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Phylogenetic analysis of microRNA biomarkers for amyotrophic lateral sclerosis

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Abstract: Amyotrophic lateral sclerosis (ALS), also called Lou Gehrig's disease, is an irreversible disease that is caused by the degeneration and death of motor neurons. Approximately 5–10% of cases are familial ALS (fALS), and the other cases are sporadic ALS (sALS). Gene mutations have been identified both in fALS and sALS patients. In this study, we discuss the four ALS-related genes, C9orf72, SOD1, FUS, and TARDBP, and review the microRNAs (miRNAs) that are associated with ALS and other neurological disorders from the literature. A phylogenetic analysis is used to explore potential miRNAs that can be taken into account when studying the difference in pathology for ALS induced by the four genes and other neurological diseases such as frontotemporal dementia, spinal muscular atrophy, and narcolepsy. We found several miRNAs that can be taken into account to study the difference in pathology between ALS and other neurological disorders.

Introduction

Amyotrophic lateral sclerosis (ALS) is an irreversible disease that may begin with limb weakness or with difficulty swallowing or speaking and gradually lead to the loss of voluntary muscle movement. This disease was discovered in the 19th century (Rowland, 2001; Visser *et al.*, 2008). It is also called Lou Gehrig's disease because the American baseball player Lou Gehrig was diagnosed with ALS in the 1930s. Not all ALS patients experience the same disease course, but progressive paralysis is commonly experienced. The mean survival time with ALS is less than five years, but 14% of the cases live longer than five years (Mateen *et al.*, 2010). The motor neurons in the brain are called upper motor neurons, and those in the spinal cord are called lower motor neurons. Motor neurons control muscle movement. The upper motor neurons transmit nerve impulses to lower motor neurons, and the lower motor neurons send nerve signals to muscles. In ALS, both the upper motor neurons and the lower motor neurons degenerate or die and stop sending messages to the muscles.

Approximately 5–10% of cases, called familial ALS (fALS), are inherited from family members, and they are caused by genetic mutations (Kurland and Mulder, 1955). Around 90% of patients are called sporadic ALS (sALS). Gene mutations have been identified in fALS and sALS patients (Sreedharan *et al.*, 2008; Vance *et al.*, 2009; DeJesus-Hernandez *et al.*, 2011; Chen *et al.*, 2013), especially the mutations of the four genes chromosome 9 open reading frame 72 (C9orf72), superoxide dismutase 1 (SOD1), fused in sarcoma (FUS), and TARDBP. Genetic defects occur in about 20–30% of fALS cases (Maruyama *et al.*, 2010). Among those, 20% are caused by a mutation in the SOD1 gene, 4–5% are the results of mutations in TARDBP and FUS genes, more than 30% are associated with C9orf72 mutations, and the rest are associated with other known or unknown genes (Chen *et al.*, 2013). Most sALS cases are caused by unknown factors. A small fraction of sALS is caused by the four genes C9orf72, SOD1, FUS, and TARDBP (Turner *et al.*, 2013). Besides gene mutations, environmental factors contribute to disease liability (Oskarsson *et al.*, 2015).

In addition to gene mutations involved in the pathology of ALS, microRNA (miRNA) biomarkers have been identified for ALS. A miRNA is a small single-stranded non-coding RNA that functions in the epigenetic control of gene expression (Wu *et al.*, 2010). miRNAs were shown to be linked to many diseases, including cancer, periodontal disease, neurodegenerative diseases, hematological diseases, and autoimmune diseases (Alevizos and Illei, 2010; Lee *et al.*, 2011; Grasedieck *et al.*, 2013; Maciotta Rolandin *et al.*, 2013; Hsieh *et al.*, 2014; Wang, 2016a; Takuse *et al.*, 2017; Ricci *et al.*, 2018; Taguchi and Wang, 2018a; Taguchi and Wang, 2018b; Chen and Wang, 2020b; Wang, 2020).

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Dysregulation of miRNAs might play an important role in the pathogenesis of multiple forms of human ALS (Emde *et al.*, 2015). In this study, miRNA biomarkers for ALS and other neurological diseases are discussed. Then, we cluster these miRNA biomarkers to find potential miRNAs that can be used to investigate the difference of pathology between ALS and other neurological diseases.

The method used in this study is first to find miRNA biomarkers from the literature for ALS and other neurological diseases, respectively. These neurological diseases include frontotemporal dementia, Parkinson's disease, Alzheimer's disease, spinal muscular atrophy, Prader–Willi syndrome, Niemann–Pick disease, neurofibromatosis, narcolepsy, Friedreich's ataxia, and ataxia-telangiectasia. Then, we cluster the biomarker sequences for ALS and neurological diseases by plotting phylogenetic trees based on different evolutionary models. Since a large proportion of fALS can be linked to one of the four genes (Turner *et al.*, 2013), we explore potential miRNAs to study the difference in pathology for ALS induced by these four genes and other neurological diseases. The phylogenetic tree is one of the useful tools of the phylogenetic analysis (Graur and Li, 2000; Wang and Hung, 2012), which has been successfully used in finding cancer miRNA biomarkers (Wang, 2016b). The phylogenetic tree combined with the microarray analysis can increase the accuracy of the miRNA biomarker prediction of cancer compared with the method only using microarray analysis (Wang, 2016b).

Four genes related to ALS

Many genes were shown to be associated with ALS. In this study, we mainly focus on the four genes C9orf72, SOD1, FUS, and TARDBP. We briefly describe these genes.

The C9orf72 gene is located on the short arm of chromosome 9, open reading frame 72. The protein is abundant in neurons of the brain and motor neurons of the spinal cord. The mutation of C9orf72 was found to be associated with both ALS and frontotemporal dementia (FTD) (DeJesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011; Majounie *et al.*, 2012). The mutation is a hexanucleotide repeat expansion of the six nucleotides GGGGCC. Healthy subjects carry 2-10 hexanucleotide GGGGCC repeats in the C9orf72 gene, while more than a few hundred repeats represent a risk for ALS. Mutations in C9orf72 account for 20–40% of fALS. It occurs more often in patients older than 50 years old.

The SOD1 gene is located on chromosome 21. SOD1 is an enzyme that can destroy free superoxide radicals in the body. In 1993, genetic mutations in the SOD1 gene were found to be linked to fALS (Rosen *et al.*, 1993). This is the first discovery of a genetic link to ALS. More than 160 different disease-associated mutations have been found in SOD1. Toxic effects caused by the mutations in the SOD1 gene are involved in ALS pathogenesis (Sangwan and Eisenberg, 2016).

The FUS gene is located on chromosome 16. The RNA-binding protein fused in sarcoma/translocated in sarcoma (FUS/TLS) is encoded by the FUS gene. Mutations in the FUS/TLS gene were discovered to cause fALS (Kwiatkowski *et al.*, 2009; Vance *et al.*, 2009). Mutations in FUS account for 5% of fALS (Shang and Huang, 2016). In addition, FUS is related to FTD for sporadic cases or familial cases (Neumann *et al.*, 2009; Zhou *et al.*, 2014; Bradfield *et al.*, 2017).

TARDBP is a gene located on chromosome 1, which encodes TAR DNA-binding protein 43 (TDP-43). Frontotemporal lobar degeneration is associated with tau, TDP-43, or FET protein accumulation (Mackenzie and Neumann, 2016). TDP-43 proteinopathy is associated with chronic traumatic encephalopathy (CTE) (McKee *et al.*, 2010; Jayakumar *et al.*, 2017). The abnormalities of TDP-43 are correlated with the clinical features of Alzheimer's disease (Tremblay *et al.*, 2011). Evidence suggests a pathophysiological link between TDP-43 and ALS (Sreedharan *et al.*, 2008). Mutations in TARDBP account for 5% of fALS.

MicroRNA biomarkers

Several ALS miRNA biomarkers related to C9orf72, SOD1, FUS, and TARDBP were discussed in the literature. From a regulatory network analysis, TDP-43 and C9orf72 are possible targets of miR-142-3p (Matamala *et al.*, 2018). In cerebrospinal fluid samples of sALS patients, five TDP-43 binding miRNAs, miR-132-5p, -132-3p, -143-3p, -143-5p and -574-5p, were significantly dysregulated (Freischmidt *et al.*, 2013). Downregulation of miR-132-5p/3p and miR-574-5p/3p was evident in TARDBP, FUS, and C9ORF72, but not SOD1 mutant patients (Freischmidt *et al.*, 2013). Let-7b levels are significantly reduced in both FUS and C9ORF72 mutant immortalized lymphoblast cell lines (Freischmidt *et al.*, 2013). The survival time of SOD1-G93A mice was significantly extended by treatment with anti-miR-155 compared with control cases (Koval *et al.*, 2013). Mature miR-206 was increased in fast-twitch muscles in the SOD1-G93A mice model (Toivonen *et al.*, 2014). miR-124a is reduced in the spinal cord tissue of SOD1-G93A mice (Morel *et al.*, 2013). miR-27a was highly expressed in ALS subjects compared with healthy control subjects (Butovsky *et al.*, 2012b).

In addition to miRNAs related to the four genes, we also discuss miRNAs that may not directly relate to these four genes but are shown to be associated with ALS. These miRNA biomarkers were predicted in Taguchi and Wang (2018b) and other studies, including miR-1290, miR-1246, miR-181a-5p, miR-4701, miR-4485, miR-455, miR-26a, miR-23a, miR-146a* and miR-1825. miR-1290 and miR-1246 were down-regulated in sALS (Wakabayashi *et al.*, 2014). The receiver operator characteristic (ROC) curve analyses indicated that miR181a-5p may be used as a prognostic and disease progression biomarker of sALS (Benigni *et al.*, 2016). miR-4701 and miR-4485 had significantly different expression levels in sALS patients compared with healthy controls (Chen *et al.*, 2016). The expression levels of miR-455 and miR-26a are different in ALS and controls (Jensen *et al.*, 2016). ALS patients had lower levels of skeletal muscle peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) mRNA compared with healthy control subjects, and miR-23a had a reduction in PGC-1α levels (Russell *et al.*, 2013). Furthermore, miR-146a* could contribute to the selective suppression of low molecular weight neurofilament (NFL) mRNA observed in sALS (Campos-Melo *et al.*, 2013).

miR-1825 was significantly down-regulated in sALS patients' plasma (Takahashi *et al.*, 2015).

Furthermore, several miRNA biomarkers were suggested in other studies. Overexpressing miR126-5p in SOD1-G93A mice muscles inhibits the neurodegenerative process that might identify a non-cell-autonomous neurodegeneration process in ALS (Maimon *et al.*, 2018). miR-374b-5p, miR-206, and miR-143-3p of sALS patients were shown to be decreased compared to controls (Waller *et al.*, 2017). miR-206 and miR-424 are potential prognostic markers in spinal onset ALS (de Andrade *et al.*, 2016). miR-132 and miR-125b were upregulated in ALS patients (Kovanda *et al.*, 2018). In addition to miR-206, miR-143-3p, and miR-132, which were mentioned above as related to the four genes, miR126-5p, miR-374b-5p, and miR-424 are potential biomarkers that can be investigated in a future study.

Materials and Methods

miRNA biomarkers of ALS and other neurological disorders

To investigate the relationship between ALS and other neurological diseases through miRNA biomarkers, we also discuss miRNA biomarkers for other neurological disorders. Tab. 1 lists miRNA biomarkers of ALS caused by the four genes and other neurological disorders, such as Williams syndrome, Parkinson's disease, Alzheimer's disease, etc. We select these neurological disorders listed in Tab. 1 because, from our analysis, we can determine at least one miRNA biomarker for each of these neurological disorders such that these miRNAs can be used to investigate the difference in pathology between these neurological diseases and ALS. In the results section, we discuss more details of this analysis result.

For the diseases in Tab. 1, since FTD is closely related to ALS and both share some common miRNA biomarkers, we discuss miRNA biomarkers of FTD here. A validation study confirmed the downregulation of miR-663a, miR-502-3p, and miR-206 in FTD patients (Grasso *et al.*, 2019); a mechanism involving miR-124 and AMAPRs was identified in regulating social behavior in FTD (Gascon *et al.*, 2014); miR-132 significantly differentiates frontotemporal lobar degeneration with TDP-43 inclusions (FTLD-TDP) and control brains (Chen-Plotkin *et al.*, 2012); and qRT-PCR analyses showed that miR-922, miR-516a-3p, miR-571, miR-548b-5p, and miR-548c-5p were significantly dysregulated in cerebellar tissue samples of progranulin (PGRN) mutation carriers for FTLD-TDP patients (Kocerha *et al.*, 2011).

TABLE 1

miRNA biomarkers for several neurological disorders and amyotrophic lateral sclerosis (ALS) related to the four genes

Disease	miRNA	References
Amyotrophic lateral sclerosis	miR-142-3p, miR-132-5p/3p, miR-574, let-7b, miR-155, miR-206, miR-124a, miR-27a, miR-143-3p/-5p	(Butovsky *et al.*, 2012a; Freischmidt *et al.*, 2013; Koval *et al.*, 2013; Morel *et al.*, 2013; Toivonen *et al.*, 2014; Matamala *et al.*, 2018)
Frontotemporal dementia	miR-663a, miR-502-3p, miR-206, miR-124, miR-132, miR-922, miR-516a-3p, miR-571, miR-548b-5p, miR-548c-5p	(Kocerha *et al.*, 2011; Chen-Plotkin *et al.*, 2012; Gascon *et al.*, 2014; Grasso *et al.*, 2019)
Parkinson's disease	miR-133b,miR-92a-3p,miR-16-5p, miR-615-3p,miR-877-3p, miR-100-5p, miR-320a, miR-877-5p, miR-23a-3p, miR-484, miR-23b-3p, miR-15a-5p, miR-324-3p, miR-19b-3p, miR-505-3p	(Kim *et al.*, 2007; Khoo *et al.*, 2012; Heman-Ackah *et al.*, 2013; Prajapati *et al.*, 2015; Hoss *et al.*, 2016; Leggio *et al.*, 2017; Chen *et al.*, 2018; Taguchi and Wang, 2018a)
Alzheimer's disease	miR-107, miR-9, miR-124a, miR-125b, miR-128, miR-26b, miR-144, miR-29, miR-34, miR-181, miR-106, miR-146a, miR-132, miR-153	(Kim *et al.*, 2007; Lukiw, 2007; Wang *et al.*, 2008; Absalon *et al.*, 2013; Cheng *et al.*, 2013; Banzhaf-Strathmann *et al.*, 2014; Gupta *et al.*, 2017)
Spinal muscular atrophy	miR-9, miR-206,miR-132,miR-183,miR-335-5p, miR-431, miR-375	(Kye *et al.*, 2014; Wang *et al.*, 2014; Bhinge *et al.*, 2016; Wertz *et al.*, 2016; Magri *et al.*, 2018)
Prader–Willi syndrome	miR-24-3p, miR-122, miR-23a-3p, miR-764, miR-1264, miR-1912	(Zhang *et al.*, 2013; Magri *et al.*, 2018; Pascut *et al.*, 2018)
Niemann–Pick disease	miR-196a, miR-98, miR-143, miR-155	(Ozsait *et al.*, 2010; Pascut *et al.*, 2018; Pergande *et al.*, 2019)
Neurofibromatosis	miR-29c, miR-34a, miR-214, miR-10b, miR-204, miR-21, miR-486-3p	(Chai *et al.*, 2010; Gong *et al.*, 2012; Sedani *et al.*, 2012; Masliah-Planchon *et al.*, 2013)
Narcolepsy	miR-188-5p, miR-4499, miR-1470, miR-4455, miR-30c, let-7f, miR-26a, miR-130a	(Holm *et al.*, 2014; Mosakhani *et al.*, 2017)
Friedreich's ataxia	miR-886-3p,miR-15a-5p, miR-26a-5p, miR-29a-3p, miR-223-3p, miR-24-3p, miR-128-3p, miR-625-3p, miR-130b-5p, miR-151a-5p, miR-330-3p, miR-323a-3p, miR-142-3p	(Mahishi *et al.*, 2012; Dantham *et al.*, 2018; Seco-Cervera *et al.*, 2018)
Ataxia telangiectasia	miR-18a, miR-421	(Hu *et al.*, 2010; Guibinga *et al.*, 2012; Wu *et al.*, 2013)

Phylogenetic tree analysis

We adopted a phylogenetic tree analysis to find potential miRNAs that could be used to investigate the difference in pathology between other neurological diseases and ALS induced by the four genes. These miRNA biomarkers were clustered based on the phylogenetic tree analysis. Since the similarity of two nucleotide sequences can be measured using different evolutionary models, we plotted phylogenetic trees based on different evolutionary models using the MATLAB software (Mathworks, 2014).

To perform this method, we needed to find miRNA biomarkers of ALS and other neurological disorders, respectively. The miRNA biomarkers are listed in Tab. 1. To plot the phylogenetic tree of these miRNAs, we used the stem-loop sequences of these biomarkers because they may provide more information than the mature -5p sequence and mature -3p sequence. The stem-loop sequences can be accessed from the miRBase (http://www.mirbase.org/) (Kozomara and Griffiths-Jones, 2013). To classify these miRNA sequences, we first needed to calculate the distances for any two miRNA sequences. Next, we classified the sequences such that sequences with a small distance can be clustered into a group. In the study, we applied the phylogenetic tree method to classify these sequences. Thus, using the MATLAB software requires two steps: The first is to select an evolutionary model to calculate the distance between two nucleotide sequences; after calculating all the distances of any two sequences, the second step is to find a clustering method to build a tree.

The distance model method in the MATLAB software includes the p-distance, Jukes–Cantor distance, alignment score distance, etc. The clustering method (linkage function) in the MATLAB software includes the median method, the single method, and the average method, and so on. In this study, we used the Jukes–Cantor distance (or the alignment score distance) to calculate the distances and the median method (or the average method) as the linkage functions to build a tree.

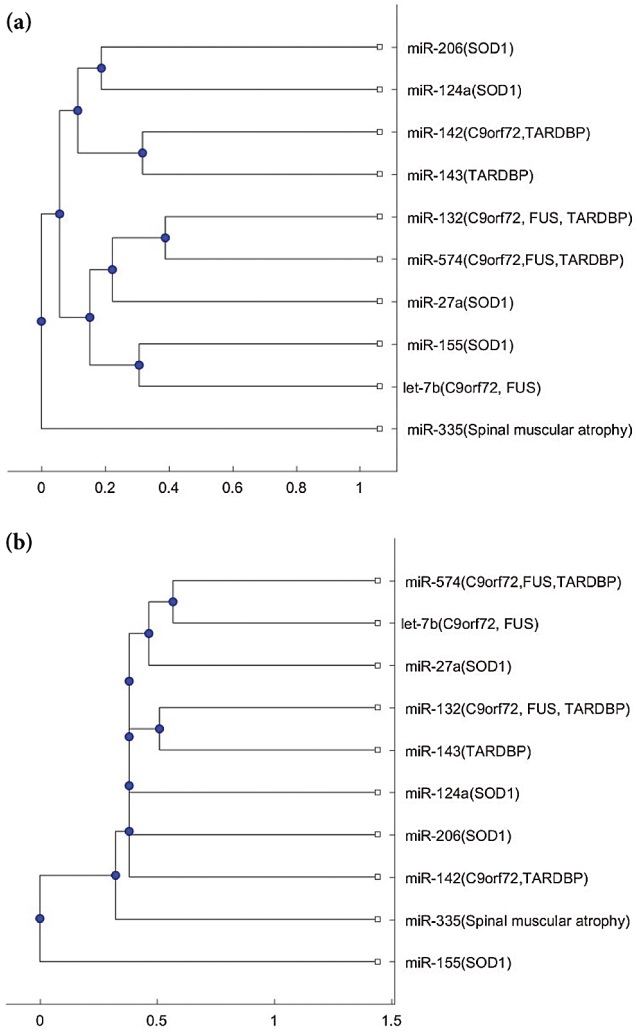

FIGURE 1. (a) The phylogenetic tree of miR-335 and the 9 ALS miRNA biomarkers based on the Jukes–Cantor distance and the average linkage method. (b) The phylogenetic tree of miR-335 and the 9 ALS miRNA biomarkers based on the alignment-score distance and the median linkage method.

Results

miRNAs

We plotted the phylogenetic trees of the 9 ALS miRNA biomarkers in Tab. 1 and each biomarker for other neurological disorders. Fig. 1 shows the phylogenetic trees based on the 9 miRNA biomarkers of ALS and miR-335, which is a biomarker of spinal muscular atrophy. Figs. 1a and 1b were plotted based on the Jukes–Cantor distance and the average linkage function method, and the alignment score distance and the median linkage function method, respectively. From Fig. 1a, miR-335 is in another branch of the tree based on the Jukes–Cantor distance. In addition, except for the biomarker miR-155 of ALS, miR-335 is also in a separate branch of the tree in Fig. 1b, based on the alignment score distance. miR-155 is a biomarker of ALS related to the SOD1 gene. We present more discussions of miR-155 in the discussion section. From this phylogenetic tree analysis, we found that miR-335 may be a useful biomarker to discriminate spinal muscular atrophy and ALS. As a result, miR-335 can be a potential miRNA to investigate the difference in pathology between spinal muscular atrophy and ALS.

Fig. 2 shows the phylogenetic trees of miR-1470 (narcolepsy miRNA biomarker) and the 9 ALS miRNA biomarkers. From Figs. 2a and 2b, miR-1470 is in a separate branch of the two trees. Thus, miR-1470 may be a potential miRNA for investigating the difference in pathology between narcolepsy and ALS.

Fig. 3 shows the phylogenetic trees of miR-548b (FTD miRNA biomarker) and the 9 ALS miRNA biomarkers. Unlike miR-335 and miR-1470, which are always in a separate branch of the trees, in Fig. 3a, miR-548b cannot be clustered to a different group. In Fig. 3b, miR-548b is in a separate branch of the tree compared with the other 8 ALS biomarkers, excluding miR-155. Since miR-548b is clustered in a separate group in one of the two trees, we may consider that miR-548b is a potential miRNA for investigating the difference of pathology between FTD and ALS.

In addition to the three miRNAs, miR-335, miR-1470, and miR-548b in Figs. 1–3, Tab. 2 lists other miRNAs of Tab. 1 that have the potential to be used in investigating the difference of pathology between the other neurological

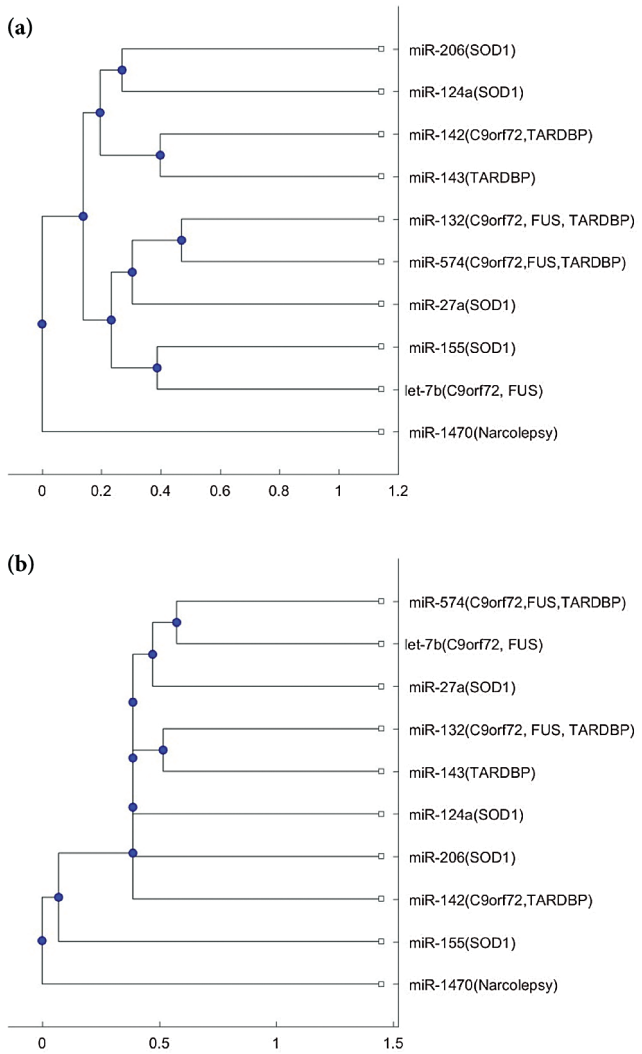

FIGURE 2. (a) The phylogenetic tree of miR-1470 and the 9 ALS miRNA biomarkers based on the Jukes–Cantor distance and the average linkage method. (b) The phylogenetic tree of miR-1470 and the 9 ALS miRNA biomarkers, based on the alignment score distance and the median linkage method.

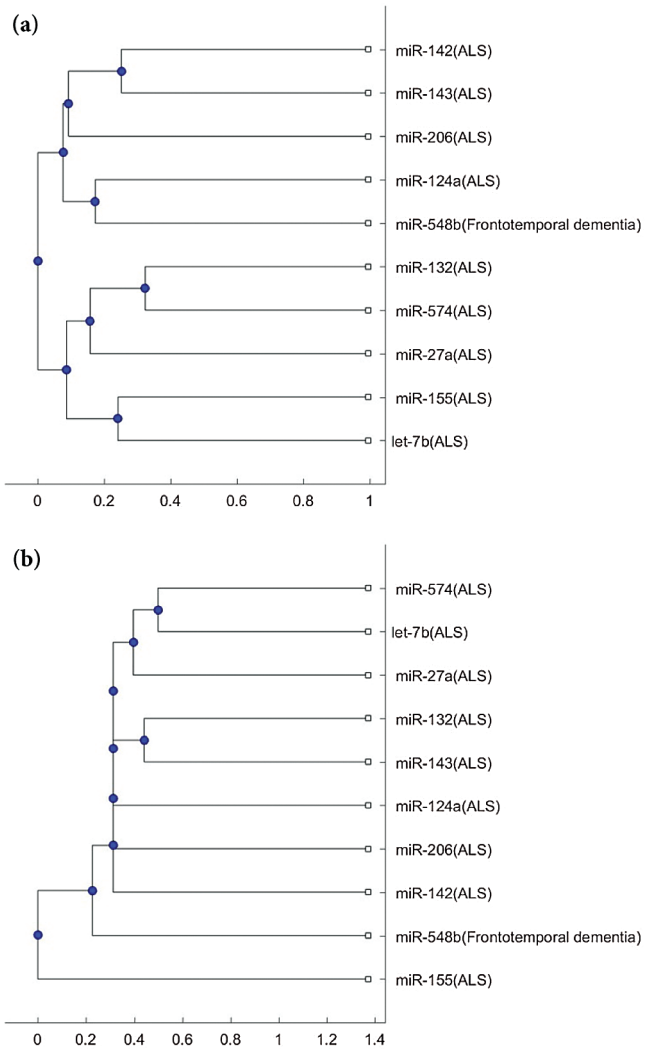

FIGURE 3. (a) The phylogenetic tree of miR-548b and the 9 ALS miRNA biomarkers based on the Jukes–Cantor distance and the average linkage method. (b) The phylogenetic tree of miR-548b and the 9 ALS miRNA biomarkers based on the alignment-score distance and the median linkage method.

TABLE 2

Potential miRNAs for investigating the difference of pathology between other neurological diseases and ALS induced by the four genes

Disease	miRNA
Frontotemporal dementia	miR-548b, miR-548c
Parkinson's disease	miR-133b
Alzheimer's disease	miR-29, miR-181
Spinal muscular atrophy	miR-335, miR-375
Prader–Willi syndrome	miR-24
Niemann–Pick disease	miR-98
Neurofibromatosis	miR-10b
Narcolepsy	miR-4499, miR-1470, miR-4455
Lesch–Nyhan syndrome	miR181a
Friedreich's ataxia	miR-886, miR-29a, miR-24
Ataxia telangiectasia	miR-18a

disorders of Tab. 1 and ALS. For each miRNA in Tab. 2, either one of the two trees or both trees shows that the miRNA is in a separate branch of the tree. Fig. S1 provides the trees in which the miRNAs are in a separate branch based on the Jukes–Cantor distance and the average linkage method. Fig. S2 provides the cases for the alignment score distance and the median linkage method.

In Figs. 1b and 3b, a SOD1 biomarker, miR-155, is alone in a separate branch. In addition to these two Figs., this phenomenon frequently occurs in the phylogenetic trees of other miRNAs, listed in Tab. 2.

Validation

To confirm these identified miRNAs, we use the Human MicroRNA Disease Database (HMDD) to provide a validation of these results. HMDD is a database that provides experiment-supported evidence for human miRNA and disease associations (Huang *et al.*, 2018). We use HMDD to validate our results from three aspects. The first one is to check whether the miRNAs listed in Tab. 2 are associated with ALS. Since the miRNAs listed in Tab. 2 are used to classify ALS with the other diseases, they should not be associated with ALS. We uploaded the miRNAs listed in Tab. 2 to HMDD. For the 18 miRNAs, none of them are associated with ALS in HMDD. Therefore, we validate that the selected miRNAs are not ALS-related miRNAs.

The second aspect is to check whether the selected miRNAs are associated with the corresponding diseases listed in Tab. 2. In HMDD, we have confirmed that miR-548b and miR-548c are associated with FTD, miR-133b is associated with Parkinson's disease, miR-29 and miR-181 are associated with Alzheimer's disease, miR-335 is associated with spinal muscular atrophy, and miR-18a is associated with ataxia-telangiectasia. Therefore, 7 of the selected miRNAs are confirmed to be related to their corresponding diseases, but not ALS.

The third aspect is to check the miRNAs listed in Tab. 1. The result is presented in Tab. 3. The second column of Tab. 3 lists miRNAs that are confirmed in HMDD to be related to the diseases but not ALS; the third column of Tab. 3 lists the miRNAs in the second column that are selected by the phylogenetic method. From Tab. 3, it can be seen that there are many miRNAs selected by this method. Therefore, this phylogenetic tree method can select miRNAs that may be potential to be used in studying the difference of pathology for ALS and other neurological diseases. This also reveals that these identified miRNAs listed in Tab. 2 have the potential to be used for studying the difference of pathology for ALS and their corresponding neurological diseases (Fig. 4).

In addition, the above mentioned phylogenetic trees were plotted based on the stem-loop sequences. We can compare the stem-loop sequence analysis result with the 3p- or 5p-miRNA sequence analysis result using the 7 selected miRNAs of the third column in Tab. 3. But the 3p- or 5p-miRNA sequence of miR-206 can not be obtained from miRBase. Thus, we use the other 8 miRNA biomarkers of ALS in the 3p- or 5p- miRNA sequence analysis. Compared with the stem-loop sequences, the 3p- or 5p- miRNA sequences analysis only selected the 3 miRNAs, miR-335, miR-548c, and miR-133b, among the 7 miRNAs. Figs. S3 and S4 show the phylogenetic trees of the 3p- or 5p-miRNA sequences analysis for these 3 miRNAs. More comparisons of the stem-loop sequence and 3p- (or 5p-) miRNA sequence will be investigated in a future study.

Discussion

Phylogenetic tree analysis has been widely used in the study of miRNAs. Phylogenetic tree-informed miRNA analysis has uncovered conserved and lineage-specific miRNAs in Camellia during floral organ development (Yin *et al.*, 2016); the phylogenetic analyses highlighted the potential of miRNAs to become an invaluable tool to resolve previously intractable nodes within the tree of life (Tarver *et al.*, 2013), and the evolution of the two disease resistance-related miRNAs, miR-482 and miR-1448, was inferred using the phylogenetic analyses (Zhao *et al.*, 2012). The phylogenetic age of miRNAs was computed in a study that concluded that older miRNAs were significantly more likely to be associated with disease than younger miRNAs (Patel and

TABLE 3

miRNAs related to the corresponding disease but not ALS from the Human MicroRNA Disease Database (HMDD), and miRNAs selected by the phylogenetic tree method

Disease	miRNA related to the corresponding disease but not ALS	Selected miRNAs by the method
Frontotemporal dementia	miR-922, miR-516a-3p, miR-571, miR-548b-5p, miR-548c-5p	miR-548b-5p, miR-548c-5p
Parkinson's disease	miR-133b, miR-19b-3p	miR-133b
Alzheimer's disease	miR-107, miR-29, miR-181, miR-153	miR-29, miR-181
Spinal muscular atrophy	miR-206, miR-335-5p,	miR-335
Prader–Willi syndrome	–	–
Niemann–Pick disease	miR-196a	–
Neurofibromatosis	–	–
Narcolepsy	–	–
Friedreich's ataxia	hsa-miR-625-3p, hsa-miR-330-3p, hsa-miR-323a-3p, hsa-miR-142-3p	–
Ataxia telangiectasia	miR-18a	miR-18a

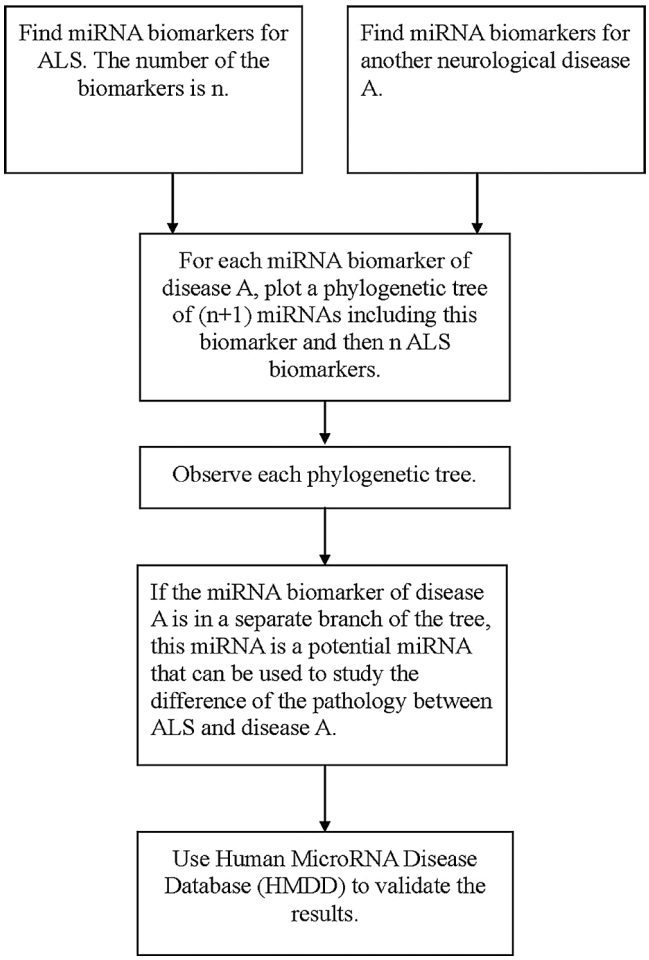

FIGURE 4. The flowchart of finding miRNAs that are potential to be used in studying the difference of pathology for ALS and another neurological disorder.

Capra, 2017). Furthermore, a combination of the phylogenetic tree analysis with a bioinformatics method can increase the accuracy of miRNA cancer biomarker prediction compared with only using the bioinformatics method alone (Wang, 2016b). The phylogenetic relationship of the miRNA biomarkers has been used to investigate the association between anti-NMDA receptor encephalitis and vaccination (Wang, 2017). In addition, the phylogenetic analysis has been used to explore the association between anti-NMDA receptor encephalitis and tumors based on miRNA biomarkers (Wang, 2019) and the association between two diseases (Chen and Wang, 2020b). These studies showed that phylogenetic analysis is a useful tool to explore miRNA functions.

Compared with the 9 ALS miRNA biomarkers, the miRNAs listed in Tab. 2 can be clustered to a separate group of the tree or be clustered with miR-155 to a separate group of the tree either using the alignment-score distance or the Jukes–Cantor distance. Thus, these identified miRNAs may be taken into account when studying the difference in the mechanisms for these diseases. Among these ALS miRNA biomarkers, miR-155 related to the SOD1 gene is different from the other biomarkers because it is often in a separate branch of the trees. There are several situations, such as miR-155, alone in a separate branch, or miR-155, with a disease biomarker in a separate branch. Therefore, more studies into the miR-155 regulatory mechanism may be useful in understanding the difference of pathologies for ALS and other neurological disorders.

In addition, miRNA studies can be used to investigate the comorbidities of diseases (Wang, 2019; Chen and Wang, 2020a; Chen and Wang, 2020b; Wang, 2020). I might use miRNA biomarkers to explore the comorbidities of ALS in a future study. Neurological disorders and cancer were shown to be the comorbidities of ALS in the literature. FTD was shown to be associated with ALS in many studies. ALS is developed in about 15% of patients with FTD (Van Mossevelde *et al.*, 2017). The repeat expansion of C9orf72 is a major cause of FTD and a cause of other neurodegenerative diseases, including ALS (Dobson-Stone *et al.*, 2013). As a result, the hexanucleotide repeat expansions of the C9orf72 gene link FTD to ALS (Renton *et al.*, 2011; Benigni *et al.*, 2016). Mouse models of C9orf72 hexanucleotide repeat expansion to relate these two diseases were explored (Liu *et al.*, 2016; Batra and Lee, 2017; Ji *et al.*, 2017).

The ALS drug, Riluzole, was found to be an anti-cancer drug in various cancers, including breast cancer, brain tumor, prostate cancer, osteosarcoma, and melanoma (Akamatsu *et al.*, 2009; Le *et al.*, 2010; Dolfi *et al.*, 2017; Liao *et al.*, 2017; Sperling *et al.*, 2017). ALS might be inversely associated with cancer because a lower risk of cancer was observed in ALS patients, but a higher risk of ALS was observed in cancer patients compared with controls (Fang *et al.*, 2013; Freedman *et al.*, 2013). A microarray and survival analysis showed that ALS is related to cancer (Taguchi and Wang, 2017).

Conclusion

ALS is a complex disease, and its pathogenesis remains unknown. Several genetic factors, including C9orf72, SOD1, FUS, and TARDBP, have been discovered to be associated with ALS. Genes related to ALS may also be associated with other neurological disorders such as FTD. miRNA biomarkers of ALS and other neurological disorders have been discussed in the related literature. To the best of our knowledge, there have not been any studies exploring the difference of pathology between two neurological disorders using their miRNA biomarkers based on the phylogenetic tree analysis.

In this study, we applied the phylogenetic tree to analyze miRNA biomarkers of ALS and other neurological diseases. Using this method, we found a number of miRNAs that can be taken into account when studying the difference of pathology of ALS induced by the four genes and other neurological disorders such as frontotemporal dementia, Parkinson's disease, Alzheimer's disease, spinal muscular atrophy, Prader–Willi syndrome, Niemann–Pick disease, neurofibromatosis, narcolepsy, Friedreich's ataxia, and ataxia-telangiectasia. In addition to these neurological disorders, this method can be used to explore the difference in pathology between ALS and other diseases. This may provide a useful direction in exploring the pathology of diseases based on miRNA biomarkers.

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Supplementary Materials

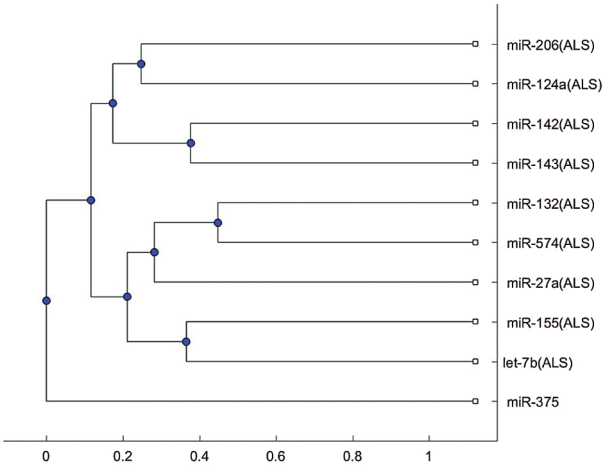

FIGURE S1. Phylogenetic tree based on Jukes-Cantor distance and the average linkage method.

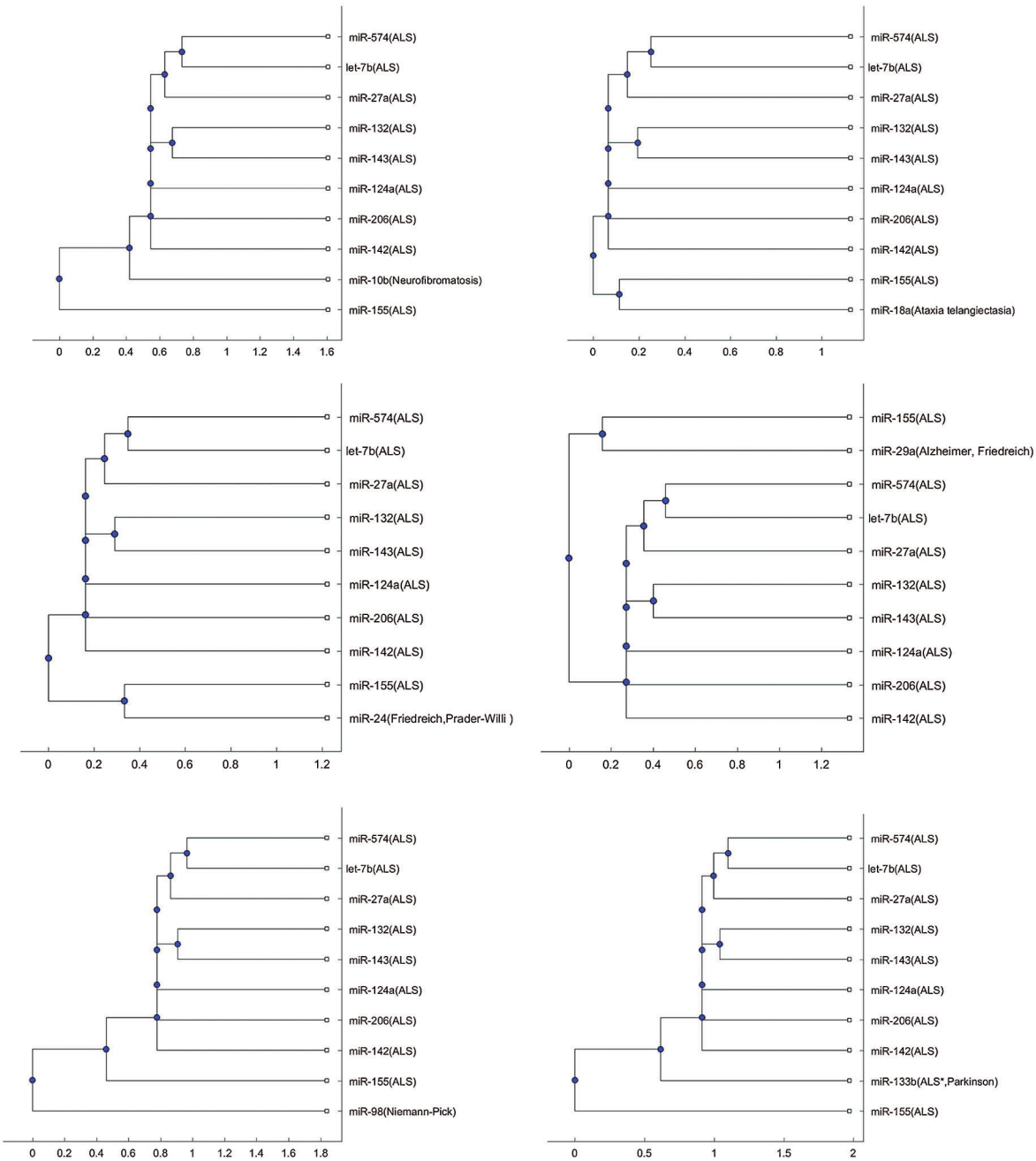

FIGURE S2. (continued)

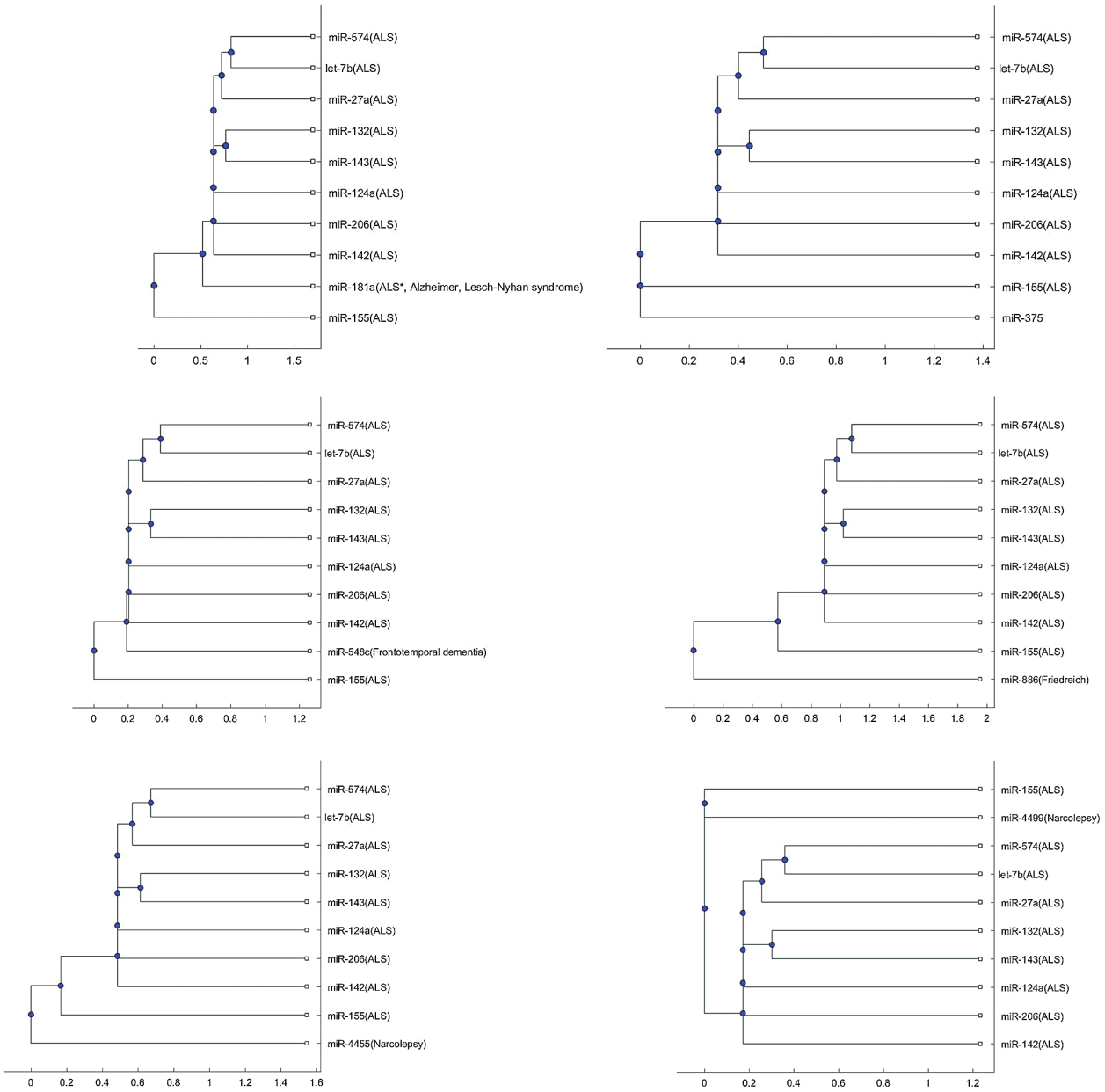

FIGURE S2. Phylogenetic tree based on alignment-score distance and the median linkage method.

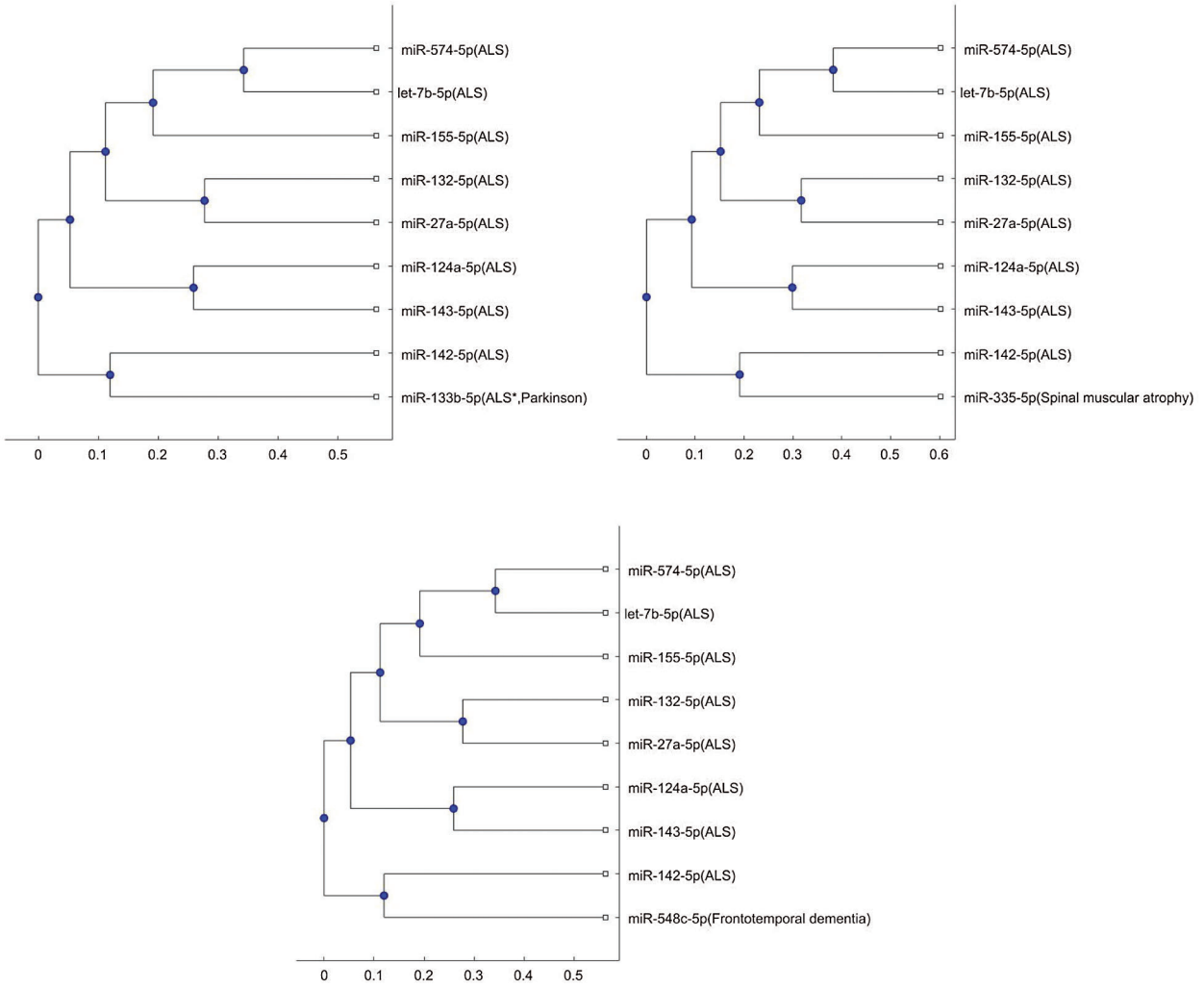

FIGURE S3. Phylogenetic tree for 3p- or 5p- miRNA sequences based on Jukes-Cantor distance and the average linkage method.

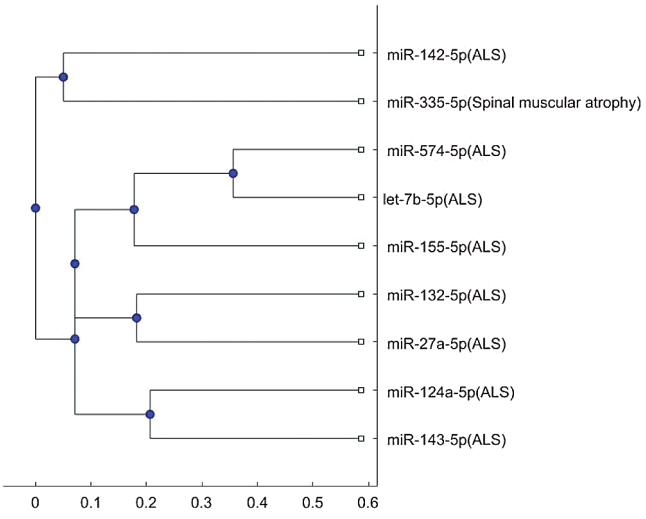

FIGURE S4. Phylogenetic tree for 3p- or 5p- miRNA sequences based on alignment-score distance and the median linkage method.