Characterization of human sporadic ALS biomarkers in the familial ALS transgenic mSOD1\textsuperscript{G93A} mouse model

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder of motor neurons. Although most cases of ALS are sporadic (sALS) and of unknown etiology, there are also inherited familial ALS (fALS) cases that share a phenotype similar to sALS pathological and clinical phenotype. In this study, we have identified two new potential genetic ALS biomarkers in human bone marrow mesenchymal stem cells (hMSC) obtained from sALS patients, namely the TDP-43 (TAR DNA-binding protein 43) and SLPI (secretory leukocyte protease inhibitor). Together with the previously discovered ones—CyFIP2 and RbBP9, we investigated whether these four potential ALS biomarkers may be differentially expressed in tissues obtained from mutant SOD1\textsuperscript{G93A} transgenic mice, a model that is relevant for at least 20% of the fALS cases. Quantitative real-time PCR analysis of brain, spinal cord and muscle tissues of the mSOD1\textsuperscript{G93A} and controls at various time points during the progression of the neurological disease showed differential expression of the four identified biomarkers in correlation with (i) the tissue type, (ii) the stage of the disease and (iii) the gender of the animals, creating thus a novel spatio-temporal molecular signature of ALS. The biomarkers detected in the fALS animal model were homologous to those that were identified in hMSC of our sALS cases. These results support the possibility of a molecular link between sALS and fALS and may indicate common pathogenetic mechanisms involved in both types of ALS. Moreover, these results may pave the path for using the mSOD1\textsuperscript{G93A} mouse model and these biomarkers as molecular beacons to evaluate the effects of novel drugs/treatments in ALS.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is an adult-onset, incurable progressive neurodegenerative disorder characterized by selective death of motor neurons in the motor cortex, the motor nuclei of the brainstem and the anterior horn cells of the spinal cord, which leads to fatal paralysis (1–4). Most cases of ALS are sporadic (sALS) and of unknown etiology. Approximately 5–10% of patients have an autosomal dominant inherited familial form of the disease with a clear family history (FALS) (5,6) which also can be inherited by an autosomal recessive trait (7,8). FALS is clinically and pathologically indistinguishable from sALS (1), suggesting similar disease mechanisms. ALS has a worldwide incidence that ranges from 1.5 to 2.7 per 100 000/year (9,10), with higher male incidence (9). At least 14 genes and loci have been identified to be mutated in ALS (11–13). Superoxide dismutase 1 (SOD1) gene is the most common one and accounts for 20% of FALS and apparently for 5% of sALS (14). Other examples include the TAR DNA-binding protein 43 (TARDBP-43) and Fused in Sarcoma (FUS) genes which account for 5–10% of FALS (15). Recently, GGGGCC repeat expansions in the gene C9ORF72 were found to be present in 23.5% of FALS tested

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(13). It is unknown how these genes are involved in the pathogenesis of ALS. The causes for most cases of sALS are unknown and the clinical course is highly variable, suggesting that multiple factors are involved in the mechanisms mediating the progression of the disease (2). This fact underlines the need to use a multidisciplinary approach to isolate and characterize specific biomarkers in biological samples from sALS patients. We have previously (16) used human bone marrow mesenchymal stem cells (hMSC) obtained from several ALS patients (ALS-hMSC) and succeeded in identifying two genes, namely the CyFIP2 and the RbBP9, that showed a significant decrease in post-transcriptional A-to-I RNA editing, compared with hMSC from healthy individuals. Moreover, at the transcriptional and translational levels, these two genes showed abnormal expression in ALS-hMSC, suggesting a defect in the regulation of the expression of these genes in ALS patients. To strengthen this view, we tested by quantitative real-time PCR (qRT-PCR) the expression of these genes in peripheral blood leukocytes (PBL) isolated from blood samples of sALS patients and found that the CyFIP2 and RbBP9 levels of mRNA expression were significantly different compared with PBL from healthy donors. Here we show two additional potential biomarker genes that are differentially expressed at the mRNA level in bone marrow hMSC isolated from sALS patients, namely the human secretory leukocyte protease inhibitor (SLPI) and the TAR DNA-binding protein 43 (TARDBP-43 or TDP-43). We subsequently investigated whether these four identified ALS biomarkers, CyFIP2, RbBP9, TDP-43 and SLPI, are differentially expressed in tissues obtained from mutant SOD1G93A transgenic mice. We measured the mRNA expression levels of these four genes in brain, spinal cord and muscle of the mutant SOD1G93A mice and littermate controls at various time points during the development of the neurological disease.

RESULTS

Differential expression of TDP-43 and SLPI in hMSC obtained from sALS patients

As shown previously for the CyFIP2 and RbBP9 genes, we followed the rationale to identify ALS biomarkers in hMSC derived from bone marrow samples of sALS patients and detected two additional candidate genes that were differentially expressed in ALS-hMSC, TDP-43 and SLPI (Fig. 1): TDP-43 is known as a major component of cytoplasmic ubiquitinated protein inclusions in neurons and glial cells of both sALS and fALS patients, resulting in substantial loss of TDP-43 from the nucleus (5,17). Missense mutations in TDP-43 do not seem to be solely responsible for the pathogenicity of TDP-43 inclusions (18,19), since in the majority of ALS patients tested, no TDP-43 mutations were identified, suggesting that abnormal regulation of TDP-43 may be involved in this specific disease process (5,20). We, therefore, looked more closely on the TDP-43 expression profile in ALS-derived cells. qRT-PCR analysis shows significantly altered expression levels of TDP-43 being lower in ALS-hMSC (n = 10) compared with non-ALS-hMSC (n = 7) controls (Fig. 1A). We subsequently examined the expression levels of SLPI. SLPI was originally identified in a complementary DNA (cDNA) microarray comparative analysis that we had performed between two ALS and two non-ALS-hMSC as a gene with a highly differential expression in ALS (data not shown). SLPI is an 11.7 kDa serine protease inhibitor involved in the protection against elastase-induced tissue damage at the site of inflammation (21,22); yet, its implication in ALS remains unknown. qRT-PCR analysis showed a significant alteration in the SLPI expression, being significantly higher in ALS-hMSC (n = 7) than in non-ALS-hMSC (n = 6) controls (Fig. 1B), confirming thus the finding of higher SLPI mRNA expression in ALS cells observed in the cDNA microarray.

Characterization of ALS biomarkers expression in mouse tissues

Having in hand four potential biomarkers that were identified in hMSC obtained from sALS patients, we wanted to further evaluate their significance, and potential diagnostic/prognostic value, by investigating the changes in their expression along with the progression of the neurological disease. For that we used the very well studied transgenic SOD1G93A mouse model. The use of the genetic mouse model allows us to test these genes longitudinally throughout the development of the neurological disease. We examined whether these ALS biomarkers genes were spatiotemporally expressed differently in mutant SOD1G93A transgenic mice compared with non-transgenic littermates (referred to as WT) controls. To this end, we tested the mRNA expression levels of the four genes CyFIP2, RbBP9, TDP-43 and SLPI in the brain, spinal cord and muscle tissues during a time frame of 120 days. Each tissue was obtained from at least three different mice (transgenic and WT; male and female), sacrificed at the age of 30, 60, 90 and 120 days (P30, P60, P90 and P120, respectively), along with the development of the neurological disease (starting from the early pre-symptomatic stage (P30) and ending to the severe symptomatic stage (P120)). RNA was extracted from the tissue samples to produce cDNA, and the qRT-PCR analysis of the specific four biomarker genes was performed. GAPDH expression served as a house-keeping gene reference for each reaction. The multiple samples obtained in these experiments produce a high complexity for the data set analysis and their graphic presentation. In order to simplify the data, the results are presented at two different levels of analysis:

(i) Tissue level: Analysis of the gene expression in each tissue per time point both in males and females. At each time point, we compared the gene expression of each biomarker between mSOD1G93A and WT in each tissue sample (Fig. 2).

(ii) Disease progression level: Analysis of the gene expression pattern in a time-dependent manner in each tissue, both in males and females. We compared gene expression in mSOD1G93A tissue samples at all time points relatively to the respective gene expression in the WT tissue samples (Fig. 3).

Analysis of the gene expression in tissues at specific time points

To determine the differences between WT and mSOD1G93A at the mRNA expression level of each biomarker, in each tissue sample at pre- and symptomatic stages, we performed qRT-PCR analysis and calculated at each time point the relative quantification (RQ) of each gene in the brain, spinal cord and muscle tissues. The
results from these experiments are represented in a heat map for gene expression levels of the four biomarkers in mSOD1\textsuperscript{G93A} tissues relatively to the tissues from WT mice at different age time points, creating a specific molecular spatiotemporal signature (Fig. 2). The representative significance of the gene expression levels in each tissue per time point are indicated in the graph. Overall, the results reveal that each gene is expressed differently in the tissues of WT and mSOD1\textsuperscript{G93A} mice in at least one time point. Furthermore, these results reveal that the tissues express the genes at different levels in pre- and symptomatic stages. Specifically, at the early P30 and P60 stages, the relative gene expression in mSOD1\textsuperscript{G93A} tissues was mainly lower than in WT, whereas at later stages (P90 and P120), the relative gene expression in mSOD1\textsuperscript{G93A} tissues was mainly higher in the WT tissues. Remarkably, two genes, RbBP9 and TDP-43, had shown significant differences at P120 in all three tissues, both in males and females. These results suggest that at a specific time point, each gene presents a different expression pattern in the tissues. The significant differences in gene expression are only relevant to the tissue and at a respective time point.

Analysis of the gene expression in tissues during disease progression

To investigate whether the biomarker expression differences detected in tissues are relevant to disease progression, we examined the expression pattern of the four genes during the time-course period of 120 days by calculating the RQ of each gene expression in WT and mSOD1\textsuperscript{G93A} in each tissue. To identify significant differences, we performed a two-way analysis of variance (ANOVA) in RQ results in which the variables were defined as group (WT versus mSOD1\textsuperscript{G93A}) and time (data from the four time points P30, P60, P90 and P120) and tested the correlation between the group and the specified time point, calculating the RQ value pattern with time progression in males or in females. This evaluation of various correlation levels/patterns aims to help us to determine whether the differences are related to the outcome of the disease or to the intrinsic gene expression pattern behavior over time. The results from this analysis are summarized in a table in Figure 3 indicating in which of the tissues in males and females the expression pattern of the genes was significantly affected by the course of the disease during the 120 days. The respective graphs for each of the genes with significant pattern changes are shown in the results section of Supplementary Material (see also Supplementary Material, Fig. S1).

DISCUSSION

In this work, we show that the previously described genes CyFIP2 and RbBP9, along with the TDP-43 and SLPI, which were identified in the current study, found in hMSC obtained from patients with sALS are also differentially and spatiotemporally expressed in the brain, spinal cord and muscle of the mSOD1\textsuperscript{G93A} mouse model of fALS. These expression differences were found both at pre-symptomatic and at symptomatic stages of the disease. These results support the possibility of a molecular link between sALS and fALS and may indicate common pathogenetic mechanisms involved in both types of ALS. Moreover, these data may pave the path for the utilization of the mouse transgenic model as a reliable tool to investigate the molecular mechanisms of the disease in a time- and tissue-dependent manner.

Our first characterization of these ALS biomarkers found them to be differentially expressed in cultured hMSC of 7–10 sALS cases. The gene expression differences between ALS-hMSC and control-hMSC are kept throughout passages in culture, suggesting that an intrinsic molecular mechanism should be responsible for these differences in gene expression in the sALS cells. Further experiments with more bone marrow ALS-hMSC samples or probably with skin fibroblasts as less invasive material
source are needed to reveal the meaning of this ALS phenotype in non-neurons and its relevance in diagnosis, prognosis and disease mechanism. To this end and supported by our previous findings with CYFIP2 and RbBP9 in blood leukocytes samples of ALS patients (16), it will be important to follow the biomarkers expression including TDP43 and SLPI in blood leukocyte samples of many ALS patients to assess individual expression pattern changes with time or disease progression. Our results indicate that each potential ALS biomarker can be viewed at several different levels and in this way we can assess the gene expression of each biomarker and its possible involvement in the disease mechanism in the mSOD1G93A mouse model. Significant differences were observed for each one of the four genes at the tissue level in at least one tissue at any time point. This type of analysis can be used in the future for efficacy evaluation of potential drug compounds for ALS where a local/tissue drug effect could be assessed at a specific time point, where a significant difference in the biomarkers expression between mSOD1G93A and WT is observed. When looking at the correlation between the gene expression and the time course of the disease (Fig. 3 and Supplementary Material, Fig. S1), we noticed that each gene which is significantly affected by the disease progression in mSOD1G93A shows a trend of increased mRNA levels in muscle. Surprisingly, in both genders, the muscle, and not the brain, nor the spinal cord, showed the highest number of significant differences in the gene expression of the four genes. These results of the muscle tissue may support the ‘dying-back’ hypothesis, which proposes that the pathogenetic mechanism of ALS is initiated within the muscle cells, leading subsequently to the degeneration of the motor neurons, which is possibly mediated by a deficiency in motor neurotrophic factor (15) or by the activation of a retrograde stress signal (23). Interestingly, supporting this possibility, different reports show that RbBP9, CyFIP2 and SLPI are in someway involved in apoptosis in diverse cell types and in cancer (24–28).

From these results, we still cannot determine whether the observed differences in the biomarkers’ expression between mSOD1G93A and WT are caused by the disease or are causing the disease. Nevertheless, our data support the view that these biomarkers could serve as molecular beacons to evaluate the effects of potential ALS drugs on disease progression in the mSOD1G93A mouse model. To prove this concept, it will be

Figure 2. Heat map for the gene expression of the four biomarkers in mSOD1G93A tissues relative to WT at different age time points by qRT-PCR analysis. The mRNA levels in the brain, spinal cord and muscle of the genes CyFIP2, RbBP9, TDP-43 and SLPI were determined by qRT-PCR analysis at different age time points (P30, P60, P90 and P120) in males and females of mSOD1G93A and WT littermates. Color-coded heat map shows the RQ levels in mSOD1G93A relative to WT at each time point in any of the three tissues tested. RQ >1 represents upregulated gene expression in mSOD1G93A at the same time point, whereas RQ <1 represents downregulation of the gene expression. RQ values were normalized to GAPDH house-keeping gene. The white dots overlay represents significant differences between mSOD1G93A and WT obtained at the specific time point by Student’s t-test, P < 0.05.

Figure 3. Effects of disease progression on the biomarkers expression in the brain, spinal cord and muscle of male and female mSOD1G93A. A summary table with the results of significant differences (gray boxes) in the expression pattern of the four biomarkers in the brain, spinal cord and muscle with disease progression. Graphs with the gene expression patterns are shown in Supplementary Material, Figure S1. Analysis of the gene expression pattern of the four biomarkers in tissues in relation with the disease progression was performed by calculating the RQ of each gene expression in WT and mSOD1G93A in each tissue during the time-course period of 120 days. Significant differences were determined separately in males and females by two-way ANOVA on RQ results in which the variables were defined as group (WT versus mSOD1G93A) and time (data from the four time points P30, P60, P90 and P120) and tested the interaction between group and time, which consider the RQ value pattern in each tested tissue with time progression. Statistical significance P < 0.05.
most useful to test, for instance, the effects the drug Riluzole\textsuperscript{TM}, which is known to delay the onset of the disease for few days in the mSOD1\textsuperscript{G93A} mouse model (29) [and modestly slows the progression of ALS for few months in human patients (30,31)], has on the expression pattern of the biomarkers.

Furthermore, our results also suggest that there are strong gender differences in the expression of our identified biomarkers, as evidenced in the WT and mSOD1\textsuperscript{G93A} mice. The gene expression levels of the biomarkers, in general, were higher in females, both in WT and mSOD1\textsuperscript{G93A} mice, with the exception of the expression in the brain (Fig. 2). Our data support the possibility that the gender is a variable that significantly affects the progression of the disease, and that the pathogenetic mechanisms of ALS in males and females could be different. Although several microarray analyzes were performed on mSOD1\textsuperscript{G93A} brain, spinal cord and muscle tissues (32–34), none of them distinguished between males and females, and therefore a microarray transcriptome analysis of the gene expression in the mSOD1\textsuperscript{G93A} mice will be needed to root into this important gender-dependent issue in ALS. Altogether, we have found four novel ALS potential biomarkers in non-neural tissues from sALS patients that may have direct diagnostic and pathological implications in the disease. Understanding the role of these biomarkers in the disease will enable to find targets for future ALS drugs and ALS therapies.

**MATERIALS AND METHODS**

**Bone marrow samples**

Bone marrow samples were obtained under general light anesthesia by aspiration from the iliac crest of seven healthy donors (age range: 20–56 years old) and eight ALS patients (age range: 43–56 years). All volunteers in this work had signed for consent before sample donation according to the guidelines of the ethics committee of the Laniado Hospital and The Hadassah Medical Centre supervised by the Israeli Health Ministry Ethics Committee conforming with The Code of Ethics of the World Medical Association (Declaration of Helsinki), printed in the British Medical Journal (18 July 1964).

**Isolation and culture of hMSC**

Bone marrow hMSC were isolated and cultured as described before (16). hMSC at passages 3–7 were used in this study. Briefly, the isolated cells were phenotypically characterized by FACS analysis for the presence or absence of a battery of specific cellular markers and by their multipotency differentiation potential as previously described (35).

**Mice**

B6SJL-TgN(SOD1\textsuperscript{G93A})1Gur/J transgenic male and female mice expressing mutant hSOD1\textsuperscript{G93A} and Non-carrier (NCAR) littermates (n = 48) were purchased from The Jackson Laboratories (Bar Harbor, ME, USA) and kept in the university animal care facility until the time of sacrifice at the age of 30, 60, 90 and 120 days. The Institutional Animal Care Committee at the Tel Aviv University approved all the animal protocols in this work.

**RNA isolation in hMSC and animal tissues**

The hMSC were cultured until reaching 80% confluence and kept in DMEM/F12 without serum for 72 h before the experiments. RNA isolation and cDNA synthesis were performed in these cells as described previously (16). Brain, spinal cord and the gastrocnemius muscle tissue samples were dissected from transgenic mice and NCAR littermates at defined pre-symptomatic (postnatal day 30 and day 60— P30 and P60, respectively) and symptomatic (P90 and P120) stages of the neurological disease. Total RNA was isolated using TRIzol\textsuperscript{®} reagent (Invitrogen, USA) according to the manufacturer’s instructions. The concentration of total RNA was measured using Nano Drop Spectrophotometer (Nano Drop Technologies, USA).

**qRT-PCR analysis**

Total RNA (1000 ng per 20 µl) was reverse-transcribed into cDNA with High Capacity cDNA RT Kit (Applied Biosystems, CA, USA) according to the manufacturer’s protocol. PCR reaction was carried out using Taq 2 \times Master Mix Purple (Lambda Biotech, Inc., USA) according to the manufacturer’s instructions. qRT-PCR was carried out using TaqMan\textsuperscript{™} Gene Expression Assays (Applied Biosystems) according to the manufacturer’s protocol using StepOnePlus\textsuperscript{TM} Real-Time PCR System (Applied Biosystems). qRT-PCR primers (TaqMan\textsuperscript{®} probes) were HPRT (Mm01545399_m1, Hs99999909_m1), GAPDH (Mm99999915_g1), TARDBP (Mm00523866_m1, Hs006065_22_m1), CyFIP2 (Mm00460148_m1), RbBP9 (Mm00489397_m1), SLPI (Mm00441530_g1, Hs00268204_m1).

**Data and statistical analysis**

Analysis of qRT-PCR data was conducted by the RQ method. One-way ANOVA, two-way ANOVA (for data with more than two variables) or Student’s t-test was used when suitable. A P-value of ≤ 0.05 was considered statistically significant.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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