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Transcriptomics in amyotrophic lateral sclerosis

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1. ABSTRACT

Amyotrophic lateral sclerosis (ALS) is an adult-onset, incurable neurodegenerative disease characterized by the selective death of upper and lowers motor neurons in the spinal cord, brainstem and motor cortex, which ultimately leads to paralysis and death within 2–3 years of onset. ALS is poorly understood, although multiple studies have been proposed to explain the pathophysiological mechanisms of the disorder. The development of microarray technology, for simultaneous analysis of the transcriptional expression of thousands of genes, has provided new possibilities to get better insights into the pathogenesis of ALS, and most important, potential new candidate targets for novel treatments. The present review illustrates current evidences from transcriptomic studies in animal models and human samples, related to ALS pathogenesis in parallel to molecular targets associated with the disease progression. Additionally, alteration of RNA metabolism was identified as a major dysregulated pathway in ALS and via this study, new insights into the contribution of altered transcriptional profiles of microRNAs and ALS-associated ribosomal binding proteins have been investigated, in an effort to understand the functional consequences of widespread RNA dysregulation in the disease's pathological mechanism.

2. INTRODUCTION

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is one of the most common devastating and fatal neurodegenerative disorders, characterized by the progressive and relatively loss of motoneurons, paralysis, atrophy of muscle tissues and death. There is no treatment that could relieve the disease burden because of incomplete understanding of ALS etiology (1). Currently, there is only one FDA-approved compound; riluzole does not resolve the disease, but slows progression and extends survival with modest effects (2, 3). The genetic causes of ALS is still under investigation; approximately 10% of ALS cases are inherited (familiar ALS), associated to dominant mutations in or deletion of the cytosolic Cu/Zn superoxide dismutase 1 gene (4), but 90% of them are sporadic or originated from unknown genetic factors (5,6). Other genes implicated in ALS are TAR DNA-binding protein (7–9), fused in sarcoma protein (10, 11), ALS2/alsin (12), ALS4/senataxin (13), or ALS8/vesicle-associated membrane protein-associated protein B (14), neurofilament heavy peptide (15), angiogenin (16), ubiquilin 2 (17), optineurin (18) and C9ORF72 (19, 20). Several molecular mechanisms have been proposed to elucidate the pathophysiological pathways contributing to motor neuron degeneration in ALS, including oxidative stress, glutamate excitotoxicity, mitochondrial dysfunction, dysregulation of RNA processing, protein aggregation, disordered axonal transport and inflammation, abnormal neurofilament function (21–27). Gene expression microarray technology is characterized as a powerful high throughput tool capable of monitoring the expression of thousands of genes in an organism simultaneously (Figure 1). In biomedical research, DNA microarrays initially were designed to measure the transcriptional levels of RNA transcripts derived from thousands of genes in different cell types and tissues (28, 29). More recent implications of DNA microarrays are useful in SNPs detection, RNA splicing and biomarkers determination (30, 31). A significant number of microarray studies has been tried to identify

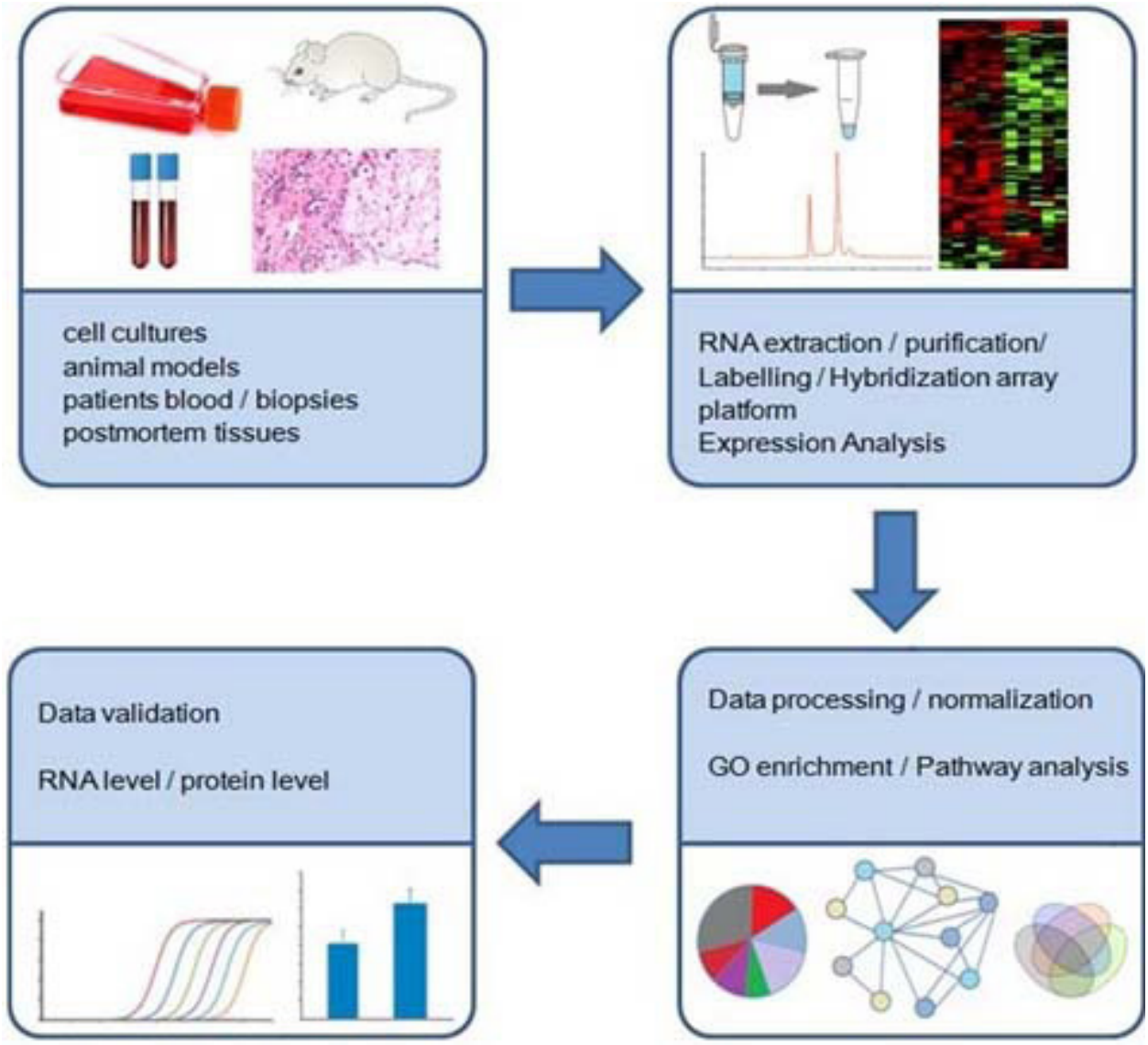

Figure 1. Schematic representation of a general workflow of a microarray study.

transcriptome alterations in ALS disease, investigating the role of new novel genes in this specific pathological process neurodegenerative disorder. This article reviews recent findings from gene expression profiling studies in amyotrophic lateral sclerosis examining molecular signatures related to ALS pathogenesis and potential therapeutic targets identification.

3. MICROARRAY TECHNOLOGY IN ALS: A POTENTIAL TOOL TO INDENTIFY DIFFERENCIALLY EXPRESSED GENES

3.1. ALS microarrays studies related to pathways in disease pathogenesis and progression

Gene expression profiling studies using microarrays represent an excellent tool to analyze the complex of disease's pathobiology as a significant method employed for genome-wide transcriptome profiling. In ALS case, a numerous studies have been implemented both in cell cultures, patients biopsies, animal models and postmortem central nervous tissues attempting to shed light to the cascade of molecular events underlying the syndrome and identifying novel relevant gene targets for therapeutic interference (32–40).

ALS is a complex and multifactorial disease characterized by the involvement of several pathological processes (Figure 2). Among the most well-characteristic pathogenic mechanism of ALS are included axonal transport dysfunction, apoptotic mechanisms, neuroinflammation, protein aggregation and mitochondrial function (41–45). Mitochondria are the major source of intracellular

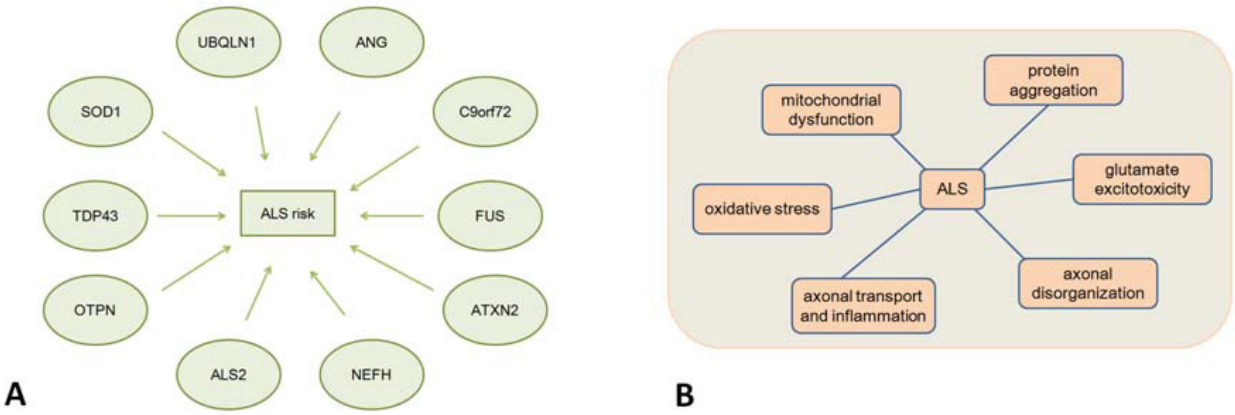

Figure 2. A) Contribution on known genes to ALS pathogenesis. B) Potential mechanisms associated with the pathophysiology of ALS.

reactive oxygen species, such as superoxide anion, hydroxyl radical, hydrogen peroxide and peroxynitrite. Upon mitochondrial stress in the majority of ALS models, SOD1 may undergo oxidative activation, competing with cytochrome C for superoxide released in the mitochondrial intermembrane space, leading to increased ROS production among with increased lipid peroxidation and DNA/RNA oxidative modifications (46–48). The mechanism mutant SOD1 causing direct and glia-mediated neurotoxicity is not fully understood, with NADPH oxidase activation and superoxide production are belonged to the most studies contributors to motoneuron death (49–51). Previous studies have demonstrated the link between oxidative stress and cell death in ALS, such as the factional role of transcriptional factor 53 in motor neuron death in mice (52) or alterations in genes involved in regeneration and tissue degradation (53). Using Mouse Genome 430 2.0. Array from Affymetrix, different mechanisms of neuronal death under oxidative stress or excitotoxic stress were analyzed in two independent cultures of primary cortical neurons of SOD1G93A animals or nontransgenic in response to the cellular stress induced by the NMDA or hydrogen peroxide (54). In SOD1G93A cortical neurons, proteasome-targeting factors such as ubiquitin associated protein 2-like, ubiquitin-conjugating enzyme E2W or ubiquitin-conjugating enzyme E2I subjected to NMDA and autophagosomal protein or proteasome subunit (PSMA6) subjected to NMDA and cytokine transcripts were found upregulated related to nontransgenic one. Utilizing SOD1G93A neurons subjected to hydrogen peroxide, microarray analysis identified altered transcriptional profiling in genes related to controlling actin-associated cytoskeletal remodeling and exogenesis, wnt signaling pathway regulators, trophic factors or ion transport (ARAP2, KIF17, Dickkopf homolog 2, IGFBP4, FGF17, PTGER3, KCNA5, and TRPV1). In another study by Zhang group, Affymetrix GeneChip Drosophila Genome 2.0. arrays were applied to investigate the cell-specific expression of mutant SOD1 in neuronal cells of flies utilizing young and old flies (5 days and 25 days respectively) with $SOD1^{G85R}$ (G85R) expression in motoneurons and glia (55). Depending on the age of flies and cell specific expression of G85R; motoneuron, glia or both, an altered expression of G85R was observed, affecting pathways related to oxidative stress, lipid metabolism, signaling genes and development of nervous system. Focusing on oxidative stress, numerous genes related to pentose-phosphate pathway, glutathione transferase activity and NADP metabolism were found down-regulated, especially in models expressing G85R in motoneurons. Older flies expressing G85R were characterized by increased stimulation to hydrogen peroxidation in motoneuron and glia demonstrating oxidative stress as a potent contributor to the pathology of ALS (55).

In the outstanding work by the Shaw group, Health *et al.* attempted to summarize in depth the applications of the transcriptomic technology in order to examine changes associated among ALS tissues. Gene expression was examined at the level of the tissue and individual cell types in both sporadic and familial forms of the disorder elucidating the mechanisms associated to motor neurons death (56). In a second work by the same group, in order to identify pathways in disease pathogenesis and potential therapeutic targets, microarray human gene expression profiling studies in mixed-cell samples, laser capture microdissection cell samples and peripheral tissue have been summarized, unraveling characteristic key-molecules related to neuroinflammation, RNA splicing and cytoskeleton involvement (57). The importance of microarray-based transcriptomics analysis was also evaluated to identify strategies related to pharmacological targeting interpreting altered pathways and networks (58), while the major findings of numerous studies that have been analyzed using global gene expression in tissues and cells from biopsy or post-mortem specimens of ALS patients or specific animal models (59). These studies corroborated the implication of previously described

disease pathways, and investigated the role of new genes in the pathological process.

The significant role of cytoskeleton-related genes in motor neurons and Schwann cells in the presymptomatic stages of ALS disease was identified for the first time using Agilent Whole Mouse Genome Oligo 4×44 K (60). Differentially expressed genes were identified in the spinal cord from 40 and 80 days old SOD1^{G93A} mice respectively comparing with altered genes in the sciatic nerve from 60 days old mice. GO enrichment analysis was performed and the cellular component GO terms related to microtubule cytoskeleton, actin cytoskeleton and microfilament cytoskeleton were over-represented. Gene expression profile related to ALS indicated *Kif3a* downregulated and *Kif1b* upregulated in the spinal cord of 40 days old mice, *Actg1*, *Adora*, *Akt1*, *App*, *Dctn1*, *Kif1a*, *Sirt2*, and *Stmn1* deregulated in the spinal cord of 80 days whereas in the peripheral nerve of 60 days Aif1, *Aif1*, *Ccnb1*, and *Mapt* have been found downregulated and *Actn3*, *Als2*, *Kif5a*, *Kif5c*, *Nos2, Nos3*, and *Tmod3* upregulated. Gene expression profiling demonstrated differentially regulation of *Kif1b* in the sciatic nerve Schwann cells (downregulation) and spinal cord motor neurons (upregulation) of 40 days old presymptomatic SOD1^{G93A} mice, significant occurrence in ALS pathogenesis. In another study a microarray analysis was performed to identify, for the first time early, molecular alterations in the presyptomatic stage (40 and 80 days) in the lumbar spinal cord of transgenic SOD1^{G93A} mice, using Whole Mouse Genome Oligo 4x44 K from Agilent Technologies (61). KEGG analysis related to ALS mechanism indicated seven common pathways among 40 and 80 days, including regulation of glutamatergic synapse (*Gnai1*, *Slc17a6*), oxidative phosphorylation (*Ndufb11*, *Ndufb8*), endocytosis (*Wwp1*, *Cxcr4*, *Acap2*), ubiquitin mediated proteolysis (*Wwp1*, *Nedd4*, *Ubr5*), chemokine signaling pathway (*Cxcr4*, *Pik3r1*, *Wasl*) and tight junction (*Gnai1*, *Kras*), demonstrating that early neuromuscular abnormalities precede motor neuron death in ALS. The Ube2i expression in astrocytes from 40 and 80 days old SOD1^{G93A} mice, indicates the participation of astrocytes in the early stage of ALS molecular mechanism and motor neuron cell death regulation (61).

The important role of the astroglial glutamate transporter EAAT2 in motor neuron degeneration was demonstrated, indicating the disability in EAAT2 activity as part of the molecular mechanism in inherited and sALS (62). Utilizing Affymetrix GeneChip Mouse Genome 430A 2.0. Arrays, *Eaat2*, *Fus/Tls*, netrin-1, and nestin were observed statistically significant altered in astrocytes of SOD1^{G93A} mutant mice (sumoylated proteolytic fragment). Netrin-1 which was founded the top upregulated among the secreted differentially expressed genes, is associated with axon migration involvement (62). The GPNMB was identified as an ALS-related factor using Agilent Mouse GE 4x44K v1 Arrays in the spinal cord of SOD1^{G93A} mice (63). GPNMB expression was increased in the motor neurons and astrocytes in the spinal cords of SOD1^{G93A} mice even if different phenotypes were observed. This newfound evidence which was confirmed further with qRT-PCR, immunohistochemical analyses and siRNA against GPNMB, indicates that GPNMB inhibits motor neuron death and contributes in motor neurons survival, revealing this transmembrane glycoprotein as a potential therapeutic target for ALS. Expression of Wnt signaling components in the spinal cords of ALS transgenic SOD1^{G93A} mice at different stages has been identified using transcriptional microarray analysis identified (Yu *et al* 2013). Wnt signaling takes part in brain development and spinal cord, playing a significant role in neurogenesis and neurodegeneration. A plethora of canonical and non-canonical Wnt signaling molecules such as Wnt1, Wnt7b, Wnt8b were found depending on the different disease's stages, evidences which were confirmed also at protein level. According to the analyses, the levels of *Ccnd1*, *Ccnd2*, *Ccnd3*, *Ep300*, *Fosl1*, *Nlk*, and *Pitx2* as parts of the Wnt target genes were found increased, indicating the significant role of Wnt signaling in ALS pathogenesis (64).

Nardo *et al.,* using GeneChip Mouse Genome 430 2.0. (Affymetrix), examined an extended comparison of the gene expression profiles of laser captured motor neurons from two separate SOD1^{G93A} mouse strains with different phenotypes like C57-SOD1^{G93A} mice and 129v-SOD1^{G93A} one , indicating transcriptional alterations in mitochondrial regulation, axonal transport pathways and protein degradation (65). The tendency of motor neurons to activate immunological defense mechanisms in response to SOD1 cell stress induced by the presence of mutant SOD1 was revealed, such as motoneurons of C57-SOD1^{G93A} mice, which display up-regulation of MHC class I genes, demonstrating the importance of the study in novel therapeutic intervention (65). Postmortem human material additionally to SOD1^{G93A} ALS and P20L Tau frontotemporal dementia mouse models were exploited to indicate common molecular mechanisms associated to motor neuron degeneration, using Agilent whole mouse genome microarrays (66). The association of altered genes to crucial motor neuron biological processes has been revealed especially on muscle contraction, immune system, stress response, signaling and protein/protein modification regulation. Common genes among animal models and human tissues were identified (Cnga3, *Crb1* and *Otub2*) associated with motor neuron degeneration, explaining similarities between mice's phenotype (66). Whole genome expression profile studies of lumbar spinal cord with peripheral blood and tibialis anterior muscle in SOD1^{G93A} mice at presymptomatic and early symptomatic have been compared performing analysis on Illumina MouseRef8

v1.1. microarray BeadChips (67). *Taldo1*, *Uqcr10* and *Atp6v1d* genes pathway have been observed in all three tissues with highly expression in mice spinal cord and strong regulation in oxidative phosphorylation and pentose phosphate. The last evidence which is also implicating into mitochondria function and oxidative stress regulation indicates a strong overlap between blood and spinal cord gene expression profile in $SOD1^{G93A}$ mouse model, factor able to demonstrate peripheral blood as a potential material for new ALS diagnostic biomarkers development (67).

The alterations in gene expression among oculomotor and spinal cord motor neurons in post-mortem neurologically human midbrain and spinal cord biopsies were ascertaining using GeneChip Human Genome U133 Plus 2.0. Array from Affymetrix (23). A significant number of deregulated genes related to mitochondrial oxidative phosphorylation, immune system functions, transcriptional regulation, and ubiquitin-mediated protein degradation have been revealed with emphasis on GABAergic and glutamate receptor subunits mediated transmission, indication which rationalizes the strong connection between ALS progression and oculomotor neurons (23). Bernandini *et al.,* using Gene Chip Human Genome Focus Array (Affymetrix), tried to maintain a system based on muscle expression profiles in ALS disease (68). Genes linking with human skeletal muscle structure and metabolic pathways have been observed significantly down-regulated like myosin, myogenin, collagen, *Eno3*, *Fbp2*) or up-regulated (forkhead box O, myogenic factor 4, cAMP-dependent protein kinase regulatory subunit RI1 alpha) respectively, additionally to mitochondrial genes like *Actn3 a*nd *Chrna1* which consist members of oxidative phosphorylation pathway (68). Gene groups associated with cytoskeletal and mitochondrian dysfunction in the motor cortex of patients with sALS have been identified (69). In a further study, microarray data from five dependent muscular tissue diseases, namely ALS, acute quadriplegic myopathy, mitochondrial encephalomyopathy, polymyositis, lactic acidosis and stroke-like episodes and dermatomyositis, have been evaluated trying to provide unique molecular markers for each human muscular disease (70). Analyzing ALS patients data from Affymetrix HG-U133A Platform GPL96 performing a variance modeling approach, myofibril genes like nebulin and alpha F-actin, tropomyosins (*Tpm-1/2/3*) and troponins (*Tnn-c1/c2/i2/t1*) were identified significantly downregulated, while actin-capping proteins like *Capza1*, *Capzb* and *Tmod1* upregulated respectively. These results indicate the unique role of myofibril gene dysregulation in ALS as a result of actin-myosin interaction inability (70). Whole genome expression profiles of sALS patients motor cortex samples were performed, identifying potent alterations in selectively genes implicated in cell cycle phases, iron regulation homeostasis, cytoskeleton structure development and synaptic plasticity molecular pathways (71). In a recent study, genes implicated in glutamate metabolism, ER stress, activation of chaperones and endoplasmic reticulum response have been indicated differentially expressed among ALS patients with motor neuropathy human motor nerve biopsies (72).

3.2. ALS microarrays studies associated with post-transcriptional regulation of gene expression

Alternative splicing of mRNA transcripts with emphasis on the nucleo-cytoplasmic transport, translational silencing and RNA degradation, provides an important mechanism for gene regulation and proteomic diversity generation, diversifying in parallel protein modular functions, with neuronal circuits development and synaptic function and plasticity significant impact (73–75). Dysregulations in RNA metabolism, a strong attribute of ALS at multiple levels, contribute to the pathogenesis of the disease, including changes in miRNA biogenesis, spliceosome integrity and RNA editing (76, 77). Mutations in *Tardbp* gene have been found in about 3 to 4% of fALS cases and in about 2% of sALS patients (78, 79). Evidence of the role of mutations in ribosomal proteins TDP-43 and FUS/TLS have been indicated for their contribution in messenger RNA processing and splicing regulation due to their interaction with splicing factors (80, 81). TDP-43, as a highly conserved heterogeneous nuclear ribonucleoprotein, aggregates in the cytoplasm and nuclear compartments of neurons and glial cells, being often accompanied by nuclear clearance of the protein (82, 83). The availability of genomic technologies provides the convenience to unravel disease mechanisms. Gene expression profiling studies using microarrays have been used in order to investigate the transcriptome profiles of the ribosomal proteins regulation in different ALS-animal models or cell lineages. Applying Affymetrix GeneChip Mouse Gene 1.0. ST arrays on C57BL/6J mouse brain, TDP-43 target genes associated with synaptic function and development were identified, depending on their localization at the presynaptic membrane of axon terminals (84). GO analyses revealed that TDP-43 may be a potent RNA regulator of genes involved in synaptic transmission process such as syntaxins, syntaxin binding proteins, synapsin and synaptophysins (84). An extended microarray analysis on brain of GMR-Gal4/UAS-TDP-43 transgenic Drosophila model indicated numerous altered genes implicated mainly in cellular oxidative homeostasis and cell cycle regulation. *Ucp4b* gene profile has been detected upregulated in transgenic flies, while notch genes related to prion disease have been founded upregulated, suggesting that TDP-43 investigates alteration effecting Notch neuronal regulation and intercellular communication pathway in ALS pathogenesis (85).

In parallel, mutations in *Fus* gene have been observed repeatedly in ALS, sharing many common characteristics with TDP-43 as binding proteins with RNA recognition motifs. FUS protein localized into the nucleus of neurons and glial cells, founded in complex with RNA polymerase II along with several transcription factors like YB-1, PU.1 and NF-κB, establishing FUS aggregation as the most prominent pathological feature in both FTLD-FUS and ALS-FUS (10, 86–88). Applying Affymetrix GeneChip Mouse Exon 1.0. ST exon array, gene expression changes and alternative splicing occasions have been examined to elucidate the significant role of this gene in primary *Fus*-deficient motor neurons, cerebellar neurons, cortical neurons and glial cells (89). Comparing *Fus*-mediated gene expression profiles of motor and cortical neurons no significant alterations have been observed contrary to neuronal cells and cerebellar neurons samples. Additionally, motor and cortical neuronal cells profiles were found similar to glial cells but not in alternative exon profiles. A group of differentially motor- and cortical neuron-specific splicing incidents like *Mapt*, *Digap4* and *Snap25* have been identified. Channel-associated genes *Synj1*, *Scn8a* and *Rims1* have been revealed as conceivable *Fus*-regulated motor neuron-specific alternative splicing targets with motor neuron degenerative contribution along with *Kcnip*, *Stxbp1* and *Fmr1* as cortical-neuron-specifc splicing events (89). Extending the previous study, the same group investigated alteration in gene expression and alternative splicing profiles of TDP-43-silenced primary cortical neurons (90) comparing additionally the previous transcriptome profiles with *Fus*-silenced neurons prolifes provided by Fujioka *et al.* Utilizing Affymetrix GeneChip Mouse Exon 1.0. ST Array, 25% of genes with altered expression levels additionally to 10% of genes with differentially spliced exons were similar to the transcriptome profiles of both *TDP-43*-silenced primary cortical neurons and *Fus*-silenced primary cortical neurons (90). These results indicate a significant overlap in gene expression alterations sharing also common molecular pathways, suggesting in summary that both TDP-43 and FUS proteins may affect common downstream RNA-regulated cascades which potentially may be associated with the ALS mechanism.

sALS-associated epigenetic marks have been investigated using Illumina Human Methylation 27 DNA BeadChip array, resulting in aberrant gene expression (91). This study examined ALS-dependent methylation dysregulation of several genes previously implicated in neuronal development, differentiation, and proliferation either mutations in genes associated with mental retardation and neurodegeneration, providing a better understanding of disease pathogenesis and facilitate the discovery of new therapeutic targets. In a different study, the transcriptome profile of spinal cord and cerebellum of TIA-1 depleting mice was dissected using GeneChip HT Mouse Genome 430 2.0. Array Plates (Affymetrix), with emphasis in lipid storage and membrane trafficking, demonstrating the role of TIA-1 protein as a potential effector on mRNA lipid homeostasis regulation in the brain (92).

4. microRNAs IMPLICATION IN AMYOTROPHIC LATERAL SCLEROSIS

Several studies indicate miRNAs as important contributors in motor neuron diseases associated with the central nervous system development, neuronal differentiation and pathogenesis of neurodegeneration (93–95). MiR-34b and miR-9 are playing a potential role in Huntington's disease (96, 97), whereas miR-206, miR-29, miR-132 and miR-153 implicate in Alzheimer's disorder (98–100). In the midbrain of patients with Parkinson's disease, the levels of miR-133b have been founded increased, suggesting this agent contribution as a negative regulator of dopaminergic neuron development (101). Additionally, miR-7 and miR-153 have been identified to regulate alpha-synuclein levels post-transcriptionally (102, 103). In ALS a number of microarrays studies have identified the essential contribution of microRNAs as significant biomarkers, pathogenesis regulators or potential therapeutic targets as well like mir-9, -23a, -29b, -455, -106, -338–3p and -451 (104–108). MiR-155 is presented as a well-promising therapeutic target in ALS supporting pro-inflammatory pathways through interactions with anti-inflammatory molecules like inositol phosphatase SHIP1 and protein kinase phosphatase-1 (109–111). TDP-43 plays an important role in miRNA pathway, while mutations in this protein have been characterized as a common attribute in disease pathogenesis, perturbing miRNAs biogenesis or causing altered expression profiling of mature miRNAs as well (112). The skeletal muscle-specific miR-206 slows ALS progression by sensing motor neuron injury and promoting the compensatory regeneration of neuromuscular synapses, indicating as a promising candidate molecular marker of this motor neuron disorder (113, 114).

The miRNAs transcriptome profiling of $SOD1^{G93A}$ mice brain cortex has been analyzed using mouse miRNA microarrays 8x15K V2 from Agilent Technologies (115). From a pool of significant hybridized miRNAs of mouse brain microglia the same amount was observed also in the whole immune system and brain whereas in overexpressed transgenic mice a numerous of different miRNAs were founded upregulated. Microarray analysis revealed that miR-155, -146b, -22, -365, -125b, -214 have been identified as key immune system contributors which could control neuroinflammatory pathways, suggesting the strong connection between immune system and brain microglia (115). The differential profile of miRNAs has been investigated at the spinal cord of $SOD1^{G93A}$

transgenic mouse model using miRCURYTM LNA array v.18.0. from Exiqon, illustrating a huge number of significant deregulated miRNAs (116). Elevated levels of miRNA-9 three months upon animal death, suggest this molecule as a prominent event in ALS mechanism (116). In order to determine viable miRNA therapeutic targets for ALS, miRNA transcriptome changes in both $SOD1^{G93A}$ rat and $SOD1^{G93A}$ mouse spinal cord tissue have been measured using Affymetrix Mouse Genome 430 2.0. array (109). A plethora of miRNAs profiles were found altered in the end-stage ALS mice and rats spinal cord, focusing mainly on miR-155 expression and the potential anti-miR-155 positive treatment contributing this molecule as a novel well-promising therapeutic target for ALS disorder (109). Toivonen *et al.* investigated miRNAs alterations in the skeletal muscle of $SOD1^{G93A}$ mice upon hybridization on Affymetrix GeneChip miRNA 2.0. chips (113). Significantly altered miRNAs like miR-1, -133a, -133b, -145, -21, -24 and -206 were identified from extensor digitorum longus muscles and plasma of animals, with muscle-enriched miR-206 being the only one with increased expression among male and female groups at neonatal, pre- or late- symptomatic state of ALS progression. This expression pattern has been evaluated also in human ALS patient's serum, prompting miR-206 as a promising candidate biomarker for this selective motor neuron disease.

The expression profiles of human miRNAs have been analyzed using Miltenyi Biotec PIQO miRXplore microarrays in peripheral leukocytes of sALS partients in an earlier stage of the disease (107). Numerous microRNAs were revealed down-regulated compared to healthy patients, whereas miR-338–3b was indicated significantly up-regulated in sALS patients' blood, evidence which was found also in brain from ALS patients (117). These specific microRNAs, which were associated for the first time with sALS, play an important role in PI3K/AKT pathway, like miR-451 or miR-638 in nervous system regulation. Raman *et al.*, exploting Applied Biosystems TaqMan Low Density Arrays, determined gene expression transcriptome changes on miRNA levels in human fibroblast cultures, suggesting fibroblasts as a potential disease model for sALS- and PLS-pathophysiological mechanisms study and therapeutic targeting (75). Comparative analysis identified hundreds of significantly differentially expressed transcripts either in sALS or in PLS fibroblasts respectively, with a variety of genes being implicated in miRNA biogenesis along with transcription, metabolism, RNA processing stress response and signaling (75).

5. DISCUSSION

ALS is a complex and multifactorial disease characterized by the involvement of several pathogenic conditions. The underlying pathophysiology of ALS is not clearly understood causing by several molecular mechanisms proposed to explain the neuronal degeneration in ALS. Large scale gene expression microarray analyses represent an excellent tool aiming to clarify the disease's molecular pathways complexity either to elucidate the contribution of distinct biochemical pathways of this specific disorder (57). These pathways, as presented in Table 1, encompass predominantly oxidative stress, endoplasmic reticulum stress, mitochondrial dysfunction, axonal disorganization, glutamate excitotoxicity, abnormal neurofilament function, protein misfolding, accompanied by the impairment of RNA processing and aggregated proteins accumulation, demonstrating important ALS regulatory key features (56, 118).

In order to extend microarrays measurements focusing on ALS pathogenetical mechanisms being primarily examined complementary to the transcriptome, the implication of RNA-seq is necessary. Next-generation sequencing technologies are revolutionizing our ability to characterize diseases at the genomic, transcriptomic and epigenetic levels (119, 120). RNA-Seq constitutes a developed deep-sequencing technology approach providing an accurately measurement of the level of transcripts (30, 119). As described earlier, RNA-mediated neurodegeneration is acutely implicated in ALS. Significantly mutated RNA-binding proteins *i.e.* TDP43, FUS, SOD1, ubiquilin and optineurin have been found in aggregates into the motor neurons cytosol in ALS patients, leading either to their misfolding and mislocalization or to perturbed RNA metabolism. Dysregulation of RBPs has been recently emerged as a prominent pathogenic mechanism, which follows the discovery of cytoplasmic mislocalization and RBPs aggregation in afflicted sALS neurons (73, 78, 79). RNA-Seq analysis performed by Illumina Genome Analyzer tried to interpret the effects of *Fus* associated with ALS pathogenesis either on wild-type or R521G and R522G mutated or knocked-down one, displaying alternatively splicing patterns (122). DAVID Functional Annotation Tool analysis revealed significant changes in ribosomal-related genes expression in wild-type *Fus* overexpressed samples along with spliceosome-related genes effectiveness upon *Fus* silencing. Extended data interpretation gave prominence to genes associated with RNA-binding motif regulation and endoplasmic reticulum targeting. It should be highlighted that a compelling number of differentially expressed genes has been identified increased both in R521G and R522G *Fus* mutant forms, demonstrating that FUS alterations may contribute to the disease mechanisms (122).

RNA-seq methodology has been demonstrated that TDP-43 plays a significant role as an ion channel regulator, in synaptic transmission and release, in neurotransmitter 123). TDP-43

Table 1. Recent gene expression profiling studies in ALS animal models and patient's samples associated with critical mechanisms in ALS pathogenesis and progression

Samples	Mechanism associated with the pathogenesis of ALS	Significant Findings	Reference
$SOD1^{G85R}$ Drosophila model	Oxidative stress	Cell-specific expression of both dSOD1 and G85R influencing lifespan, hydrogen peroxide sensitivity and lipid peroxidation levels alteration	55
$SOD1^{G93A}$ mouse model	Cytoskeleton-related genes in motor neurons and Schwann cells in the presymptomatic stages	Differentially regulation of *Kif1b* gene in the sciatic nerve Schwann cells along with spinal cord motor neurons	60
$SOD1^{G93A}$ mouse model	Tight junction, antigen processing and presentation, oxidative phosphorylation, endocytosis, chemokine signaling pathway, ubiquitin mediated proteolysis and glutamatergic synapse at both pre-symptomatic ages	Initial triggering for neuronal degeneration and muscle adaptation keeps function before the onset of ALS symptoms. The *Ube2i* expression in astrocytes on the early mechanisms in ALS	61
$SOD1^{G93A}$ mouse model	axon migration	Netrin-1, astroglial glutamate transporter EAAT2 regulation	62
$SOD1^{G93A}$ mouse model	Extracellular matrix	GPNMB as a potential therapeutic target for ALS	63
$SOD1^{G93A}$ mouse model	Wnt signaling pathway	Upregulation of Wnt signaling components and target genes involved in growth regulation and proliferation	64
$SOD1^{G93A}$ mouse model	Immunological, angiogenic activation, and anti-oxidative processes seems to promote neuroprotective effects that are associated with slower disease progression.	Increased major histocompatibility complex I expression by motor neurons	65
human oculomotor and spinal motor neurons, rotent oculomotor and spinal cord	Synaptic transmission, ubiquitin-dependent proteolysis, mitochondrial function, transcriptional regulation, immune system functions, and the extracellular matrix	Enhanced GABAergic transmission associated with ALS progression	23
human ALS patients	Skeletal muscle damage, oxidative metabolism	Correlation of *Prkr1a*, *Foxo1*, *Trim32* and *Actn3*, with sarcomere integrity to mitochondrial oxidative metabolism	68
Motor neurons from patient muscle biopsies	unique downregulation of major thin and thick filament in ALS	myofibril gene dysregulation as a result of loss of actin-myosin interaction.	70
Drosophila	dysregulations in RNA metabolism	Expression of TDP-43 specifically in neurons elicited significant expression differences in genes and pathways	85
central nervous system primary cells	Dysregulations in RNA metabolism	*Mapt*, *Stx1a*, *Scn8a*, identification regulated by *Fus* as potential therapeutic targets for ALS/FTLD.	89
Mouse primary cortical neurons	Dysregulations in RNA metabolism	RNA targets of TDP-43 and FUS as a common pathway in ALS / FTLD neurodegenerative processes	90
Mouse motor neurone-like cell mode, patient-derived fibroblasts	Dysregulations in RNA metabolism	loss of nuclear TDP-43 is associated with RNA processing abnormalities in ALS motor neurones	127
Tia-1 KO mouse nervous tissue spinal cord and cerebellum	Dysregulations in RNA metabolism	Genetic ablation of the *Tia-1* assosiacted with mRNAs encoding lipid homeostasis factors in the brain	92
astrocytes from non-transgenic and TDP-43M337V transgenic cells	Dysregulations in RNA metabolism	Pathogenic TDP-43 affects the expression of secretory proteins (Chi3L1) contributing to non-cell-autonomous neuron death	128
$SOD1^{G93A}$ mouse model	Gene expression regulation at post-transcriptional level	miR-365 and miR-125b interfere, respectively, with the interleukin-6 and STAT3 pathway determining increased tumor necrosis factor alpha (TNFα) transcription	115
$SOD1^{G93A}$ mouse model	Gene expression regulation at post-transcriptional level	miRNA-9 significant role in the pathogenesis of $SOD1^{G93A}$ transgenic mice	116
SOD1(G93A) rat, SOD1(G93A) mouse, ALS patients	Gene expression regulation at post-transcriptional level	miR-155 is indicated as a potential therapeutic target extending survival and disease duration in the SOD1G93A mouse	109

Skeletal muscle and plasma of $SOD1^{G93A}$ mice, serum from human ALS patients	Gene expression regulation at post-transcriptional level	miR-206 as promising candidate biomarker for ALS	113
Leukocytes from ALS patients	Gene expression regulation at post-transcriptional level	mir-338–3p increased levels in sALS patient blood	107
sALS patients, fibroblasts cultures	Gene expression regulation at post-transcriptional level	fibroblasts as potential cellular models for ALS pathophysiological mechanisms study	75
sALS patients	Whole-genome expression profiles of human motor cortex	Significant molecular pathways associated with ALS like iron regulation homeostasis, cytoskeleton strudture development and synaptic plasticity	71
ALS patients	Gene expression changes in human motor nerve diagnostic biopsies obtained from	Significant ALS molecular pathways related to endoplasmic reticulum unfolded protein response, chaperone activity and glutamate metabolism	72

regulates important genes while loss of function or overexpression could exhibit strong effects like altered splicing and clustered annotation of misspliced genes associated with nervous system development, cell projection morphogenesis, ATP-dependent chromatin and regulation of dendrite morphogenesis. Using HITS-CLIP technology coupled with RNA-seq, the pivotal role of *Fus* in neurodegeneration has been revealed as a neuronal transcriptome regulator (124). Sequencing human brain samples and mouse neurons differentiated from embryonic stem cells respectively followed by gene ontology enrichment analysis, has been indicated that *Fus* is participating in an enormous network of cross-regulation of RBPs *i.e.*TAF15, EWS together with FUS constitute the FET family of RBPs, characterized by a strong enhancement in genes responsible for neuronal projection, controlling synaptic, neuronal recognition progress and function (124). Whole transcriptome profiling study of laser capture microdissected motor neurons was performed using the transgenic $G85R^{SOD1\text{-}YFP}$ mouse model at a presymptomatic state, developing a compelling number of differentially expressed genes among G85R and wild-type motor neurons which are linked mainly to neuronal function (125). More precisely, GO analysis illustrated enrichment of genes controlling neurite outgrowth, axon formation, calcium metabolism, calcium sensing, ion homeostasis and mitochondrial function. RNA-Seq analysis of motor neurons in these transgenic $G85R^{SOD\text{-}YFP}$ mice identified slightly mRNA profile alterations. Post-translational effects, could involve interactions between mutant SOD1 misfolding and cellular cytosolic or membrane proteins, affecting conceivably their role in macromolecular trafficking and or synaptic organelle function (125). RNA-seq data analysis at the spinal cord of transgenic FUS^{R521C} mice observed numerous enriched functional annotation groups which regulate extracellular matrix, including members of the collagen and cadherin gene families, phagocytosis, chemotaxis, immune-mediated processes, ion channel, synaptic specificity and neuron outgrowth (126). The transcriptome data indicated also microglia-specific molecular markers associated either with CNS cell types or peripheral myeloid immune cells like *Olfml3*, *Tmem119*, and *Siglec-H*. Simultaneously, RNA-seq data demonstrated that that $SOD1^{G93A}$ microglia expressed fundamental neurotoxic factors, like *Mmp12*, *Optn*, *Spp1*, *TNF-a*, IL-*1b*, *IL-a* along with receptors for type 1 *Ifnar1* and *Ifnar2* and the proinflammatory oxidase NOX2, evidences that attribute a complicated transcriptional profile, revealing neuroprotection and neurotoxicity concurrently (126).

In ALS pathogenesis, it has been assumed that damaging a selective population of motor neurons leads to disease onset, duration and length of survival. It is essential to understand comprehensive disease's etiology to identify novel neuroprotective agents that might postpone either allow disorder's furtherance in parallel with the urgency of compelling therapeutic strategies able to reduce the burden of motor damage (129–132). Further research's direction should be focused on deepening our comprehension on cellular and pathological mechanisms causing ALS. The potential of transcriptomic analysis helps to define candidate genes and novel prognostic biomarkers for future investigation, disease course and treatment response. All new insights contribute to unravel transparently ALS pathogenesis demonstrating likewise perspicuous targets for future therapies.

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Abbreviations: fALS, familiar ALS; sALS, sporadic ALS; *TDP-43,* TAR DNA-binding protein; *TBPH,* TAR DNA-binding protein homolog; FUS, fused in sarcoma protein; VAPB, vesicle-associated membrane protein-associated protein B; SOD1, superoxide dismutase 1; *NEFH*, neurofilament heavy peptide; ANG, Angiogenin; UBQLN2; Ubiquilin 2; ATG4D, autophagosomal protein; GPNMB, nonmetastatic melanoma protein B; SNPs, novel single nucleotide polymorphisms; ACTN3, alpha-actinin-3; CHRNA1, cholinergic receptor, nicotinic, alpha 1; reactive oxygen species, ROS; AQM, acute quadriplegic myopathy; PM, polymyositis; MELAS lactic acidosis and stroke-like episodes; DM, dermatomyositis; FTLD, frontotemporal lobar degeneration; PLS, primary lateral sclerosis; GO, gene ontology; RBPs, ribosomal binding proteins; TGF-beta, transforming growth factor beta; CNS, central nervous system; Mmp12, matrix metalloproteinase 12; Optn, optineurin; TNF-a, tumor necrosis factor a; Spp1,osteopontin.

Key Words: Neurodegenerative disease, Microarray technology, Transcriptomic studies, Mitochondrial stress, ROS production, RNA metabolism, Review

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