Chapter 8

Tiny microRNAs Fine-Tune Amyotrophic Lateral Sclerosis Regulation

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Additional information is available at the end of the chapter

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Abstract

Progressing muscle wasting and dramatic neurodegeneration of upper and lower motor neurons are the initial symptoms of amyotrophic lateral sclerosis (ALS) that eventually cause aetiology or death in quick succession. The functional mechanism of ALS is non-cell autonomous but it strongly influences on non-neural cells including microglia, astrocyte muscles and T cell. In ALS, neurodegeneration is triggered by at least four gene mutations that are not related to any classical signalling pathways, molecular mechanism or known cellular ingredients. MicroRNA is endogenous tiny non-coding RNA, which is required for fine-tuning or micromanaging protein expression post-transcriptionally. In this review, we identified numerous microRNAs and their possible targets in ALS-related genes. These microRNAs misprocess ALS-related protein-coding genes via microRNA-gene circuits. This result sheds a strong link between microRNA and ALS genes. The mechanistic insight of multiple microRNAs related to ALS is required to treat neuro-inflammation and neuro-degradation. It is proposed that the micro-regulation of multiple microRNAs is involved in generation of unique neuroprotective agent against ALS. Therefore, a classical and novel microRNA-mediated therapy might unravel an alternative strategy for ALS-related neurodegeneration. This strategy indeed implicates real promises to illustrate a unique impact for ALS cure.

Keywords: amyotrophic lateral sclerosis (ALS), small microRNA, hotspot, microRNA-mediated therapy, neurodegeneration

1. Overview and introduction

Amyotrophic lateral sclerosis (ALS) is an important neuromuscular disease [1]. ALS was recognized back in 1850 by the English neurophysiologist Augustus Waller for the appearance of shrivelled nerve fibres. In 1869, in a scientific literature it was described and named ALS by the French neurologist Jean-Martin Charcot [2]. ALS, otherwise known as Lou Gehrig's disease, caused a baseball player, Lou Gehrig, to retire from his peak season. This New York Yankees player was called the iron horse for his contribution in the field (adapted from http://www.hopkinsmedicine.org/neurology_neurosurgery/centers_clinics/als/conditions/als_amyotrophic_lateral_sclerosis.html, http://www.biography.com/people/lou-gehrig-9308266).

Gradual degeneration of motor neurons in the brain and spinal cord is sought after to characterize this disease [1]. After the disease contracts, the motor neurons can no longer deliver impulses to the muscles, resulting in atrophy of muscles and muscle weakness (**Figure 1**). ALS does not disrupt a person's intellectual reasoning, vision, hearing or sense of taste, smell and touch. Mostly, ALS does not affect a person's bowel, sexual or bladder functions. ALS is often referred to as a neurodegenerative syndrome because the disease becomes evident in various

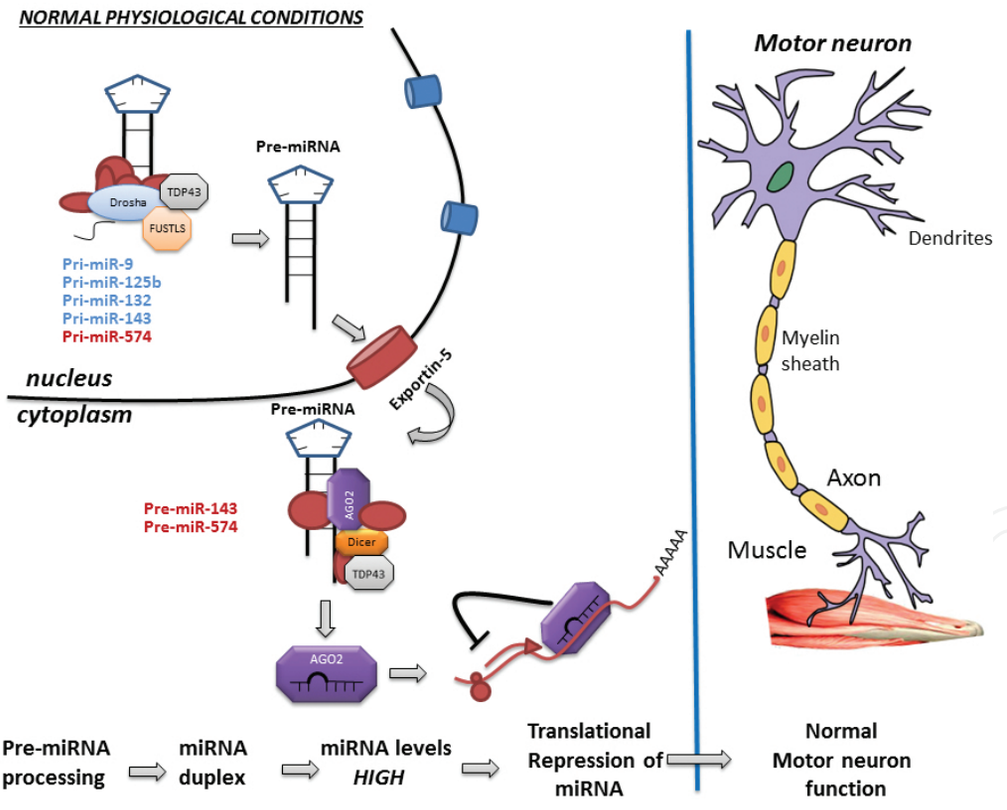

Figure 1. Under normal physiological condition, pri-miRNAs are processed in nucleus by Drosha along with its partner DGCR8. Recently it has been revealed that interactions with TDP-43 and FUS–TLS enhance miRNA biogenesis. In this diagram the pri-miRNAs, regulated by TDP-43, have been shown in blue colour, and the pri-miRNAs, regulated by FUS–TLS, have been shown in red colour. It has also been demonstrated that TDP43 associates with Dicer complex and helps in the processing of pre-miR-143 and pre-miR-574 into mature miRNAs in cytoplasm (adapted from Bicker S, Schratt G. MicroRNAs in ALS: small pieces to the puzzle. EMBO J. 2015;34[3]:2601–3 and Ref. [21]).

patterns. ALS occurs scarcely and spontaneously. Currently, there is no straightforward and classical cure for ALS (adapted from http://www.hopkinsmedicine.org/neurology_neurosurgery/centers_clinics/als/conditions/als_amyotrophic_lateral_sclerosis.html, http://emedicine.medscape.com/article/1170097-clinical).

MicroRNAs are the tiny endogenous non-coding RNA that represses protein synthesis post-transcriptionally by coupling at the 3′ untranslated leader sequences of target mRNAs [4]. MicroRNA micro-manages a broad range of biological process including developmental decisions, cellular differentiation, programmed cell death for pattern formation, and many pivotal roles in different human diseases [5]. At least 500–1000 microRNAs are found in vertebrate [6]. However, each microRNA can target numerous mRNAs [7]. It is suggested that 30–40% of human transcriptome is under control of different miRNA-gene circuits.

Biogenesis, processing and functional mechanism of microRNA are unique. Four different functional processes are involved in microRNA-mediated gene expression where (1) co-translational protein synthesis is disrupted, (2) translation elongation is inhibited, (3) translation product is terminated prematurely and finally (4) translation initiation is disrupted. MicroRNA inhibits their functional targets by sequestering to target mRNAs [8].

Basically, microRNA can be used as a fine-tuner for different gene regulatory networks. Especially numerous microRNAs are expressed in different sites of the brains [9]. They participate in the functional mechanism of brain development. They have important roles in brain morphogenesis, neuronal differentiation, dendrite and spine developments, synaptic structure formation and neuronal plasticity, etc. [10]. Multiple clusters of microRNA play important parts in different acute and chronic pathological disorders in the brain. In this review we have mentioned a set of microRNA that produce a distinct cluster in different ALS-related genes. The dysregulation of these microRNAs collectively misregulates multiple mRNAs related to ALS and other neurological diseases. Multiple microRNAs can simultaneously control several endogenous mRNAs because 3′ UTR of an mRNA can have complementary sites for a cluster of microRNAs; conversely a single microRNA can regulate multiple mRNAs [11]. In protein homeostasis, different cellular events participate with different microRNAs. However, different microRNAs may involve various mediators to control gene expression [12].

2. Symptoms of ALS

"ALS is like a lit candle: it melts your nerves and leaves your body a pile of wax. Often, it begins with the legs and works its way up. You lose control of your thigh muscles, so that you cannot support yourself standing. You lose control of your trunk muscles, so that you cannot sit up straight. By the end, if you are still alive, you are breathing through a tube in a hole in your throat, while your soul, perfectly awake, is imprisoned inside a limp husk, perhaps able to blink, or cluck a tongue, like something from a science fiction movie, the man frozen inside his own flesh. This takes no more than 5 years from the day you contract the disease". Courtesy from "Tuesdays with Morrie" by Mitch Albom.

Each individual patient may experience symptoms differently. Symptoms may include:

- Twitching and cramping of muscles, especially those in the hands and feet
- Loss of motor control in the hands and arms
- Impaired use of the arms and legs
- Weakness and fatigue
- Tripping and falling
- Dropping things
- Uncontrollable periods of laughing or crying
- Slurred or thick speech and difficulty in projecting the voice

As the disease progresses, symptoms may comprise:

- Shortness of breath
- Difficulty in breathing
- Difficulty in swallowing
- Paralysis (adapted from http://www.hopkinsmedicine.org/neurology_neurosurgery/centers_clinics/als/conditions/als_amyotrophic_lateral_sclerosis.html)

3. Symptom statistics

ALS occurs between the ages of 40 years and 70 years, but the disease can occur at a younger age also. It affects throughout the world without any ethnic, racial or socioeconomic boundaries. ALS is responsible for almost five of every 100,000 deaths in people aged 20 or older. The frequent age for ALS is after 60 years age. The incidence of ALS is five times higher than Huntington's disease and almost equal to multiple sclerosis. Fifty percent of affected patients live at least three or more years after diagnosis, 20 percent live 5 years or more and up to 10 percent will survive more than 10 years (adapted from http://www.hopkinsmedicine.org/neurology_neurosurgery/centers_clinics/als/conditions/als_amyotrophic_lateral_sclerosis.html).

4. Medical classification of ALS

Making a proper diagnosis in ALS is complicated because symptoms can vary in each patient. Based on the symptom, ALS can be classified in five broad ranges based on the disease symbol:

Classical ALS – characterized by the deterioration of upper and lower motor neurons [nerve cells]. This symptom generally affects more than two-thirds of patients with the disease.

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Primary lateral sclerosis [PLS] – in which case the upper motor neurons deteriorate. If the lower motor neurons are not affected within 2 years, the disease usually remains a pure upper motor neuron disease. It is the rarest form of ALS.

Progressive bulbar palsy [PBP] – this condition starts with difficulties in speaking, chewing and swallowing due to lower motor neuron [nerve cell] deterioration. It affects about 25% of those with ALS.

Progressive muscular atrophy [PMA] – in which the lower motor neurons deteriorate. If the upper motor neurons are unaffected within 2 years of contracting the disease, the disease usually remains a pure lower motor neuron disease.

Familial – that affects more than one member of the same family (adapted from http://www.hopkinsmedicine.org/neurology_neurosurgery/centers_clinics/als/conditions/als_amyotrophic_lateral_sclerosis.html).

5. Tiny small RNA or microRNA

It seems the world of non-coding RNAs is expanding like the universe, with the progress of science. After the discovery of miRNA in 1993, our knowledge about miRNA is increasing exponentially. *Lin-4* was the first miRNA to be discovered, in 1993, by the joint efforts of Victor Ambros's and Gary Ruvkun's laboratories [13]. After that, *let-7* is a heterochronic gene of *Caenorhabditis elegans* and was the second miRNA to be discovered, in 2000, 7 years after the finding of the first miRNA [14]. MicroRNAs [mi-RNAs] are 18–25 nt RNAs produced from a cellular RNA with a stem-loop structure. These evolutionarily conserved, naturally abundant, small, regulatory non-coding RNAs can inhibit gene expression at the post-transcriptional level in a sequence-specific manner [8]. It can be (i) intronic and (ii) intergenic [15].

All miRNAs undergo 5′ capping and 3′ polyadenylation, which are common events in case of mRNA processing also [16]. Intronic miRNAs are produced under host gene promoter [15]. Intergenic miRNAs have own promoter [15]. In the nucleus, genes encoding miRNA are generally transcribed by RNA polymerase II (Pol II) into large primary miRNA transcripts (pri-miRNA) (sized >1 kb) [15–17]. The pri-miRNA is maximally 2.2 kb long [15, 17]. Processing of pri-miRNA:pri-miRNA > pre-miRNA > miRNA [15]. miRNAs bind to the 3′ UTR region of target mRNAs [8]. Once the miRNA binds to a completely complementary region of target mRNA, mRNA gets degraded [18]. miRNA-mediated regulation does not require to have a perfect match with its target-binding region [19]. Only seven-base sequence between second and eighth nucleotides from the 5′ end is called "seed region (sequence)" and a complete match of these sequence is required for degradation of target mRNA[s] [20]. It is believed that the strength of the inhibition varies depending on the sequences [20]. A single miRNA may directly affect the expression of hundreds of proteins at one time and several miRNAs can also target the same mRNA and result in enhanced translational inhibition. It causes post-transcriptional gene control [8].

6. Link between miRNA and ALS

In last few decades, neuroscience has made a remarkable progress. This has led to accumulation of humongous information including neuronal signalling and neuronal circuits. Neuroscience itself is complex and RNA interference (RNAi) molecules have made it much more intricate. The finer details are being searched to cope up with the neurodegenerative disorders. In the postgenomic era, we are still searching mysteries related to non-coding RNA. These small RNA molecules are the controller of our genome.

The microRNA molecules are acting as nodes and links of gene expression. These experts work in various biological functions. Using complementarity against cognate mRNA, this 21–22 nt stretch plays havoc. A miRNA can target several mRNAs, and in combination with other regulatory molecules, it can alter a cell's expression and activity. Neurology includes networking between the cells of nervous system and other cells of the organism. Direct and indirect targets of one miRNA make a cell's system much more complex. ALS is a motor neuron disorder and it has been proved in various reports that ALS can be caused due to dysregulation of miRNAs and thus misexpression of proteins in the cells (**Figure 2**). This chapter deals with the miRNAs which are culprits behind ALS [21].

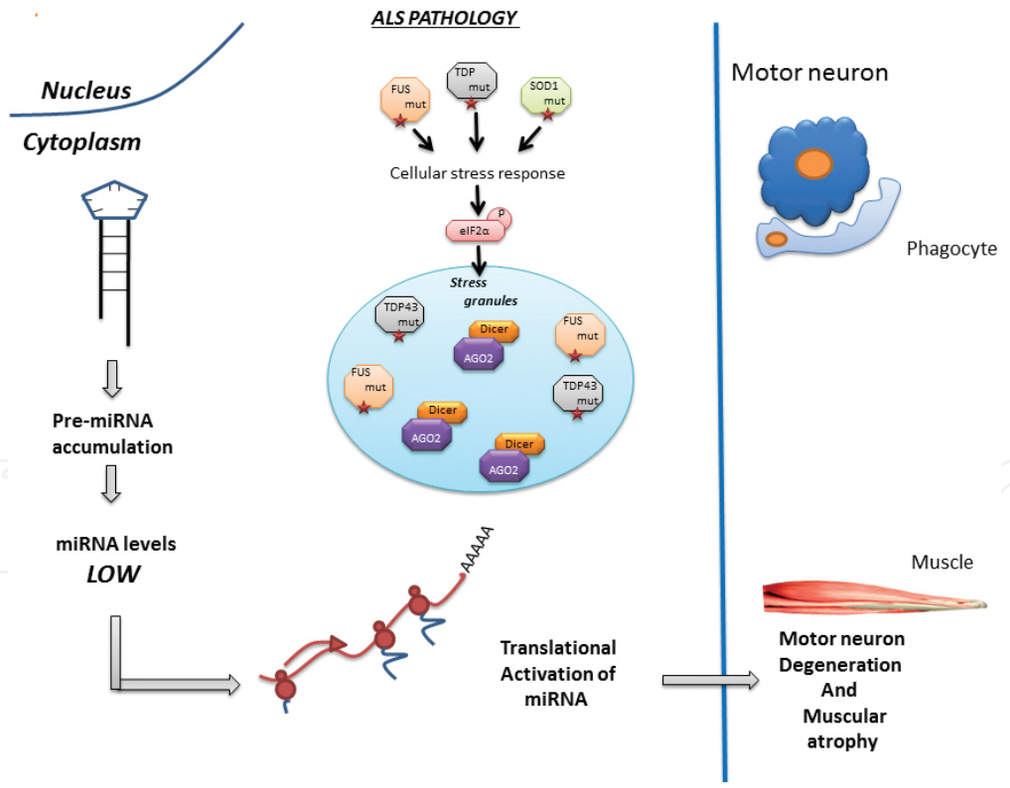

Figure 2. In ALS, there are mutations in multiple genes like TDP-43, FUS–TLS and SOD1 and these results in activation of stress response involving phosphorylation of eIF2α and stress granule formation. In ALS, reduced pre-miRNA processing and decreased levels of different miRNAs are caused by remodelling of Dicer complex in cytoplasm. Thus, the translation of target mRNAs is relieved, resulting in degeneration of motor neurons (adapted from Bicker S, Schratt G. MicroRNAs in ALS: small pieces to the puzzle. EMBO J. 2015;34[3]:2601–3).

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7. MicroRNA and its biogenesis pathway

miRNAs, after being discovered in 1993 [13], has come a long way and established itself as one of key regulators at the post-transcriptional level. It gets expressed in wide varieties of organism from plants to humans. Many miRNAs are conserved across several species. These regulatory molecules are of 21–25 nt in length. Many components of miRNA-processing machinery can be found in Archaea and Eukarya, which supports its ancient presence [3]. Databases of different miRNAs are increasing day by day because of its association with several biological processes and diseases. miRNAs account for >3% of all human genes [22, 23]. At first it gained its initial importance because of its involvement in developmental biology. Now it has been noticed that normal miRNA population can be dysregulated in different diseases, and thus, they are being used as biomarker to detect diseases. Sequence-specific regulation by miRNA makes it more specific but it can diversify its function by binding to 3′ UTR sites of several mRNAs.

The miRNA genes can be operated from their own promoter or promoter of another gene [15]. Primary miRNA transcripts are transcribed by RNA pol II in nucleus [16, 17]. Primary transcripts are of multiple hairpin loops. In nucleus Drosha along with DGCR8 cleaves at 3′ end to form pre-miRNAs. Pre-miRNAs have a hanging 3′-OH end [24]. 70 nt structure pre-miRNAs get transported to cytoplasm from nucleus by Ran-GTP and Exportin-5 [24]. In the cytoplasm, RNase-III Dicer enzyme cut at the loop structure of the pre-miRNA to produce passenger strand and guide strand [24]. The thermodynamically unstable strand, i.e. guide strand, works as a mature miRNA [25]. These mature miRNAs get loaded into RISC complex [RNA-induced silencing complex]. RISC complex contains several proteins which are needed for ribonucleotide binding and cleaving the target mRNA. Argonaute protein, GW182 are some of the factors of RISC [8]. Once RISC factor is assembled, the miRNA guides the complex to its target on the basis of complementary base pairing. The complementarity is generally restricted to the second to eighth base from 5′ end of miRNA, which is called “seed” region [25]. This seed region binds to 3′ UTR of target mRNA [19, 20]. The fate of target mRNA depends upon the extent of base pairing to the cognate miRNA [19, 20]. The miRNA will direct the destruction of the target mRNA, who has the perfect or near-perfect pairing [19, 20]. If there is multiple partially complementary site, the accumulation of protein from target mRNAs will be inhibited.

8. miRNA in mammals

miRNAs can inhibit translation in many ways like co-translational protein degradation, inhibition of translation elongation, premature termination of translation, inhibition of translation initiation, deposition of mRNAs in cytoplasmic P-bodies and premature ribosome drop-off [8].

9. Post-initiation mechanisms

Studies in the worm *C. elegans* and in mammalian cell cultures present evidences that miRNAs repress protein synthesis after translation is initiated. The contradictory observation that the targets of miRNAs appear to be actively translated while the corresponding protein product remains undetectable prompted the proposal that the nascent polypeptide chain might get degraded co-translationally. The identity of protease is unknown. Proteasome was excluded as a possibility because proteasome inhibitor does not restore the protein. Several other evidences lead to the suggestion that premature ribosome dissociation is caused by miRNAs [8].

10. Inhibition of translation initiation

There was an observation by a group, where it was observed that the central domain of Argonaute proteins has sequence similarities to the cytoplasmic cap-binding protein *eIF4E* [eukaryotic translation initiation factor 4E], which is necessary for the cap-dependent translation initiation in the cell. *eIF4E* binds to the m7Gppp-cap structure of mRNAs by stacking the methylated base of the cap between two tryptophans. At the equivalent position of the tryptophans in eIF4E, Argonaute proteins have phenylalanines that could mediate a similar interaction between the molecules. Consistently, Kiriakidou et al. [26] showed that human Argonaute 2 (AGO2) binds to m7GTP present on Sepharose beads. It was shown that substituting one or both AGO2 phenylalanines with valine residues suspended the silencing activity. Using human cells it was shown that AGO2 associates with both *eIF6* and large ribosomal subunits. By binding to the large ribosomal subunit, *eIF6* prevents this subunit from joining with the small ribosomal subunit prematurely. *AGO2* recruits *eIF6*, thus stopping the association of the large and small ribosomal subunits, causing translation repression [8, 27].

11. miRNA-mediated mRNA decay

Argonaute proteins prevent the formation of the closed-loop mRNA configuration by a murky mechanism that includes de-adenylation. MicroRNAs trigger de-adenylation and de-capping of the target mRNA. Proteins required for this process are components of the major de-adenylase complex [CAF1, CCR4 and the NOT complex], the de-capping enzyme DCP2 and several de-capping activators. Thus, signal for translation is lost.

P-bodies or cytoplasmic processing bodies are nothing but ribosome-depleted areas inside a cell. Recent studies support the evidence that the mRNAs silenced by miRNAs are localized to P-bodies for storage or degradation. Induced by miRISC and RNA helicase activity, remodelled mRNPs may modify the translation initiation complex at the 5′ end of target mRNAs, which may cause translation repression and localization of mRNAs in P-bodies. P-bodies may facilitate the access of the de-capping factors to the cap structure, thus facilitating

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mRNA degradation. However, with the help of appropriate signals, stored mRNAs residing in P-bodies could be released and returned to the translational machinery of the cell [8, 28].

12. miRNAs in different parts of the human brain

One of the important processes is microRNA-mediated gene regulation, which is fine-tuning the expression of genes essential for the pathway. Using bioinformatics approach, one can easily understand that the cells of human brain are finely tuned by miRNAs. Prediction of miRNA target sites using bioinformatics approach can give us a whole bunch of gene names. But a major weakness of in silico analysis is the lack of experimental validation. For circumventing such problem, a combinative approach can be implemented where target sites are predicted using multiple programmes. Out of miRNAs, which get expressed in the midbrain, cerebellum, hippocampus and frontal cortex, majority of them are from midbrain while almost equal proportions of microRNAs are expressed in the hippocampus and frontal cortex. The microRNAs in the cerebellum are less in number compared to miRNAs expressed in other regions considered. The target sites of the microRNA expressed in different parts of the brain had been predicted on the 35 genes related to ALS initially. The result obtained from miRanda [version 1.0] indicates that 477 target sites were predicted for mRNAs expressed in midbrain as compared to the target sites [411] predicted for miRNAs expressed in the hippocampus (**Figure 3**). However, total target sites predicted for miRNAs expressed in the cerebellum and frontal cortexes were 175 and 395, respectively (**Figure 3**). The number of targets for miRNAs expressed in cerebellum is considerably less comparing to binding sites for miRNAs expressed in other areas of the brain. Surprisingly, microRNA targets were distributed in the different regions of the genes and are not limited to only in 3′ UTR. Total 1456 target sites were predicted using miRanda for miRNAs considered in the study. Target sites in the 3′ untranslated region are comparatively less. A significant number of targets were also found in the 5′ UTR of the genes. Earlier, there was occurrence of miRNA target sites in coding region and 5′ UTR of genes was considered as exception in animals. But numerous recent evidences have established that microRNAs can target different regions of a gene and microRNA-based regulation is not confined only to 3′ UTR [9, 29]. Experiments aimed at identification of specific parameter or factor for effective targeting of 3′ UTR by miRNAs have failed considerably. Conversely, the existence of miRNA target sites in ORF of Nanog, *OCT4* and *SOX1*, during induction of stem cell pluripotency, reinforces the existence of miRNA-binding sites in other sites of the genes in animals. These results established the notion that microRNA-target sites are not only restricted to 3′ UTR in animals. Such variability in regions also adds cues for anticipating the mode of action especially their role in transcriptional, post-transcriptional and translation inhibition. As the majority of the algorithms developed for miRNA target site prediction consider only 3′ UTR, confirmation of 3′ UTR target site prediction using a combinatorial approach will be helpful. Target sites on ALS-related genes were classified according to the microRNAs expressed in four different regions of the brain (**Figure 3**). As there were less miRNAs expressed in cerebellum, this can explain small number of target sites predicted on genes associated to ALS. Analysis of miRNA targets in each gene associated with ALS showed

that there are no target sites that could be detected in 3′ UTR for mRNAs expressed in midbrain for *CASP1*, *GRIA1*, *GPX1*, *DAXX*, *CAT*, *SOD1*, *NEFM*, *NEFL* and *NEFH* genes. Furthermore 5′ UTR lack any target sites of same miRNAs in *CASP1*, *GRIA1*, *GPX1*, *DAXX*, *CAT*, *SOD1*, *NEFM*, *NEFL*, *NEFH*, *RAC1* and *TNF* genes. The miRNAs expressed in midbrain showed the least number of target sites in *RAC1* and *CASP1* genes, whereas 29 target sites were predicted in *NOS1*. Similar results were there for the miRNAs expressed in the cerebellum for the same genes. For instance, *RAC1* has on one target site whereas *NOS1* has 11 target sites. For miRNAs expressed in the hippocampus, *CASP1* and *GRIA1* genes have a few numbers of predicted sites [4] and *MAP3K5* was predicted to possess maximum target sites [30]. Similarly, *CASP1* and *SOD1* have two target sites each, while 23 target sites had been predicted for *TNFRSF1A* gene for miRNAs, expressed in the frontal cortex. On the other hand, target sites could not be predicted for six genes, namely, *GPX1*, *CYCS*, *CHP*, *CASP3*, *SOD1* and *RAB5A*, for miRNAs expressed in the cerebellum. No target sites could be predicted in 3′ UTR of *ALS2*, *BID*, *CASP1*, *GRIA1*, *DAXX*, *CCS*, *CAT*, *CASP9*, *TOMM40*, *TNF*, *SOD1*, *RAC1*, *NEFM* and *NEFL* genes. No target site could be predicted in *BCL2*, *CASP1*, *GRIA1*, *DAXX*, *CYCS*, *CASP3*, *TNF*, *RAC1*, *NEFM* and *NEFH* genes for miRNAs expressed in the cerebellum. These results demonstrate that the majority of the ALS genes lack any target site for miRNAs expressed in the cerebellum. miRNA targets were found in most of the genes, but surprisingly few genes, *CASP1*, *GRIA1*, *DAXX*, *CAT*, *SOD1* and *NEFM*, lack any miRNA target site in their 3′ UTR. This shows that complexity of regulations and numerous members of this play give the brain another level of entanglement [9] (**Figure 4**).

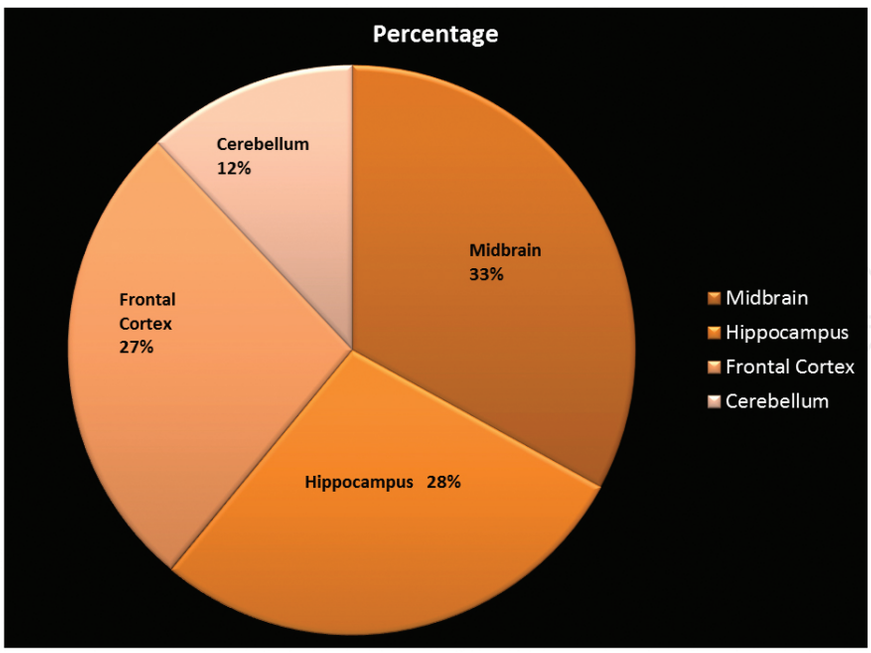

Figure 3. Percentage of miRNAs, expressed in the brain. From this diagram we can have an idea that the regulation of genes by miRNAs is widespread in various parts of the human brain (adapted from Ref. [9]).

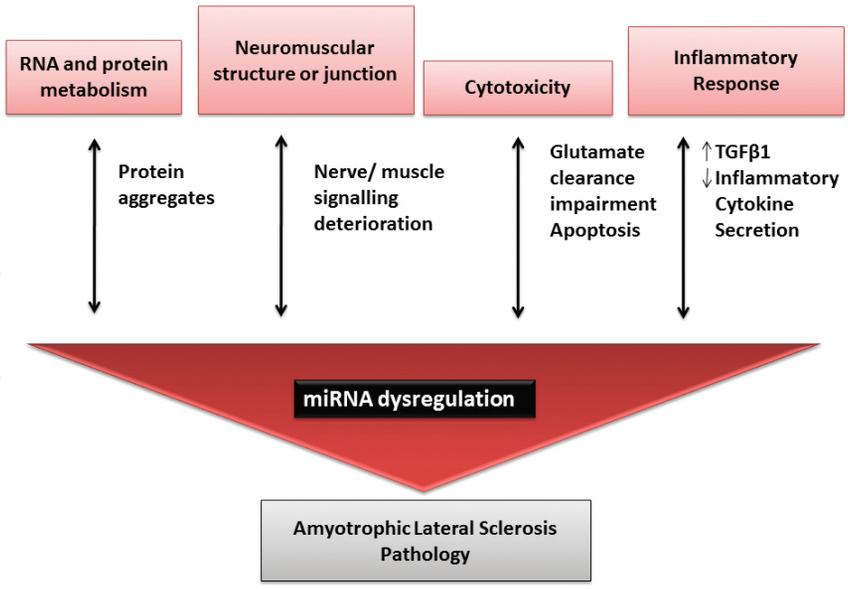

Figure 4. Either dysregulation of miRNA biogenesis and function might result into ALS pathogenesis or ALS pathogenesis can cause dysregulation of miRNA biogenesis and function. Disrupted signalling at the neuromuscular junction, caused by cytotoxicity associated with the faulty glutamate clearance or an overactive inflammatory response, results in neuromuscular degeneration. Dysregulation of key miRNAs triggers the altercations in cell physiology, resulting in ALS pathology (adapted from Ref. [84]).

13. Causative factors in ALS

Most of the cases are sporadic ALS [sALS]. Only 10–15% are usually inherited as an autosomal dominant trait and defined as familial [fALS]. They show comparable etiopathology and they affect same neuronal population [31]. Several ALS genes (*SOD1*, *Alsin*, *SETX*, *SPG11*, *FUS/TLS*, *VAPB*, *ANG*, *TARDBP*, *FIG4*, *OPTN*, *ATXN2*, *VCP*, *UBQLN2*, *SIGMAR1*, *CHMP2B*, *PFN1*, *SQSTM1*, *C9ORF72*) and additional chromosomal loci have been identified [32, 33]. Almost two-thirds of the familial cases are due to mutations of *C9ORF72*, *SOD1*, *TARDBP* and *FUS* genes. Twenty percent of fALS is because of the mutation of *SOD1* gene, coding for the superoxide dismutase one mitochondrial and cytoplasmic copper/zinc enzyme, responsible for metabolizing naturally occurring, but harmful, superoxide radicals to molecular oxygen and hydrogen peroxide. A massive hexanucleotide-repeat expansion (GGGGCC)n in the first intron of *C9ORF72* gene on the chromosome 9p21is the most frequent cause of ALS identified to date [30, 33]. After identifying several causal genes, it has been hard to find the clear evidence of the pathogenic role of some mutations. The most extensively studied gene, SOD1, still lacks pathogenic feature of the reported missense, nonsense and deletion mutations. Studies suggest that there are multiple genetic factors which contribute to develop ALS ultimately or show phenotypical features. Though the contributions of environmental factors have not been proved, ALS is a combination of genetic and environmental factors. It has been clearly noted that with increasing age, one develops higher risk of ALS. Some reports tell that heavy metals, pesticides and exposure to electromagnetic field might cause ALS [34]. The use of neurotrophic

factors like IGF-I, GDNF, VEGF, ADNF-9, colivelin and angiogenin showed potential in therapy of ALS. Talampanel (AMPA antagonist), ceftriaxone (antibiotic), pramipexole/dexpramipexole (dopamine agonists) and arimoclomol (activator of molecular chaperons' protein repair pathway) are amongst the existing drugs to treat ALS. Lately stem cell and immune therapies have been suggested [35]. A comprehensive listing of trials in the USA can be found at ClinicalTrials.gov.

A wealth of evidence supports that ALS is a multifactorial and multisystemic disease, characterized by overlapping different mechanisms, transduction pathways and multicellular crosstalk. Transgenic animals expressing human mutant SOD1 (mSOD1) have helped to reveal that perishing of motor neurons is not the only reason behind ALS but there is active contribution of non-neuronal cells like microglia, astrocytes and muscle and T cells, which differently participate to the different phases of the disease [36, 37]. Expression of mSOD1 within the most vulnerable motor neurons primarily causes for disease onset; synthesis of the mutant protein by interneurons also positively participates to disease initiation [38, 39]. Speeding of disease progression is caused by moSOD-1-mediated injury and neighbouring astrocytes and microglia. Schwann [40] and muscle cells play as direct partners of the injured motor axons, either working as recipients or initiators of the initial damage. These characteristics thus properly earned to ALS the definition of non-cell-autonomous disease [41]. Chimeric mice formed by expressing both mutated and non-mutated SOD1 proved that wild-type nonneuronal cells prolong the survival of motor neurons carrying mSOD1 protein. Neighbouring astrocytes, microglia, oligodendrocytes and Schwann cells can cause the degeneration of motor neurons [42, 43]. Compelling evidence indicates that primary mSOD1-expressing astrocytes from mouse, rat and humans cause motor neurons death by releasing toxic factors into the media while sparing interneurons [44–46]. Astrocytes deriving from neuronal progenitor cells, gathered from fALS and sALS cases, exhibit features of non-cell-autonomous toxicity [47]. There are very few studies of non-cell-autonomous toxicity. Astrocytes expressing mutant TDP43 [TDP43-M337V] participate in ALS pathology only through cell-autonomous processes. It has been studied that conditioned medium from astrocytes that express SOD1-G93A, or SOD1-G86R, or TDP43-A315T leads to extensive motor neuron death via non-cell-autonomous mechanisms. Still, there is a long way left to travel and to unravel mystifying stories behind ALS [21] (**Figures 1** and **3**).

14. The miRNAs involved in ALS regulation

14.1. Effects of miRNA biogenesis related but mutated factors in ALS

Several factors are there, which can affect CNS homeostasis and thereby facilitate neurodegenerative and neuroinflammatory diseases. Perturbation in intricate miRNA network can disrupt neuronal function in cell-autonomous or non-cell-autonomous manner [48–50]. It has been shown that there is cell-type-specific deletion of miRNA-processing enzyme, Dicer in cerebellar Purkinje, or striatal, retinal, spinal and cortical neurons, which leads to degeneration and ataxia [51–54]. Neurodegeneration also occurs after targeted deletion of Dicer in astro-

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cytes, oligodendrocytes and Schwann cells, perhaps because the glial cells have some effects on neuronal survival [55–58] (**Table 1**). Experimental results show that deletion of Dicer directly in spinal motor neurons mimics most of the clinical (e.g. progressive paralysis) and pathological (e.g. astrocytosis and signs of axonopathy) traits of ALS [54]. Several groups have proved that TDP43 and FUS/TLS are involved in multilayered steps of RNA processing and miRNA biogenesis pathway. It has been investigated recently they have role in miRNA-related ALS [59–62]. FUS, nuclear factor complexes with Drosha along with DGCR8. This association is indispensible in miRNA processing in nucleus. Cytoplasmic TDP-43 associates with Dicer, containing TRBP. This interaction facilitates the processing of the specific pre-miRNAs by Dicer, generally a subset of the pre-miRNAs whose yield in the nucleus is regulated by TDP-43, by direct binding to their terminal loops. It was proved that TDP-43 promotes neuronal outgrowth by facilitating miRNA production. Further studies show that TDP-43 has its role in both nucleus and cytoplasm. It associates with DGCR8 containing nuclear Drosha complex in RNA-dependent and RNA-independent fashion. In nucleus, TDP-43 binds to a selected pri-miRNAs, and in cytoplasm, it binds to terminal loops of pre-miRNAs. TDP-43 facilitates neuronal outgrowth through the regulation of miRNA processing [60].

Tissue/cells	**miRNAs**	**Changes**	**Biological function**	**Model**
Skeletal muscle	miR-1/206 family	↓	Myogenic differentiation	TDP-43 mice
CNS	miR-29a	↑	ER stress-induced cell death	SOD1-G93A mice
Skeletal muscle	miR-206, miR-133b	↑	Maintenance and repair of NMJ	SOD1-G93A mice
Human blood serum	miR-206, miR-106b	↑	*Ni*	ALS patients
Lumbar spinal cord	miR-b1336, miR-b2403, miR-sb659	↓	NFL stability	sALS patients
Lumbar spinal cord	miR-b1123, miRb2948, miR-b3265, miR-b5539, miR-sb1217*, miR-sb3998	↑	NFL stability	
epSPCs	miR-124a	↑	epSPC fate	SOD1-G93A mice
epSPC fate	miR-9, miR-19a, miR-19b	↓	epSPC fate	
Brain microglia	miR-155, miR-146b, miR-22, miR-365, miR-125b, miR-214	↑	Microglia activation	SOD1-G93A mice

Tissue/cells	miRNAs	Changes	Biological function	Model
CSF	miR-132-5p, miR-132-3p, miR-143-3p	↓	*Ni*	sALS patients
CSF	miR-143-5p, miR-574-5p	↑	*Ni*	
Neurons	miR-9	↓	*Ni*	*TARDBP* A90V, *TARDBP* M337V patients
Spinal cord	miR-9	↑	Neuronal differentiation	SOD1-G93A mice
Spinal cord	miR-124a	↓	Glutamate transport	SOD1-G93A mice
Spinal cord	miR-558, miR-16-2*, miR-146a*, miR-508-5p, miR-373*, miR-551a, miR-506, miR-518a-5p, miR-518e*, miR-890	↑	NFL stability	sALS human patients
Spinal cord	miR-624, miR-520, miR-524-5p, miR-548a-5p, miR-606, miR-612, miR-647 miR-155, miR-17, miR-19b, miR-20a, miR-24-2*, miR-106a, miR-142-3p, miR-142-5p, miR-146a, miR-146b, miR-223	↓	NFL stability	
Spinal cord	miR-155, miR-17, miR-19b, miR-20a, miR-24-2*, miR-106a, miR-142-3p, miR-142-5p, miR-146a, miR-146b		*Ni*	SOD1-G93A mice
Spinal cord	miR-24-2*, miR-142-3p, miR-142-5p, miR-146a, miR-146b, miR-155		*Ni*	Human patients
Blood leukocytes	miR-338-3p	↑	*Ni*	sALS human patients
	miR-451, miR-1275, miR-328-5P, miR-638, miR-149, miR-665	↓	*Ni*	
Skeletal muscle	miR-23a, miR-29b, miR-206 miR-455	↑	Mitochondrial function	Human patients

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Tissue/cells	miRNAs	Changes	Biological function	Model
Spinal cord microglia	miR-544, miR-23, miR-203, miR-340 miR-146. miR-130/miR-301, miR-155, miR-27, miR-16/miR-497/miR-195*, miR-20a/miR-106b/miR-17-5p	↑	*Ni*	SOD1-G93A mice
Spleen monocytes	let-7, miR-15b, miR-16, miR-27a, miR-34a, miR-132, miR-146a, miR-155, miR-223, miR-451	↑	*Ni*	SOD1-G93A mice
CD14+CD16− monocytes	miR-27a, miR-155, miR-146a, miR-32-3p	↑	*Ni*	Human ALS patients
Skeletal muscle	miR-206	↑	Maintenance and repair of NMJ	SOD1-G93A mice

Ni, not investigated in the study (adapted from Ref. [21] with the permission from Bentham Science Publishers).

Table 1. Summarization of dysregulated miRNA in ALS.

One group has reported the changes that occur in the miRNA population followed by TDP-43 knockdown in cultured cells. It was observed that *let-7b* and *miR-663* expression levels are down- and upregulated, respectively (**Table 1**). It was found that both miRNAs can bind to TDP-43 directly in different positions: within the miRNA sequence itself (i.e. let-7b) or in the hairpin precursor (miR-663). Using microarray data and q-PCR, candidate transcripts are identified, whose expression levels are discriminately affected by these TDP-43–miRNA interactions. TDP-43 gets increased in ALS [61] leading to neurotoxicity from both gain and loss of functions. Neurodegeneration and ALS phenotypes are caused by a partial loss of TDP-43 function. TDP-43 toxicity in neighbouring cells has effects on motor neurons. Both dysregulation and dysfunction of TDP-43 are relevant to ALS [53]. Researchers have reported that TDP43 depletion could affect the levels of specific miRNAs in human hepatocarcinoma cells, by potentially binding to their sequence and/or precursor elements [61]. Regulation of miRNA by TDP43 is conserved amongst mammals. TDP43 associating with both nuclear and cytoplasmic microprocessor complexes actively participates in the production of miRNAs indispensable for neuronal outgrowth. Finally, it was noted that the carboxyl-terminal of TDP43, where most ALS mutations reside, is crucial for the interaction with the miRNA-processing complexes [60]. Recent discovery of mutations in the genes, encoding for the RNA-binding proteins TDP43 and FUS/TLS, has proved the key role of regulatory RNA in the pathogenesis of ALS. Both proteins are generally localized within the nucleus, but their mutated forms delocalize and form neuronal inclusions and dystrophic neurites, as well as

glial cytoplasmic inclusions. The sensory organ precursor cells of peripheral nervous system of *Drosophila melanogaster* were used as a model to demonstrate that hTDP43 clinical ALS mutations influence early neurogenesis and neuronal specification, through the regulation of miR-9a biogenesis. Further studies in human cells show that TDP43 is an essential element in miRNA biogenesis during neuronal differentiation by controlling the stability of Drosha and thus affecting miRNA production. Similar inferences were drawn after FUS/TLS silencing in human neuronal cell lines. FUS/TLS downregulation affects the biogenesis of a large class of miRNA and several of them have important roles in neuronal differentiation and synaptogenesis. Specific miRNAs, in particular miR-9, miR-125b, miR-132 and miR-143, are regulated by both TDP43 and FUS/TLS ALS-related proteins. In recent reports, TDP43 was shown to bind to and regulate the incorporation into the RISC complex of specific mature miRNAs, in both Drosophila and human systems. TDP43 interacting with miR-1/miR-206 family decreases the activity of these miRNAs, by the disruption of their association with the RISC complex. As this miRNA family is involved in muscle development and homeostasis, either depletion or overexpression of TDP43, by altering miR-1/miR-206 balance, might be involved in muscle pathogenesis of ALS. Nine specific miRNAs were selected and their levels were analysed in ALS patients, on the basis of their known misregulation after TDP43 depletion in cell lines. Samples of cerebrospinal fluid (CSF), serum and immortalized lymphoblastoid cell lines from fALS and sALS patients were analysed, and it was confirmed that there is dysregulation of miR-132, miR-143 and miR-574, whose processing is indeed regulated by TDP43 and/or FUS/TLS proteins. The sequencing of the 3'UTR region of the FUS/TLS gene in 420 ALS patients highlighted a mutation in two ALS patients with severe consequence, leading to FUS/TLS protein accumulation. This mutation maps to the seed sequence recognized by miR-141 and miR-200a in the 3'-UTR of FUS/TLS and that FUS/TLS is linked to these miRNAs by a feedforward regulatory loop, where FUS/TLS upregulates miR-141/200a, which in turn affects the FUS/TLS protein synthesis [21].

15. CNS-related miRNA dysregulations in ALS pathogenesis

miR-9, an evolutionarily conserved and multifunctional neuronal miRNA, is involved in the selection of neuronal precursors from the neuroepithelium in flies and in the specification of midbrain-hindbrain boundaries in vertebrates. Both miR-124 and miR-9 participates together in neuronal differentiation. Perturbation in their function results in the susceptibility to neuronal diseases. miR-9/miR-9* expression is significantly reduced in patients with Huntington's disease. miR-9 and miR-132 are downregulated in Alzheimer-affected brain. miR-9 has role in ALS also. There are several miRNAs, which are specific for neural cell fate and cell cycle regulation. After inducing neuronal differentiation, miR-9, miR-124a, miR-19a and miR-19b are differentially expressed in SOD1-G93A epSPCs, while miR-9 gets upregulated in SOD1-G93A differentiated cells, with respect to WT-SOD1 and control mice. Expression analysis of the predicted miRNA targets leads to identification of a functional network of Hes1. Pten, Socs1 and Stat3 genes are important for controlling epSPC fate and showing neurogenic potential in vitro. A time-course analysis and cellular distribution pattern of miR-9 were tested.

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It was inferred that miR-9 expression is spatially and temporally controlled in SOD1-G93A spinal cord and there is upregulation at presymptomatic stage through early symptomatic stages, mainly in the ventral horns of grey matter, where neurodegeneration is known to occur. There is a role of miR-9 in pathogenesis in induced pluripotent stem cell differentiated into postmitotic neurons and derived from ALS patients with the TDP43 A90V and M337V mutations. In all these cases, the levels of miR-9 are decreased with respect to control neurons. In *Drosophila* TDP43 mutants, miR-9a expression is significantly inhibited; it supports the concept that TDP43 acts through miR-9a to control neuronal specification and to assure the robustness of genetic control programmes. The steady-state level of miR-9 is required for normal neuronal functions, as both up- and downregulation can lead to neurodegeneration. Mutant SOD1 deregulates neurofilament (NF) balance in motor neurons [63]. NF proteins are one of the major intermediate filaments of neuronal cytoskeleton. They provide the structural integrity of neurons and help in maintenance of cell shape and axonal calibre. In CSF of ALS patients, low-molecular-weight NF is in abundance, which leads to a significant correlation between CSF NFL levels and disease progression. NF might be used as a marker of disease progression in ALS [64]. In ALS spinal motor neurons undergo a selective reduction in the steady-state level of NFL mRNA, which results in alteration of the stoichiometry of NF expression [63]. miR-9 is an upstream regulator of NF mRNAs and there are no such evidences in determination of regulation of NFL by miR-9 in ALS [54]. The q-PCR of sALS ventral lumbar spinal cord tissue shows that a set of additional miRNAs, predicted to control NFL, was deregulated. miR-146a*, miR-524-5p and miR-582-3p are capable of interacting with NFL mRNA 3′ UTR in a manner that is steady with the suppressed steady-state mRNA levels observed for NFL in spinal motor neurons in ALS. This demonstrates the fact that miRNAs are involved in the suppression of NFL mRNA and ALS spinal motor neuron neurofilamentous form aggregates [65]. A recent study was performed on a RNA library derived from control and sALS spinal cords, and it was shown that a panel of novel miRNAs whose sequence is comprised in the miRNA response elements (MREs) within the NFL mRNA 3′ UTR was recognized to be differentially expressed in ALS, compared to control [66]. Functional analysis shows that miR-b1336 and miR-b2403 are significantly downregulated and both stabilise NFL mRNA. In motor neurons there are several miRNAs, which have the capability of controlling NF expression at post-transcriptional level. Dysregulation of miRNA expression occurs not only in motor neurons but also in spinal cord microglia isolated from SOD1-G93A mice. Scientists did whole expression profiling of miRNA and mRNA at presymptomatic stage and at onset of symptoms, and end stage of disease and pathway analysis was used to hypothesize a correlation between top ten altered inflammatory miRNAs and dysregulated inflammatory genes in ALS [65]. To characterize the relationship between miRNA/target deregulations functionally in ALS inflammation, microarray of miRNA from neonatal SOD1-G93A mouse brain primary microglia was done and it was found that the expression of mutant SOD1is able to increase about 50% of the expressed miRNAs [67]. There is significant increase in selected immune-enriched miRNAs, miR-22, miR-155, miR-125b and miR-146b amongst those most highly modulated. By luciferase assays and lentiviral infections, a group of researchers confirmed the increase in miR-365 and miR-125b, the last known to be involved in stimulation of pathogenic inflammatory responses and neurodegeneration by inhibiting neurotrophism-

relevant genes. miR-365 and miR-125b suppress the IL-6/STAT3 pathway in ALS microglia, by targeting IL-6 and STAT3, respectively, and they have a role in overall increase of TNFα mRNA levels. TNFα treatment increases miR-125b levels in microglia; thus it results in abnormal TNFα release. IL-6, a marker of activated microglia, is downregulated after symptom onset in ALS animal models and TNFα is upregulated in G93A mice and in ALS patients. miR-365 and miR-125b dysregulations might have a hand in pathological cytokine profile of ALS. People suggest that the pathogenesis of ALS involves astroglial dysfunction, with a dramatic loss of the excitatory amino acid transporter-2/glial transporter1 (EAAT2/GLT1) in both ALS patients and animal models [68–71]. The EAAT2/GLT1, is an important atroglial transporter, dynamically regulated by neurons, and it is involved in maintenance of extracellular glutamate concentration below neurotoxic level [72, 73]. The exosomes containing miR-124a and released from neurons can be directly internalized into astrocytes, which results in increase in miR-124a and GLT1 protein levels. miR-124a is downregulated in spinal cord of mutant SOD1 mouse models at end stage of disease, and in vivo injection of miR-124a oligonucleotides into spinal cord of ALS mice results in 30% increase in EAAT2/GLT1 expression. There is very little information about miRNA expression in ALS-induced muscle impairment. There is miR-23 upregulation in skeletal muscle biopsies, collected from ALS patients, and it is correlated with reduction in peroxisome proliferator-activated receptor coactivator-1 (PGC-1alpha) mRNA and protein, observed in both mouse and human muscle diseased samples. This coactivator is involved in muscle mitochondrial biogenesis and function. There is reduction in skeletal muscle mitochondrial function in ALS. It has been suggested that there might be a relation in miR-23a inhibition of PGC-1α and its downstream signalling [74, 75].

16. Role of miR-206 in disease impediment

It was reported that miR-206, a skeletal muscle-specific miRNA, strongly induced in ALS mouse model is in coincidence with the onset of neurological symptoms. It is a modifier of disease pathogenesis. In G93A-SOD1 mice, loss of miR-206 expression does not affect disease onset apparently, but accelerates disease progression, by skeletal muscle atrophy, kyphosis and paralysis, and reduces survival. In miR-206$^{-/-}$/G93A-SOD1 mice, neuromuscular junctions (NMJs) are disorganized and reinnervation of denervated muscles by motor axons is postponed in the absence of miR-206. Transcript derived from the *miR-206/133b* locus, which was originally identified as a synapse-associated non-coding RNA called 7H4, has a role in encoding components of the postsynaptic apparatus. The reported 7H4 sequence does not include miR-206; RT-PCR shows that miR-206 sequences are included in this synapse-enriched transcript. Histone deacetylase 4 (HDAC4) mRNA is one of the strongest computationally predicted targets of miR-206. HDAC4 protein expression is upregulated in skeletal muscle of miR-206$^{-/-}$ animals in comparison with wild-type controls after denervation event. *Hdac4* mRNA levels remain unchanged in miR-206$^{-/-}$ mice. It suggests that miR-206 acts in this case by translational inhibition rather than by mRNA destabilization [21].

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17. Side effects of our immune system

The immune system has a huge role in maintenance of physiological equilibrium within the CNS and in controlling neuronal cell death after adverse effects start. Butovsky et al. [65] performed a comparative analysis of miRNA expression profile of inflammatory monocytes and microglia from SOD1-G93A ALS mice to generate novel biomarkers and possible therapeutics. Spleen monocytes, Ly6Chi, are recruited to the spinal cord where they proliferate during disease progression. They have pronounced proinflammatory profile in both miRNA and gene expression profile. This type of miRNA profile was found maintained in ALS patients and is apparently unique for ALS, when compared with other neuroinflammatory diseases like multiple sclerosis [65]. De Felice et al. [75, 76] did a miRNA expression profile of circulating leukocytes in a small sample of sALS patients and detected variation of eight distinct miRNAs. miR-338-3p expression is upregulated in brain tissue from ALS patients [31]. miRNAs can circulate in cell-free forms in body fluids like serum and plasma and act as signalling molecule between cells [77, 78]. Ever-increasing evidences support the idea that serum circulating secretory vesicles, including exosomes and shedding microvesicles, can function as intercellular shuttles of RNA and miRNA [60]. Muscle-enriched miR-206 is upregulated not only in SOD1-G93A mice muscles but also there is symptomatic increase in circulation in those mice. The whole transcriptome analysis of both mRNA and miRNA of sALS fibroblasts is done, in order to confirm whether dysregulated processes in the CNS might be reproduced in cells from peripheral tissues. The microprocessor complex gene DGCR8, the gene encoding Dicer enzyme and the RISC proteins AGO1/2 are downregulated in sALS fibroblasts with consequent decrease of multiple miRNAs. This creates a new horizon for generating new diagnostic pool for ALS [21].

18. Hotspot identification

MicroRNAs can bind to the same complementary target sites or it can bind to proximally located sites adjacent to other miRNA target sites. A hotspot is a stretch of nucleotide sequence, which is prone to target of several groups of miRNAs. miRNAs are regulated and expressed spatially and temporally inside cell (**Figure 5**). This type of regulation and expression gives miRNAs power to play a plethora of roles in various different biological processes. All the miRNAs occupying position in "hotspot" may not regulate a gene in the same pattern. Generally, predominant miRNA competitively outcasts other miRNAs and plays as a potential repressor. There is no clear evidence of the cause behind competitive outcasting and variable effectiveness. Various researchers are trying to use different standards and parameters like the same chromosomal location for more than two miRNAs, the same orientation, phylogenetic relationships and absence of interfering transcription unit.

miRNA targets are generally predicted using miRanda and then further analysed for miRNA-prone regions in all the selected genes. Generally, a region is defined as hotspot, if it has a minimum of 10 nucleotides overlapping from the starting position and occurrence of three

miRNA targets. It has been shown that there are 11 miRNA-prone regions in 35 genes associated to ALS. CYCS and MAP2K3 have one hotspot each in 3′ UTR, while single target site hotspot was in 5′ UTR in four genes (**Figure 5**), that is, BAX, DERL1, SLC1A2 and RAB5A. Studies have shown that MAP2K3 has two hotspots by miRNA cluster prediction. Target sites for three miRNAs, i.e. hsa-miR-107, hsa-miR-423-5p and hsa-miR-103, are congregated in another hotspot found in 3′ UTR. MAP2K3, having two such hotspots, is presumed to show more sensitivity towards gene regulation by microRNAs. The small number of hotspots makes the miRNAs more specific and stringent regulator. The variability in existence of such target site hotspots adds complexity in gene regulation [9] (**Figure 5**).

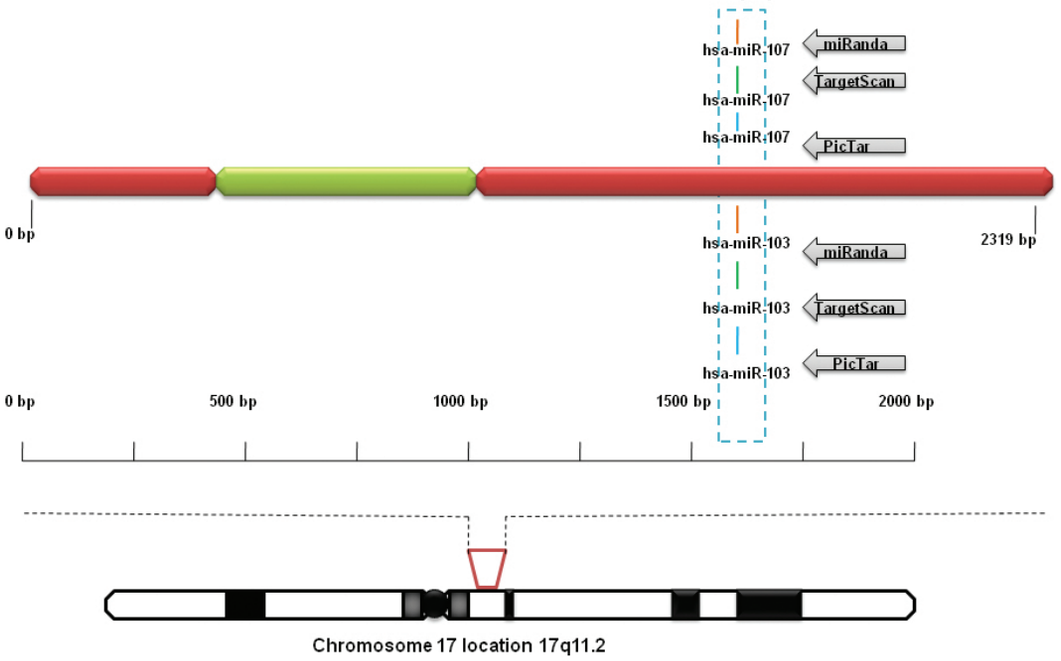

Figure 5. Schematic representation of susceptible 3′ hotspots of human MAP2K3 gene by various miRNAs like hsa-miR-107 and hsa-miR-103. To detect the susceptible sites on human MAP2K3 gene, bioinformatic tools like miRanda, TargetScan and PicTar were used (adapted from Ref. [9]).

19. Multifarious miRNA regulation

MicroRNA-mediated gene regulation adds multilayered complexity to gene expression. Distribution of target sites over several genes of one miRNA makes the picture more intricate. In principle, one miRNA can bind to more than one gene (multiplicity), and one gene can be controlled by more than one miRNA (cooperativity) [79]. Relaxed base pairing gives rise to multiplicity property. Target sites are rarely distributed on a gene. Generally they are scattered and the range of occurrence varies in numbers in a gene [80]. Often, presence of more target sites per gene indicates efficient regulation. Studies have shown that miRNAs, related to ALS, have multiple targets. After calculating multiplicity and cooperativity, scientists have shown that hsa-miR-370 showed maximum multiplicity as it showed 65 interactions with 30 gene

sequences while no target site could be predicted in five genes, namely, *CASP1*, *GPX1*, *DERL1*, *CCS* and *SOD1*. It is highly expected that the degree of repression by hsa-miR-370 would be considerably high. It has been shown in the papers that hsa-miR-874 have 52 interactions with 29 genes and have no target in *CASP1*, *CHP*, *SOD1*, *RAC1*, *RAB5A* and *MAP2K6* genes. None of the above-mentioned miRNAs display any interaction with SOD1. Documented evidences show that SOD1 is one of the core factors for ALS that encodes the free radical scavenging enzyme copper zinc superoxide dismutase in ALS pathway. Then these miRNAs might not be involved in the regulation of *SOD1* activity. Scientists have reported that *PRPH* showed high cooperativity followed by *TNFRSF1A* and *TOMM40*. It is really difficult to interpret the complex picture presented by these multifaceted interactions. *PRPH* is regulated by 10 miRNAs at 23 positions, so first 10 miRNAs demonstrate high cooperativity towards *PRPH*. Relatively very low cooperativity was shown in *BID*, *MAP2K6* and *DERL1* genes. It might be inferred that there exists a low sensitivity of these genes towards microRNA-mediated gene regulation. Simple process of selection of microRNA targets is endowed with inherent complexity and this led to the development of a complex network by two phenomena, that is, multiplicity and cooperativity. The multiplicity and cooperativity might be the deciding factors for the mode of miRNA action. How these factors work is a matter of mystery [9].

20. Complex interrelationship

Figure 6 conveys interaction map of miRNA and selected 35 genes. Interactions amongst genes and miRNAs are depicted with arrows, where 1 = hsa-miR-370, 2 = hsa-miR-874, 3 = hsa-

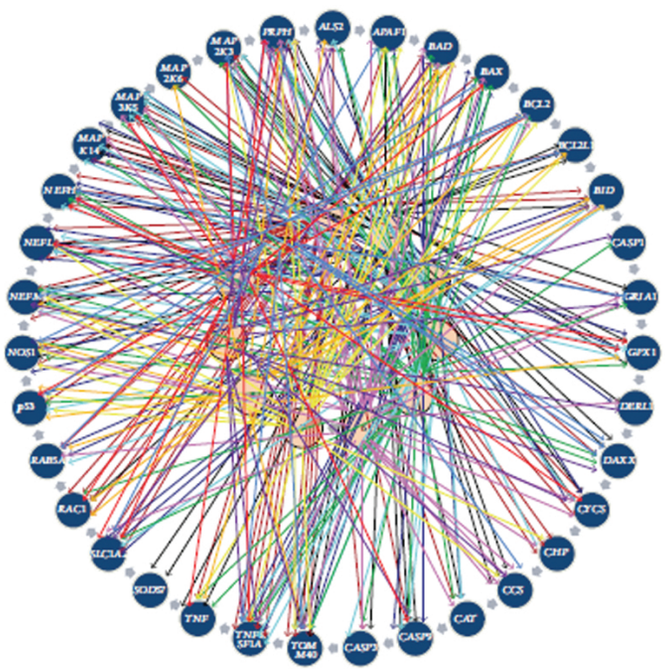

Figure 6. Interaction map of different miRNAs and selected 35 genes. Complex interrelationship amongst different genes and different miRNAs is depicted with the arrows. It is very evident from the diagram that one with miRNA can target 3′ UTR of several genes, thus adding layers to the gene regulation architecture (adapted from Ref. [9]).

miR-423-3p, 4 = hsa-miR-323-5p, 5 = hsa-miR-760, 6 = hsa-miR-149, 7 = hsa-miR-139-3p, 8 = hsa-miR-744, 9 = hsa-miR-324-3p, 10 = hsa-miR-339-3p and 11 = hsa-miR-654-5p. From **Figure 6** we can understand the multilayered regulation [9].

21. Usage of miRNAs in therapeutics

The human mind is not satisfied to know the causative factors of a disease. Our main target is to reduce ailment and to do that we need good diagnostic system along with good therapeutics. miRNAs are one of a kind. Being a regulator of gene expression, it has far-reaching effect on our physiology. They are dysregulated in several diseases. Researchers are now trying to generate therapeutic targets to treat ALS. Presently, riluzole is the only FDA-approved drug to treat ALS [81] and it modestly slows the disease progression. A phase I clinical trial of an antisense oligonucleotide [ISIS 333611] has been proven effective. This oligonucleotide targets SOD1 mRNA and represses the production of mutant SOD1 and this is effective when delivered to the CSF of patients with fALS [82]. The same kind of drug is there which targets the sense strand of the C9orf72 hexanucleotide repeat and reduces the toxicity by suppressing RNA foci formation both in vivo and in vitro [30, 33, 83]. Several delivery systems are being invented day in and day out to deliver miRNA formulations and drugs to the CNS through blood-brain barrier. The first miRNA-based therapeutic is the miR-122 antagonist SPC3649, which is currently being evaluated in phase II clinical trials. It targets the hepatitis C virus. So far, this agent has not exhibited any adverse effects.

There are two basic approaches of miRNA-based therapeutics. One is miRNA antagonists which impede endogenous miRNAs that have a harmful gain of function in diseased tissues and involve the use of an anti-miR – a chemically modified antisense RNA – to knockdown miRNA. In one of the first endeavours to use antagomirs in ALS, delivery of anti-miR-155 to *SOD1* Gly93Ala mice via ventricular osmotic pumps delayed mortality of the patients successfully. A downfall associated with this approach is the potential for nonspecific binding to other RNAs inside the cells. As one miRNA can regulate several mRNAs, it limits usage of miRNAs as therapeutics. In the second approach to miRNA therapeutics that involves miRNA mimics and miRNA replacement therapies, miRNAs are reintroduced into cells exhibiting downregulation, thus reactivating key pathways [84].

It is really difficult to dissect all the pathways, which are regulated by one miRNA. Before releasing drugs in clinics, there should be several clinical trials to eliminate any deleterious effect. Williams and group [85] showed that miRNA-206 delayed ALS progression and promoted regeneration of neuromuscular synapses effectively in mice. This group investigated pathological modifications in motor axons and nerve terminals that precede motor neuron degeneration and clinical symptoms and the role of the skeletal muscle-specific miR-206 in motor neuron-skeletal muscle fibre signalling. This miRNA is significantly upregulated in SOD1-G93A mouse model of ALS. The genetic ablation of miR-206 in these ALS mice accelerates the disease progression. miR26 might be needed for compensatory regeneration of

neuromuscular synapses after acute nerve injury and thus it decelerates ALS progression. miR-206 represses the translation of HDAC4 mRNA and counteracts its negative influence. This miRNA is also involved in neuromuscular gene expression and synapse formation. miR-206 decelerates ALS progression by detecting motor neuron injury and boosting compensatory regeneration of neuromuscular synapses by specifically inhibiting HDAC4 protein synthesis. HDAC4 has a deleterious role in ALS patients and it has been confirmed. It was shown in experiments that low progression rate in patients with ALS is associated to greater compensatory reinnervation and low HDAC4 levels. miR-133b is present in the same transcript encoding miR206, which is upregulated after denervation in the NMJs. The genetic ablation of miR-133b was studied in SOD1-G93A mice and it was noticed that the lack of miR-133b did not modify NMJ development or reinnervation after nerve injury and, overall, disease progression was not affected. miR-206 plays an important role in NMJ reinnervation specifically. miR-155 expression can be efficiently inhibited by anti-miR delivery to the CNS and periphery of SOD1-G93A mouse [86]. Inhibition of miR-155 can improve disease progression in ALS. Single intracerebro-ventricular injection of miR-29a-specific antagomir inhibiting CNS-related miR-29a expression in CNS can prolong the survival of ALS patients, thus suggesting miR-29a as a possible marker for disease progression. Anti-miR can be used as therapeutics in treating ALS but we should have an eye on the bystander effects [21].

22. Conclusion

Molecular neuropathology, genomics and proteomics related to it have made a real progress in the last few decades. Candidate genes and their involvement in molecular interactions and networks have opened new venues in therapeutics. ALS is polygenic and non-cell-autonomous disease. Due to the presence of many gene variants in this disease, it is difficult to predict the susceptible individuals. There is no such inheritance pattern. As miRNA population is dysregulated in ALS, candidate miRNAs can be used as biomarker and targets of therapeutics. Discoveries of ALS-related miRNAs are leading us to solve the intricate cellular network-related details. As of for now, we have drugs to slow down ALS. But we still have nothing to treat this disease completely. Intricacies in miRNA network are making the development of drug more difficult because of bystander effects. There are several scientist groups around the world trying to find out the possible therapeutics.

Truly, present write-ups address many milestones of biological consequences of microRNA that shed potential light on the in-depth mechanisms of ALS. It definitely explains the motor neuron development, thereby expanding our new knowledge of ALS post-transcriptionally. Apart from that, present knowledge laid a major foundation for development of therapeutic agents of ALS. Furthermore, covering specific miRNA signatures targeted by different genes of ALS are important for broad classification of ALS symptoms. This sectorization of microRNA expression might be important to improve the subclassification of ALS genes which provide a unique instructive role for therapeutic approaches.

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