

Ribosomal protein 6S mRNA is a biomarker upregulated in multiple sclerosis, downregulated by interferon treatment, and affected by season

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Abstract

Background: Multiple Sclerosis (MS) is an immune-mediated disease of the central nervous system which responds to therapies targeting circulating immune cells.

Objective: To test if the T cell activation gene expression pattern (TCAGE) we had previously described from whole blood was replicated in an independent cohort.

Methods: We used RNA-seq to interrogate the whole blood transcriptomes of 72 individuals (40 healthy control, 32 untreated MS). A cohort of 862 control individuals from the Brisbane Systems Genetics Study (BSGS) was used to assess heritability and seasonal expression. The effect of interferon beta (IFNB) therapy on expression was evaluated.

Results: The MS/TCAGE association was replicated and rationalised to a single marker, ribosomal protein S6 (RPS6). Expression of RPS6 was higher in MS than controls ($p < 0.0004$), and lower in winter than summer ($p < 4.6E-06$). The seasonal pattern correlated with monthly UV light index ($R = 0.82$, $p < 0.002$), and was also identified in the BSGS cohort ($p < 0.0016$). Variation in expression of RPS6 was not strongly heritable. RPS6 expression was reduced by IFNB therapy.

Conclusions: These data support investigation of RPS6 as a potential therapeutic target and candidate biomarker for measuring clinical response to IFNB and other MS therapies, and of MS disease heterogeneity.

Introduction

MS is an autoimmune disease of the central nervous system caused, at least in part, by pathogenic leukocytes which transit from lymphoid tissue to the brain via the peripheral circulation.¹ This paradigm is supported by the success of immunomodulatory therapies such as Natalizumab, which prevents activated T cell migration across the blood-brain barrier from the peripheral circulation²; and of Fingolimod, which blocks egress of activated T cells from the lymph nodes.³ It is also supported by studies on genetic susceptibility⁴, since the majority of identified genes associated with MS are predominantly expressed in immune cells, especially the dendritic cell/ T cell axis.⁴ Monitoring changes in the numbers and states of these cells may identify pathogenic processes, useful biomarkers of susceptibility, molecular subtypes of diseases, response to therapy, and new therapeutic targets.

Earlier, we had reported mRNA expression for all known genes in whole blood from 144 individuals, 99 of whom had untreated MS.⁵ We had used PAXgene blood collections, which capture transcriptomes immediately on blood draw, rather than peripheral blood mononuclear cell (PBMCs) methods, which are less reproducible.⁶ Analysis of gene expression and genotype indicated the dominant pattern of dysregulation of T cell gene expression in MS in these individuals is matched with the genetic associations from the GWAS⁴ pointing to T cell dysregulation in MS. We identified a **T cell activation gene expression (TCAGE)** signature in whole blood that predicted the likelihood of MS. This signature reflects excess expression of genes upregulated on T cell activation and proliferation, mainly from translation and oxidative phosphorylation metabolic

pathways.⁵ This TCAGE signature needed to be confirmed in an independent cohort, and potentially simplified. The effect of therapy on the TCAGE is currently unknown, and the heritability of this signature is also not established.

In this study, we have first tested if next generation sequencing of messenger RNA (RNA-seq) could be used to interrogate gene expression in whole blood samples by comparing expression measured by both techniques (RNA-seq and microarrays) for the same samples. We then used RNA-seq to assay whole blood transcriptomes for 40 healthy controls and 32 people with untreated MS. The TCAGE signature could be rationalized to a single gene, ribosomal protein S6 (RPS6). We have tested if RPS6 expression is affected by genotype. Finally, interferon beta (IFN β) is the most widely used MS therapy, and is commonly thought to function by immunomodulation.⁷ We have tested if RPS6 expression is altered by therapy.

Materials and Methods

Sample Collection

RNA-seq Cohort: For the RNA-seq experiment, a single PAXgene blood RNA tube (PreAnalytiX, Switzerland) was collected from people with MS who were not receiving any treatment, and had not received any immunomodulatory therapy in the previous three months, from clinics in Sydney and Adelaide. Blood was also collected from healthy controls of matching ages and not receiving immunomodulatory therapy. Demographics for patients and controls are described in Table 1.

Brisbane Systems Genetics Study (BSGS) cohort: This cohort comprises 862 individuals of European descent from 312 independent families⁸ located within the greater Brisbane region. For each cohort informed written consent was obtained from each donor, and the study was approved by the Human Research Ethics Committee (HREC) from Sydney, Adelaide and Brisbane.

IFN β therapy cohort: PAXgene blood was collected from an IFN β therapy cohort of anonymised donors who were identified as MxA bioactivity positive or negative by bioactivity testing.⁹ Approval to use these samples was also granted by SWAHS HRECS.

RNA-seq

Whole blood: Total RNA was isolated from PAXgene RNA blood tubes using the PAXgene Blood RNA Kit (Qiagen, Germany), and subsequently treated with GlobinClear (Life Technologies, NY, USA), to deplete the whole blood sample of alpha and beta globin mRNA. RNA was then prepared for sequencing on Illumina

HiSeq 2000 using the Illumina TruSeq RNA sample preparation kit V1 (Illumina, CA, USA). Samples were indexed and 6 run per lane, generating at least 10 million 50bp reads per sample. Raw sequence data was aligned to the UCSC human reference genome (hg19) using the Tophat software package.¹⁰ Aligned sequencing reads were summarised to counts per gene using the Read Assignment via Expectation Maximiation (RAEM) procedure¹¹ and reads per kilobase per million mapped reads (RPKM) values calculated in SAMMate (v 2.6.1). RPKM values were transformed by quantile normalisation prior to visualization using BRB-Array Tools.¹² Visualisation was performed by performing hierarchical clustering using 1 minus correlation and average linkage metrics. The microarray gene expression analysis has been previously described in⁵ for the ANZgene cohort, and in¹³ for the Brisbane Systems Genetic Study.

Quantitative RTPCR

Primers were designed to amplify across the last two exons of RPS6 - RPS6F:GCGGCGTATTGCTCTGAAGA, RPS6R: GCAGAGAGGAAAGTCTGCGT. cDNA was made using SuperScript™ III Reverse Transcriptase (Invitrogen, San Diego, CAL), and RTPCR was performed using Power SYBER Green PCR Master Mix (Applied Biosystems, Carlsbad, CAL) at an initial 30sec 95°C denaturation, 30sec 64°C annealing and 30sec 72°C extension for 5 cycles, then 30sec 95°C denaturation, 30sec 60°C annealing and 30sec 72°C extension for 40 cycles, followed by a melt curve of 15sec 95°C, 15sec 55°C and 15sec 95°C. Quantification was by comparison to GAPDHF:ACGCATTTGGTCGTATTGGG, GAPDHR: TGATTTTGGAGGGATCTCGC , as previously described.¹⁴

Statistical Analysis

Firstly, both the pooled ANZgene cohort and individual RNA-seq data were analysed using the statistical package, EdgeR.¹⁵ EdgeR was used to assess differences in gene expression between MS and controls. Genes expressed at >25 reads per million total reads and calculated to be differentially expressed with FDR<10% were identified (Supplementary Table 1). Secondly, using the data obtained by RNA-seq, all genes with a minimum RPKM value greater than 100 were selected for further analysis (344 genes). To account for differences in expression levels, for each gene the expression level was ranked across individuals, and ranked data used to test correlations between genes. Further details are provided in figure legends and the supplementary information section.

Results

Replication of the TCAGE in an independent cohort using RNA-seq

We interrogated whole blood gene expression using mRNA from blood collected into PAXgene tubes, and RNA-seq to assess gene expression. To test the RNA-seq protocol, we first prepared two cDNA libraries from pooled mRNA from 20 MS patients and 20 healthy controls (HC) used in the ANZgene microarray experiment reported earlier,⁵ and measured expression of the transcriptome using HiSeq2000 to 30 million reads per pool. All 38 TCAGE genes expressed at >25 reads per million reads were more highly expressed in MS, 16 of these at $p < 0.001$ (Fig. 1, Supplementary Table 1A, Fig. S1). Half of these 16 were genes encoding ribosomal proteins. We then prepared RNA-seq libraries from an independent collection of whole blood from 40 HC and 32 untreated MS patients (cohort traits detailed in Table 1), and measured transcriptomes to a depth of >10 million reads per sample. We used EdgeR to assess the relative expression of all genes expressed with at least 25 reads per million reads in all samples. Of the 26 TCAGE genes in this list 12 were ribosomal protein genes, all of which were more highly expressed in MS (12/12, $p < 0.0005$). This low p value is indicative only, since these genes are likely to be coordinately regulated. Since our second study was smaller than the first, we used a permissive FDR of 10% to define a dysregulated gene set (genes listed in Supplementary Table 1B). Ribosomal protein genes are again over-represented.

We then tested the cellular pathways that were over-represented, and the immune cell subsets tagged, by these genes; and compared these with those

identified in our previously published microarray study. Again, over-expressed genes were predominantly expressed in T cells (Fig. 2a), underexpressed genes predominantly expressed in other immune cell subsets (Fig. 2b), and the most dysregulated pathways were those involving the ribosomal protein genes (Fig. 2c).

RPS6 is a surrogate marker for ribosomal protein and other TCAGE genes

If the ribosomal protein (RP) genes are coordinately expressed, measurement of one should provide a proxy for regulation of all. From these data, we chose RPS6 as the most highly associated with MS ($p < 0.00036$, t test on RPKM ranks), and compared the rank of its expression with the sum of the ranks of all TCAGE genes in each individual. Expression of RPS6 was tightly correlated with the expression of all 12 RP genes ($R = 0.93$, $p < 1.96E-25$, Fig. 3a), with all 26 TCAGE genes ($R = 0.85$, $p < 1.65E-18$), and with the 14 nonRP genes in the TCAGE list ($R = 0.70$, $p < 1.87E-11$). By comparing the aligned reads from the RNA-seq analysis of pooled MS and HC samples, it can be seen that exon usage for RPS6 was the same in MS and HC (Fig. 3b inset). We designed primers from across the exon 4-5 boundary and compared RPS6 expression by RTPCR with the RPKM measurement from RNA-seq. Measurements from both techniques were highly correlated ($R = 0.93$, $p = 2.73E-24$, Fig. 3b).

RPS6 expression is seasonal

In the RNA-seq cohort age and gender were not associated with RPS6 expression (Fig S6), but season was (Fig. 4b). We analysed the data using month of sample collection, with months classified as Summer or Winter on the basis of RPS6 expression: In Summer - October-March – RPS6 expression was highest ; Winter - April-September, lowest expression.

Overall, in the RNA-seq cohort RPS6 expression was higher in MS than HC (Fig. 4a, $p < 0.003$). However, this difference was only evident in winter ($p < 0.00019$) with a trend in summer (Fig. 4b, $p = 0.19$). The seasonal difference for HC (Fig. 4, $p < 7.2E-8$) and MS patients ($p < 0.004$) was also highly significant. Similar differences were seen in the ANZgene microarray cohort (Fig. 4a), with a significant difference between MS and controls in summer ($p < 0.0002$). The RPS6 seasonal pattern was confirmed in a third large cohort of HC from the sub-tropical city of Brisbane ($p < 0.0016$, Fig. S2). In both the RNA-seq cohort ($R = 0.82$, $p < 0.002$) Fig. 5b) and the Brisbane cohort ($p < 0.01$, Fig. S2) the RPS6 expression correlated with UV radiation. Although RPS6 expression in the RNA-seq cohort did not correlate with Vitamin D levels ($R = 0.27$, ns, Fig5), correlation increases if two months out of phase, with RPS6 increasing two months earlier than Vitamin D ($R = 0.71$, $p < 0.011$). No diurnal effect on RPS6 expression was detected in 5 individuals assayed at 4 time points (Supplementary Methods, Figure S3).

Using Receiver Operator Curves, where the expression of RPS6 was 95% sensitive to detect MS, the specificity was 43%, with an area under the curve of 0.72, $p = 0.0012$ (Fig. 6). In winter, this specificity improves to 67%, and the area under the curve to 0.92, $p < 0.0001$. In summer RPS6 expression was not predictive of MS.

Because some studies but not others¹⁶ have indicated month of birth affects on MS risk, and RPS6 regulation might be set by imprinting, we tested if expression was associated with month of birth. There was no association.

Relative expression of ribosomal protein genes in immune cell subsets

To identify the cell subsets contributing to the difference in the RP signature between MS and HC, we interrogated transcriptomes from 23 immune cell subsets in whole blood using RNA-seq (Fig. 7, Supplementary Methods), and compared this to *in silico* data for the subsets.^{17, 18} The expression level between subsets was consistent (Figure S4). RPS6 and other ribosomal protein genes were most highly expressed in CD4 and CD8 T cells.

RPS6 expression is altered by interferon beta

We measured RPS6 expression 9-15 hours after IFNB injection in 32 MS patients, 16 of which were classified as responders to IFNB, as indicated by MxA upregulation, whilst 16 were classified as non-responders. The difference between the two is considered to be due to the development of neutralizing antibodies in the latter.⁹ RPS6 expression was lower in the IFNB responsive group when compared to the non-responsive MS patients in both summer (p=0.018) and winter (p=0.006). In addition, the RPS6 expression was lower in MS patients, classified as IFNB responders when compared to controls in summer (p=0.046) and winter (p=0.079). The non-responders exhibited higher

RPS6 expression than controls in summer ($p=0.003$) and winter ($p=0.006$), and they had similar expression of RPS6 to untreated MS (Fig. 8).

RPS6 expression is not strongly heritable

Expression profiles were collected for the 862 individuals from extended MZ and DZ twin families in Brisbane System Genetics Cohort (BSGS) using Illumina HT12 v.40 arrays.¹³ Genotypes for each of these individuals were also determined (Illumina 610-Quad Beadchip). Family relationships among individuals within BSGS were used to estimate the additive genetic component of expression variation for the two probes in RPS6 using ASREML.¹⁹ Heritabilities (h^2) for the two probes were estimated by the ratio of additive genetic variation to total phenotypic variation. Estimates of h^2 for the two RPS6 probes on this chip were zero, even after correcting for month of sample collection, suggesting that any variation in the expression levels of the two probes is due to environmental factors, at least for whole blood. None of the 528,509 SNPs on this chip were significantly correlated with RPS6 expression.

Discussion

Using an independent cohort and alternative method of transcriptome interrogation, we have replicated our original finding that a set genes predominantly expressed in T cells, notably the ribosomal protein (RP) genes, are overexpressed in MS. Measurement of expression of one gene, RPS6, could be used as a surrogate for the expression of the other RP and TCAGE genes. As well as disease state, RP gene expression was highly affected by season. Higher expression was seen in the late spring, summer months than winter and early spring, mirroring the UV light seasonal pattern. We found no evidence that the expression pattern was heritable, suggesting that the difference in expression between MS and HC was caused by disease and/or environmental factors. RPS6 whole blood expression was also reduced on IFNB therapy.

Ribosomes are made up of equimolar amounts of each of the 80 ribosomal proteins, so that co-ordinate regulation of RPs is to be expected,²⁰ and is observed in this study. However, there are extra-ribosomal functions, and variable phosphorylation of the ribosome constituent proteins, so that cell subsets have subtle but important differences in their RP transcriptomes.²¹ RPS6 is essential for ribosome biogenesis, is phosphorylated at evolutionarily conserved residues, and regulates protein synthesis, cell growth and proliferation, but also has specific features which have led to its intensive investigation as a biomarker.²² It is most highly expressed in lymphocytes, especially naïve T cells (Fig 8^{17, 18}). It controls metabolic signaling in CD8 T cells;²³ has been reported to be a predictor of resistance to therapeutic inhibition of FLT3 targeting leukemic blasts;²⁴ and predicts sensitivity to the inhibitor of

immune cell proliferation, mTOR.²⁵ Higher expression of RPS6 in PBMCs was observed in people with MS more likely to relapse.²⁶ A gene regulating RPS6 phosphorylation, RPS6KB1, is associated with MS susceptibility. Phosphorylation of RPS6 is downstream of mTOR activation, and therapeutic inhibition of mTOR by drugs such as rapamycin increase the proportion of Tregs to Th1 and Th17 cells.²⁷ These data support a role for RPS6 in MS susceptibility, pathogenesis, therapeutic response, and as a target for novel therapies.

Immune cell expression of RPS6 is higher throughout the year in untreated MS patients, suggesting a greater T cell proliferation/activation, which may be enough to increase risk of development of MS and relapses. MS onset and relapses are most common in spring/summer in both the northern and southern hemispheres.^{28,29} This is when UV light and RPS6 expression start to increase and before serum vitamin D increases. Seasonally corrected serum vitamin D levels correlate with reduced risk of onset and relapses in MS, in both hemispheres²⁹.

Demands on the immune system are clearly seasonal, and immune cell subsets numbers and proportions in whole blood fluctuate.^{30,31} Although the regulators of the seasonal pattern are not known, they could include melatonin, light sensitive hormones such as vitamin D, and UV light factors independent of vitamin D.³² The seasonal fluctuation in serum 25D levels is some two months out of phase with the RPS6 and UV light fluctuation. This could indicate RPS6 expression is immediately responsive to UV light, or that immediate vitamin D

fluctuation due to UV light affects immune cells more than serum vitamin D levels.

IFNB has been the most commonly prescribed drug for MS, with many possible mechanisms of action proposed.³³ These include regulation of the balance of immune cell subsets. We have shown that the RPS6 signal is greatly reduced on IFNB therapy. This may be affected by a number of factors: reduced T cell activation and proliferation as a therapeutic response, reduced RPS6 transcription on therapy, or to altered T cell representation in whole blood after injection due to trafficking of immune cell subsets. Reduced lymphocyte and neutrophil numbers, but greatly increased monocyte numbers were seen in whole blood after injection,³⁴ and altered immune cell state might also be expected. RPS6 whole blood levels would be expected to drop, as observed in the current cohort. Whether this reduction is correlated, or even underpins clinical response needs to be determined.

A major limitation of using whole blood is that particular immune cell subset differences are difficult to identify. In this case, although RPS6 expression is predominantly from T cells (Fig. 6), the cell subset source of the RPS6 difference between MS and controls will now need to be established using flow cytometry. Cell subsets which vary by season, and in interferon response, have previously been described,^{31,32,34} but which of these, or of other subsets, contribute to the RPS6 seasonal fluctuation and IFNB response will also need to be established through flow cytometric methods. Another limitation of this study is that the RNAseq cohort controls and some MS patients were from Sydney, and many MS

patients but no controls were from Adelaide. No confounding factors between the two regions are known eg they have similar UV light levels (Fig 4), but its possible.

High RPS6 expression, fold change between seasons, or discrepancy between RPS6 and other physiological parameters such as serum vitamin D, may indicate MS susceptibility and state, but also a molecular subtype of MS particularly likely to respond to certain therapies. Testing such possibilities is vital to developing cost-effective therapies in autoimmune diseases.³⁵

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Author Contribution

Design: DB, GP

Laboratory Work: GP,PG,SS

Sample Collection: MS: SV,PG,FM,MK,MS,JL, SB; HC:PG, FM,PV, GM,JP

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Final Version: all authors

Conflict of Interests

None

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Figure Legends

Figure 1. Heat map of TCAGE gene expression in RNA-seq and ANZgene cohorts.

Rows are expression of the most highly expressed T cell activation genes (TCAGE) identified in Gandhi et al (2010)⁶ plotted against the healthy controls and MS patients, sorted by season, for the ANZgene cohort (from pooled RNA-seq) and the RNA-seq cohort. For the latter, each individual is a column. Columns sorted by month of collection. Orange is high expression, blue is low.

Figure 2. Heat map of Immune Cell Subset Expression of Genes Dyregulated in MS in the RNA-seq Cohort. Rows are the genes identified as dysregulated in the RNA-seq cohort (expression > 25 reads per million read, FDR 10%), columns the immune cell subsets (detailed in the Methods section). **A.** Genes overexpressed in MS, **B.** Genes underexpressed in MS, **C.** Pathways overrepresented in the over-expressed genes. This is determined for the 552 genes with the lowest p values (corresponding to the number identified as differentially expressed at 10% FDR in the microarray analysis by⁵) in both cohorts, and the 10% FDR list from the RNA-seq cohort. These lists were uploaded into Metacore (GeneGo, USA) and tested for over-representation in curated biological pathways (Figure 2C).

Figure 3. RPS6 expression is correlated with expression of other ribosomal genes.

A. Rank of RPS6 expression in 72 individuals, measured by RNA-seq, and Sum of Ranks of expression of all other Ribosomal Genes for those individuals. Correlation=0.93 **B.** Primers designed from exon usage in MS and Controls (inset) and the linear correlation of RTPCR and RNA-seq measurement of RPS6 is 0.95.

Figure 4. RPS6 expression in whole blood is dependent on season and disease state.

A. Comparison of RPS6 expression in the RNA-seq cohort (this study) and the ANZgene cohort, published in Gandhi et al, 2010.⁶ RPS6 is measured by reads per kilobase per million mapped reads (RPKM) for RNA-seq, and by fluorescence on microarrays (ANZgene). Data are from normalised expression, compared using two-tailed T tests. **B.** Effect of season on RPS6 expression in the RNA-seq cohort.

Figure 5. RPS6 expression is affected by season and is correlated with UV radiation and Vitamin D levels.

A. Top graph is RPS6 expression in each month, middle graph is UV index for different localities used as the source of samples (Australian Bureau of Meteorology website: <http://www.bom.gov.au/>), lowest graph is Vitamin D levels in NSW.⁴⁸ **B.** Correlation of RPS6 expression with UV radiation (R=0.83), Vitamin D (R=0.25), and Vitamin D, two months out of phase (R=0.70). R is the correlation co-efficient, determined from a linear correlation.

Figure 6. Receiver operator curves to predict MS from RPS6 expression

Figure 7. RPS6 is predominantly expressed in T cells.

Heat map shown for immune cell subsets (columns) and most expressed ribosomal protein genes, including RPS6 (rows). Colour is proportional to expression, with orange high expression and blue low expression. Immune cell subsets are defined in Supplementary Material and Methods.

Figure 8. Effect of Interferon Treatment on RPS6 Expression.

People with MS on interferon beta without neutralizing antibodies (IFN (R)), had lower RPS6 expression than healthy controls (HC), those treated with interferon but with neutralizing antibodies (IFN (NR)), and people with MS (MS). The latter did not differ from IFN (NR). Each comparison for the IFNB treatment was assessed using the two tailed T test, with no correction for multiple testing.