000–10,000; 10% of these patients have familial ALS, and 90% have sporadic ALS (sALS)[1)]. Although genome-wide data suggest genetic factors contribute to about 21% of all sALS cases[2)], it is not known how much of the remaining sporadic disease is genetic and how much is due to other factors, such as environmental exposure, ageing, or lifestyle choices. In addition, the disease pathology of sALS is not clearly defined. Therefore, the identification of pathogenesis-related genes, which are essential for elucidating the disease pathology, has become a major problem.

It is presumed that sALS, which occurs in the greater proportion of ALS patients, is triggered by numerous disease vulnerability genes and environmental components communicating in a complex way. The pathway from obtaining distinctive proof of genes and molecules to elucidating the pathology is much more complex for sALS than it is for familial ALS. The distinctive proof of genes being identified as being related to the onset and pathology of sALS occurs very late, in contrast to that of genes related to the onset and pathology of familial ALS. However, the recent introduction of cutting-edge sequencing technology has produced distinguishing proof of novel genes and

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supersedes Sanger sequencing. Previously, a Japanese group directed a very large-scale genome-wide association study (GWAS) in 1,305 Japanese ALS patients and found another single-nucleotide polymorphism (SNP) rs2275294 that was related to susceptibility to ALS in the *ZNF512B* gene on chromosome 20q13.33[3]. This investigation involving Japanese subjects was the main disclosure of the sALS susceptibility gene in East Asians; since then, other new genes have not been identified in similar regions (East Asians). Tetsuka *et al.* likewise revealed that the *ZNF512B* gene is a prognostic factor in sALS patients[4]. In addition, one case-control study suggested that the CC genotype and C allele at rs2275294 were associated with an increased risk for sALS in Han Chinese people, particularly in females[5]. However, Ju *et al.* recently performed a case-control study to examine the possible association of rs2275294 with the risk of sALS. Their results, however, indicated that the SNP in *ZNF512B* was not associated with sALS susceptibility in their Chinese population[6]. In this article, I review the reasons for the inconsistencies in GWASs, focusing on the different results related to the effect of this new SNP on sALS between Japanese GWASs and the large Chinese cohort. Additionally, I attempt to explain the reasons for the association with a prognostic factor in sALS between rs2275294 and ALS progression by listing various factors.

Susceptibility SNPs

SNPs are sections in the human genome where nucleotides (A, T, G, or C) can vary among people. The occurrence that just a single portion of the long succession is supplanted with another nucleobase is called a SNP (Fig. 1). SNPs occur generally in every 300 nucleotides, and because there are 3 billion nucleotides in the human genome, there are approximately 10 million SNPs. SNPs with a minor allele frequency of 5% or more are called common SNPs, SNPs with a frequency of 0.5% to 5% are called rare SNPs and SNPs with a frequency of less than 0.5% are called mutation SNPs. The graph in which the frequency of the polymorphism of a SNP is plotted on the horizontal axis and how that polymorphism affects the phenotype (odds ratio as a disease) is plotted on its vertical axis is quite famous (Fig. 2)[7]. Because SNPs are hereditary and shared by individuals of common descent, they can also be used as a way to track ancestry.

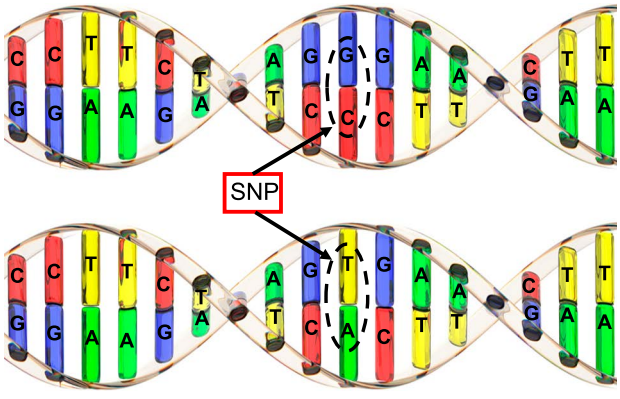

Fig. 1　The SNPs phenomenon is shown.
The upper DNA molecule differs from the lower DNA molecule at a single base-pair location (G/T polymorphism). A; adenine, T; thymine, G; guanine, C: cytosine, SNP: single nucleotide polymorphism.

The common disease-common variants hypothesis, which is based on the prediction that common disease-causing alleles or variants are found in all human populations that manifest a given disease, is commonly accepted when exploring the genetic factors of high-frequency sporadic diseases. If disease-causing mutations are derived from shared ancestral genes, it is thought that the neighbouring haplotype for the mutated chromosome currently remains and forms the locus of the causal gene and linkage disequilibrium (LD). This is a hypothesis that presupposes LD analysis. The homologous recombination of genes occurs when a chromosome is passed down to offspring. At that time, there are places where homologous recombination is likely to occur. These SNPs between hotspots act together with high probability, and SNPs that share this behaviour are said to be LD. With the aim of discovering the disease's susceptibility genes, we performed GWASs through the genome-wide analysis of common SNPs existing at frequencies higher than 5% in groups of healthy individuals. We found numerous disease-susceptibility genes via the GWAS, but we realized that their influence on disease onset was generally small and did not lead to a full understanding of pathogenesis. According to recent research, it is known that most genetic variations with large pathogenic effects occur at a low frequency.

There are three genotypes for one SNP. The reason for this is that to inherit the sequence of one from each father and mother, there are three combinations. For example, in the SNP mutation of G > T, there are three types of a polymorphism: GG, GT, and TT (Fig. 1). The risk allele is, for example, a SNP of A and G, where G is a risk for disease G against A and G is a risk allele (allele of the person who raises the risk of disease). In the abovementioned *ZNF512B* gene, because the risk allele is a C allele (rs2275294), the genotypes of ALS susceptibility SNPs are CC and CT[4].

GWASs on sALS

As a technique for distinguishing disease vulnerability genes in sporadic maladies, GWASs have been broadly performed. Association analysis, which evaluates the family of diseases that exhibit a Mendelian inheritance pattern, is a method for identifying the disease-susceptibility gene polymorphism itself or a polymorphic marker in strong LD. Due to differences in

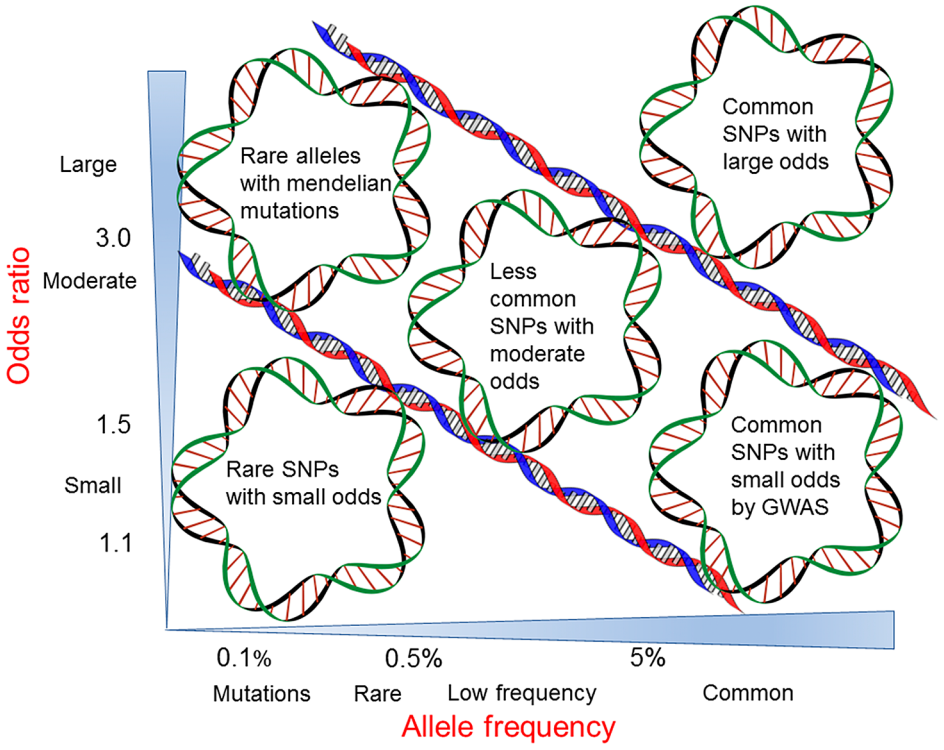

Fig. 2 Spectrum of disease allele effects (odds ratio).
Most emphasis and interest lies in identifying associations with characteristics shown within DNA lines. The range of genetic effects is visualized and divided by the odds ratio and allele frequency. Rare alleles with mendelian mutations are extremely rare and have large effect sizes (upper left). Most GWAS findings are associations of common SNPs with small effect sizes (lower right). SNP: single nucleotide polymorphism. The diagram is cited by modifying from the reference[7].

the allele frequency of the polymorphic marker between the unrelated sALS patient group and an unrelated healthy control group, the possibility exists that the disease-susceptibility gene is located in the vicinity of the polymorphic marker associated with the disease. This possibility was considered high[8]. In 2003, the whole human genome was deciphered[9], which led to the increased use of GWASs utilizing SNPs as polymorphic markers. However, because the LD detected by SNPs is limited to a narrow range of approximately several tens of kb, several SNP polymorphic markers must have high densities over the entire human genome. Furthermore, to withstand Bonferroni's correction for multiple tests among multiple markers, it is important to obtain a vast specimen measure for the objective diseases and control groups[8)10)]. Schymick *et al.* reported the first study on sALS in 2007, but it was a comparative study that was conducted using a relatively small number of sALS patients and controls[11)]. As a result of the Bonferroni correction, genes that were significantly related to sALS were not found, and the small sample size was considered a cause. Subsequent GWASs tended to increase the sample size and demonstrated the association of many genes or genomic regions, such as *ZNF512B*, *DPP6*, *ITPR2*, *UNC13A*, *FGGY*, and *KIFAP3*, with sALS[3)12)–18)]. In addition, *C21orf2*, *MOBP*, and *SCFD1* have recently been identified as new associated risk loci[19)]. There have been some achievements in utilizing GWAS in sALS (Table 1). Conversely, several analyses revealed that these results could not be reproduced by other team-conducted GWASs[20)–22)], as revealed in our studies[3)4)] and a Chinese study[6)]. The main indicators considered to be significant differences in GWASs are the odds ratio and pooled *P* value. It is generally thought that there is a significant difference when the *P* value is 5.0×10^{-8} or less in Bonferroni's correction.

SNPs as a prognostic factor in sALS patients

It is known that the age of onset, the site of symptom onset, and the period from symptom onset to diagnosis are factors related to the prognosis of sALS patients[23)–26)]. Chiò *et al.* reported that the SNP rs12608932, which is located in the intron of the *UNC13A* gene and is associated with susceptibility to sALS, significantly influences survival in Italian sALS patients[27)]; however, the mechanism of the effect of the *UNC13A* gene on survival with sALS is still unclear. In addition, Fogh *et al.* recently reported 2 loci in the *CAMTA1* gene influenced survival in patients with sALS[28)]. Conversely, *ZNF512B*, which was implicated as a gene regulating the onset of sALS, is an

Table 1　Main results reported by genome-wide in sporadic amyotrophic lateral sclerosis (ALS) Studies.

Gene	Location	SNP	Number of cases	Region or Race	Odds ratio (95% CI) or *P* Value	Ref.
ITPR2	12p12.1	rs2306677	461	Caucasian (Netherlands)	1.58 (1.30–1.91)	van Es et al. (12)
FGGY	1p32.1	rs6700125	386	Caucasian	1.35 (1.13–1.62)	Dunckley et al. (13)
DPP6	7q36.2	rs10260404	1,767	Caucasian	1.30 (1.18–1.43)	van Es et al. (14)
UNC13A	19p13.11	rs12608932	2,323	Caucasian	1.2	van Es et al. (15)
MOBKL2B	9p21.2	rs2814707	2,323	Caucasian	1.16	van Es et al. (15)
ELP3	8p21.1	D8S1820 in intron 10	1,884	Caucasian (UK)	$P = 1.96 \times 10^{-9}$	Simpson et al. (16)
KIFAP3	1q24.2	rs522444	1,821	Caucasian	$P = 1.84 \times 10^{-8}$	Landers et al. (17)
ZNF512B	20q13.33	rs2275294	1,305	Japanese	1.32 (1.21–1.44)	Iida et al. (3)
EPHA3	1q22	rs9295975 and rs28367780	117	Turkish	—	Uyan et al. (18)
C21orf2	Chromosome 21	rs75087725	1,861	Caucasian	1.45	van Rheenen et al. (19)
MOBP	3p22.1	rs616147	1,861	Caucasian	1.10	van Rheenen et al. (19)
SCFD1	14q12	rs10139154	1,861	Caucasian	1.09	van Rheenen et al. (19)

CI; confidence interval, OR; odds ratio, Ref.; reference, SNP; single-nucleotide polymorphism.

important factor in the prognosis of sALS[3]. The analysis of the *ZNF512B* gene in sALS revealed that the risk allele is independent of survival prognosis and other prognostic factors, such as sex, age of onset, period from onset to diagnosis, and site of symptom onset (bulbar/spinal cord)[4]. In addition, the mechanism of the effect of the *ZNF512B* gene on survival in sALS was indicated; specifically, the *ZNF512B* gene encodes for a transcription factor that promotes the expression of a downstream gene in the signal transduction pathway of transforming growth factor β (TGF-β), which is essential for the protection and survival of neurons. The TGF-β1, -β2, and -β3 isoforms are expressed by neurons and glial cells, and their receptors are expressed throughout the focal sensory framework. The mitigating activities of TGF-β can have a critical part in impacting neurogenic procedures, free of direct consequences for neural progenitor cells. The dysregulation of TGF-β signalling is thus recognized as a potential source of chronic inflammation. In fact, atypical TGF-β signalling and the resulting aggregation of actuated microglia in the neurogenic areas may assume a critical part in the movement of neurodegenerative disease. Several lines of evidence have shown that TGF-β signalling shielded neurons from glutamate-mediated excitotoxicity, which is a putative framework underlying the pathogenesis of ALS, and that interruption of TGF-β signalling due to the transcriptional dysregulation of its receptor is related to polyglutamine-induced motor neuron harm in spinal and bulbar muscular atrophy. TGF-β signalling is an important molecular event in the pathogenesis of motor neuron diseases[29]. In addition, the activation of the TGF-β signalling system is protective against the aggregate formation of the cytoplasmically mislocalized TAR DNA-binding protein of 43 kDa and may be a potential therapeutic approach to delay the progression of ALS[30]. Endo *et al.* showed that TGF-β, which is upregulated in the astrocytes of ALS patients and mice, is a negative regulator of neuroprotective inflammatory responses[31]. However, *ZNF512B* gene expression is reduced in sALS patients with the risk (C) allele of the new SNP, and their serum and plasma TGF-β levels are decreased, resulting in the weakening of their neuronal protection signals (Fig. 3). Thus, the survival probability of sALS patients with the risk (C) allele decreases[4].

Factors explaining inconsistent sALS results in GWASs

GWASs of ALS do not often replicate successfully, which suggests that the ALS phenotype is driven not by a single locus but by many genes that elevate risk cumulatively. The differences in the population samples or SNP datasets are considered primarily responsible for inconsistent GWAS results. Several reasons for the negative association found in the replication analyses are possible. In the first place, ALS might be a more genetically and clinically heterogeneous disease among various populaces than perceived previously[32]. Additionally,

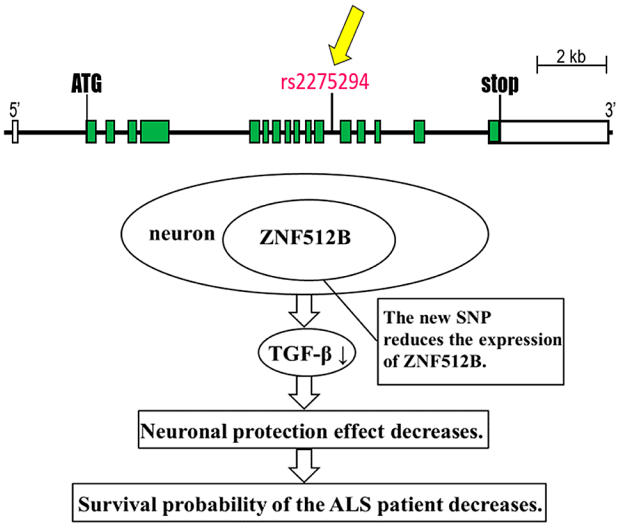

Fig. 3 The amyotrophic lateral sclerosis (ALS)-susceptibility single nucleotide polymorphism (SNP) rs2275294 was localized to intron 12 of ZNF512B.

The mechanism by which the ZNF512B gene might serve as a prognostic factor in ALS patients: In patients with the newly discovered SNP (rs2275294), ZNF512B gene expression is reduced, which results in the weakening of the neuronal protection signals by TGF-β and, therefore, a reduction in the survival probability of the ALS patients with the risk allele[4].

because of the reciprocities of unknown specific gene–gene or gene–environment interactions at different ages of onset, the influences of some risk alleles that have been identified by GWASs may be population-specific. Due to various environmental and occupational elements, the age of onset for ALS patients from Guangzhou is lower than that of ALS patients from Shanghai[33]. The sample size is essential, and a small specimen size can bring about an inadequate force, thereby producing errors in the results[34]. This is a particular problem in genetic studies of sALS because the disease is rare and because it is difficult for individual groups to achieve adequate sample sizes. There is also an issue in transethnic studies. For example, the findings for the *MOBKL2B* and *UNC13A* genes, which are most significantly associated with sALS in Caucasians, are inconclusive in Asian populations because the association of these gene with susceptibility to sALS is considered negative and has shown no evidence of association in Japanese and Chinese by GWAS[35]. In addition, the odds ratio is too low to make a significant difference (odds ratio: 1.1–1.5). Genetic studies showed that the effects of modifying genes on progression might depend on environmental factors that are potentially involved in the causation of ALS[28].

Other factors that may cause inconsistent GWAS results

Generally, obtaining a definitive diagnosis is very difficult for neurodegenerative diseases compared to other common diseases such as diabetes mellitus. For example, obtaining a definitive diagnosis of diabetes mellitus using a blood test is relatively easy. To date, more than 80 susceptibility SNPs for type 2 diabetes have been identified through GWAS[36], and the practical use of SNPs in genome and personal medical therapy has developed. In the field of genetic research of neurodegenerative diseases, SNPs are not yet used so broadly. In GWASs and other types of clinical research on ALS, ALS patients are included according to the El Escorial revised criteria[37]. Recently, these criteria have been questioned for their adequacy in diagnosing ALS, and they could be unsuitable for clinical research[38][39]. Therefore, the El Escorial criteria and their revisions are limited in scope and are not suitable for all applications. ALS also has various phenotypic classifications (Table 2), and the term ALS is used in clinical practice to both diagnose and describe a particular phenotype distinct from progressive muscular atrophy (PMA), primary lateral sclerosis (PLS), bulbar palsy, and other clinical presentations. Additionally, for the differential diagnosis of ALS, there is the potential risk of including other motor neuron diseases such as hereditary motor neuropathy, Charcot-Marie-Tooth disease, spinal-bulbar muscular atrophy, adult-onset spinal muscular atrophy type IV and even myopathies such as inclusion body myositis, although such diseases usually have slow progression. The El Escorial diagnostic criteria and their revisions describe the certainty that the phenotype is ALS as opposed to PMA or PLS, and the terms possible, probable, and definite refer to the severity of clinical presentation as a result of

Table 2 ALS phenotypes.

Type	Characteristic
ALS	Mixed UMN and LMN signs in limbs and bulbar region
PMA	Pure LMN syndrome, generally asymmetrical
Primary lateral sclerosis	Pure UMN syndrome
Progressive bulbar palsy	Isolated UMN or LMN signs, or both, confined to bulbar musculature
Flail arm syndrome	Symmetrical, proximal, largely LMN weakness of upper limbs
Flail leg syndrome	Symmetrical, distal, largely LMN weakness of lower limbs
Respiratory-onset ALS	Pure LMN syndrome, weakness of the respiratory muscles

ALS; amyotrophic lateral sclerosis, PMA; progressive muscular atrophy, UMN; upper motor neuron, LMN; lower motor neuron.

the pathology involved and not to the underlying diagnosis of ALS. Moreover, the existing diagnostic criteria of definite, probable, and possible ALS require at least one upper motor neuron (UMN) sign. However, I believe that some patients with ALS or motor neuron disease may not fulfil the criteria[39]. Cases of adult-onset motor neuron diseases that exhibit only lower motor neuron (LMN) signs can be diagnosed as PMA, for which the disease progression during the early stage of the disease is relentlessly progressive and leads to death due to respiratory insufficiency within a few years[40)–42)]. Therefore, PMA should be considered a form of ALS. Considering these concerns, it is evident that in GWASs or clinical research requiring large sample sizes, if ALS patients are not accurately selected initially, then all subsequent research is futile. For research purposes, a detailed classification of ALS is important. A precise diagnosis of neurodegenerative diseases, especially ALS, is highly challenging. Al-Chalabi *et al.* suggested that the El Escorial criteria are no longer sufficient and that a dramatic change in ALS classification is necessary[38]. Herein, I have proposed the development of a new classification system and diagnostic criteria for ALS as the first step towards conducting better clinical studies on ALS. It is generally accepted that the time from ALS onset to death due to respiratory insufficiency is 3–5 years. Tetsuka[39] showed that of the 296 patients who were diagnosed with ALS or motor neuron disease within the past 15 years and were retrospectively investigated and analysed, 108 patients (36.5%) exhibited LMN signs but not UMN signs at the early stage of the disease and were thus diagnosed with ALS or motor neuron disease (Fig. 4A). Moreover, no significant difference in the survival probability was observed between patients who exhibited both UMN and LMN signs and patients who exhibited only LMN signs within 5 years (Fig. 4B). To date, some similar original studies have been conducted[40)–47)]. Histopathologic study has shown pyramidal tract degeneration in more than half of all patients with purely LMN signs who were clinically diagnosed while alive with PMA[48]. These findings suggest that some patients with motor neuron disease exhibiting only LMN signs who die within 5 years could be diagnosed with ALS. Thus, the 5-year rule may be applicable not only to diagnosing ALS clinically but also to clinical ALS research, such as through GWAS. A limitation of this review is that there is not yet sufficient evidence; thus, the addition of some data or

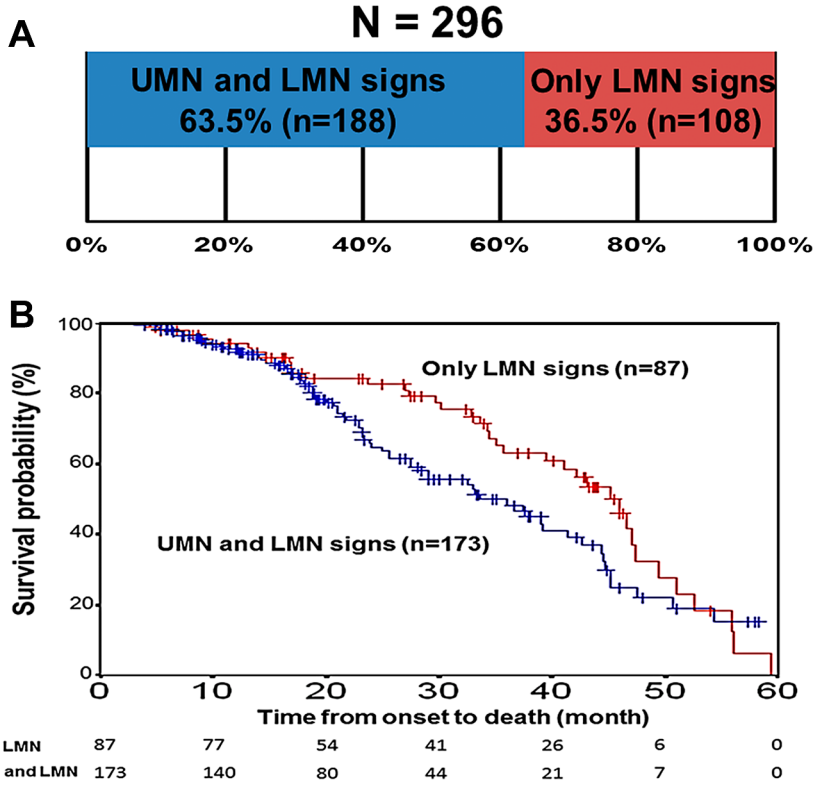

Fig. 4　The percentages of characteristic in motor neuron disease patients and Kaplan-Meier survival curves for patients with motor neuron disease.

(A) Motor neuron disease patient's percentages according to motor neuron signs (UMN: upper motor neuron; LMN: lower motor neuron). (B) Time from onset to death was limited to 5 years by examination. The curves of 260 patients (173 with both UMN and LMN signs and 87 with only LMN signs) with vs. without UMN signs did not reveal any difference (log-rank test, $P = 0.06$). The figure is cited by modifying from the reference 39.

methods to support my analysis from previous other studies on SNPs and ALS development are required. However, these analyses are my additional perspective in an attempt to define what that ALS classification system would look like.

Conclusion

Several factors may be invoked to explain why many GWAS results have not been replicated. Most ALS-causative variants are limited to a small number of patients at present. Otherwise, we must diagnose many ALS patients only using the El Escorial diagnostic criteria, without an autopsy, to conduct a GWAS, but this method may be inappropriate. The diagnostic criteria are currently fluctuating, and revisions are required. For research purposes, a detailed classification and accurate diagnostic criteria of ALS are important to enable a precise identification of the SNP associated with susceptibility to sALS. I have attempted to explain the reasons for the inconsistent association between rs2275294 and ALS progression by listing the gene–gene/gene–environment interactions, age onset, sample size, odds ratio, and inappropriate ALS diagnosis criteria to stratify this heterogeneous disease in this review.

※The author declare there is no conflict of interest relevant to this article.

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