Multiple Sclerosis and Related Disorders 36 (2019) 101407

Contents lists available at ScienceDirect

Multiple Sclerosis and Related Disorders

journal homepage: www.elsevier.com/locate/msard

Long noncoding RNAs associated with phenotypic severity in multiple sclerosis

Mehul Gupta[a], Kristina Martens[a], Luanne M Metz[a,b], AP Jason de Koning[c,d], Gerald Pfeffer[a,b,d,*]

[a] Hotchkiss Brain Institute, University of Calgary, Calgary, Alberta, Canada
[b] Department of Clinical Neurosciences, Cumming School of Medicine, University of Calgary, Canada
[c] Department of Biochemistry and Molecular Biology, University of Calgary, Calgary, Alberta, Canada
[d] Alberta Children's Hospital Research Institute, University of Calgary, Calgary, Alberta, Canada

ARTICLE INFO

Keywords:
Multiple sclerosis
Disease severity
Phenotype
Long noncoding RNA
Biomarker

ABSTRACT

Introduction: Multiple sclerosis (MS) is a disease that causes progressive neurological disability. Treatments are available that are protective against MS relapses and it is thought that reduction of early neuroinflammation may improve long term prognosis. At present there is no biomarker that can predict which patients may have a more severe disease course, and potentially benefit from more aggressive therapy. Long noncoding RNAs (lncRNAs) are emerging as potential disease biomarkers that could be of interest in prognostication of MS.

Methods: We identified a discovery cohort of 20 patients, ten of which had a mild MS phenotype and ten with severe MS phenotype according to the Age-Related MS Severity Scale (ARMSS). RNAseq was performed on RNA extracted from whole blood and bioinformatic analysis restricted to lncRNAs. Our goal was to select the most significant lncRNAs and quantify these using custom digital droplet RT-qPCR assays in a validation cohort of 44 participants (with mild or severe MS).

Results: Eight lncRNA candidates were identified from the discovery cohort. Of these, four lncRNAs remained significantly differentially expressed in the validation cohort (ENSG00000260302, ENSG00000270972, ENSG00000272512 and ENSG00000223387). Little is known about the precise roles of these lncRNAs but based on expression data they appear to be important to immune function and are of potential biological significance to MS pathogenesis.

Conclusions: This study is the first to investigate possible lncRNA biomarkers to differentiate phenotypic severity in MS. Although the findings are preliminary based on our small sample size, they are sufficient to identify hypotheses for future investigation, and give guidance regarding the design of future studies.

Introduction

Multiple Sclerosis (MS) is a significant source of disability in young adults and occurs with a prevalence of 240 per 100,000 in Canada (Beck et al., 2005). Decision-making regarding MS therapy is complex, although the general principle is that reduction of inflammation early in the disease course increases the likelihood of reduced disability during the later phase of the disease. The available disease-modifying therapies have differences in their ability to prevent clinical relapses and development of further MRI abnormalities, and in general the more efficacious agents are associated with more severe side-effects (Scolding et al., 2015). In this respect it would be preferable to use the high-potency agents in cases expected to have a more rapidly progressing disease course. Unfortunately, there is substantial clinical heterogeneity of MS, with no biomarker capable of predicting which patients will develop severe, rapid disability.

There is currently a high level of research interest in lncRNAs as biomarkers and for their potential contribution to the pathogenesis of MS. One research group used microarray analysis in six MS patients and five controls, with subsequent *in silico* analyses as well as transfections of specific lncRNAs in CD4 + cells of MS participants and controls (Zhang et al., 2016; Zhang et al., 2017; Zhang et al., 2018). Studies by this group identified overexpression of lncDDIT4 and its corresponding cis-target, DDIT4, in the peripheral blood mononuclear cells of MS patients compared to healthy controls. Moreover, silencing of lncDDIT4 in naïve CD4 + T cells enhanced Th17 cell differentiation via upregulation of the DDIT4/mTOR pathway, suggesting a potential mechanistic role of lncDDIT4 in TH17 cell differentiation. Additionally, this group demonstrated overexpression of Linc-MAF-4 in MS patients. Further *in vitro* assays suggest that Th1 cell differentiation of naïve

* Corresponding author at: HMRB 155 3330 Hospital Dr. NW Calgary, Alberta, Canada.
E-mail address: gerald.pfeffer@ucalgary.ca (G. Pfeffer).

https://doi.org/10.1016/j.msard.2019.101407
Received 29 June 2019; Received in revised form 29 August 2019; Accepted 18 September 2019

CD4 + T cells increases expression of Linc-MAF-4, with down regulation of the transcript biasing cells towards Th2 differentiation. These results suggest lncRNA transcripts may play a mechanistic role in the differentiation of T cell lineages thought to be associated with CNS autoimmunity and demyelination. Other studies have selected candidates based on previously described associations with immune disorders and used RT-qPCR to compare MS participants to controls (Dastmalchi et al., 2018; Eftekharian et al., 2017; Ganji et al., 2019; Gharesouran et al., 2018; Gharesouran et al., 2019; Gharzi et al., 2018; Santoro et al., 2016), or analysis of polymorphic variation (Mahdi Eftekharian et al., 2019; Rezazadeh et al., 2018). This latter type of study raises the possibility that noncoding sequence variants may have an important effect on lncRNA function and MS pathogenesis. Multiple differentially expressed promising candidates have been identified through these methods, however, functional characterization and validation are necessary to develop diagnostic markers for MS.

Another report quantified lnc-IL-7R based on its positional relationship to IL-7Ra, a candidate gene associated with MS in 36 MS patients and 30 healthy controls (Bina et al., 2017). Though the study was unable to identify differential expression of either the lncRNA candidate or its associated gene, significant correlation between lnc-IL-7R and IL-7Ra were seen in both MS and healthy groups. These studies suggest a potential functional role of lncRNA transcripts in regulation of gene targets in MS. Several studies using animal models have also considered the role of lncRNAs in MS pathogenesis (Duan et al., 2018; Guo et al., 2017; Li et al., 2018; Liu et al., 2018; Sun et al., 2017), or combinations of human participants and animal model studies (Masoumi et al., 2019; Pahlevan Kakhki et al., 2018). These studies have demonstrated the role of multiple lncRNA transcripts in differentiation of CD4 + T cell linages.

This study investigated whether a long non-coding RNA (lncRNA) expression signature may act as a prognostic biomarker for MS, allowing identification of patients who will develop a more severe disease course.

Methods

Ethics statement

Research ethics board approval was obtained from the University of Calgary Conjoint Health Research Ethics Board (REB17-1193). All participants provided written informed consent.

Cohort selection

Patients were identified from amongst participants of an ongoing dynamic prospective cohort study of MS at the University of Calgary MS Clinic in Calgary, Alberta, Canada. Patients were categorised as having mild or severe phenotype of MS based upon their age-related MS severity scores (ARMSSS (Manouchehrinia et al., 2017)), from at least three clinic visits. Of the 2831 study participants, the 600 patients with the lowest ARMSSS values were designated as having a mild phenotype, and the 600 patients with the highest ARMSSS values were categorised as having a severe phenotype. A subset of 64 participants: 21 severe (ARMSSS range: 5.73–9.94) and 43 mild (ARMSSS range: 0.06–3.08) participated in this pilot study. Participant blood samples were collected into Paxgene RNA tubes, and total RNA was extracted using the Paxgene RNA purification kit. 20 samples (10 mild and 10 severe) were included in a discovery cohort for candidate biomarker identification using transcriptome sequencing (RNAseq), while the remaining 44 (33 mild and 11 severe) were included in a validation cohort. We analysed differences in clinical characteristics of the cohort including MS onset age, age at recruitment, sex, EDSS, ARMSS, and disease-modifying therapy used at the time of recruitment. Significance was determined using Mann-Whitney-Wilcoxon test for ordinal variables and by Chi-square or Fisher's exact test for nominal variables.

RNA-Seq

Total RNA samples were verified by fluorimetry using an RNA specific dye (Qubit fluorimeter) and RIN analysis on an Agilent TapeStation 2200. Samples had RIN scores of 6.4–8.4. 500 ng of each sample was then subjected to rRNA depletion and Illumina library preparation using the #20020596 TruSeq Stranded Total RNA Library Preparation Kit (H/M/R) from Illumina according to the manufacturer's Low Sample (LS) protocol. This protocol used RiboZero magnetic beads to remove rRNA and single Illumina TruSeq i7 indexes. After PCR enrichment (15 cycles) and clean-up, the final libraries were validated by TapeStation analysis and qPCR library quantitation (Kapa qPCR Library Quant Kit for Illumina) and then pooled. Sequencing was then performed on an Illumina NextSeq 500 sequencer. For single-end sequencing, the 20 sample pool was sequenced on three consecutive 75 cycle high-output NextSeq V2 sequencing runs. An average of 80 million clusters PF were obtained for each sample. For the paired-end sequencing, the 20 sample pool was sequenced on a single 150 cycle (2×75 bp) high-output NextSeq V2 sequencing run to yield an average of 26 million clusters PF per sample.

Bioinformatic analysis

Resulting RNAseq data were analyzed for differential expression between mild and severe subgroups using the DeSeq2 R package (Love et al., 2014). RNAseq data were aligned to the EnsEMBL GRCh38.p12 transcript set using the FeatureCounts package. Once aligned, the transcript count matrix was analyzed using DeSeq2 to identify transcripts differentially expressed between MS phenotypes. Transcripts annotated as lncRNAs in the EnsEMBL GRCh38.p12 were selected for further inspection. Additional selection was performed for transcripts with detectable expression in at least 18 of 20 participants. Further selection identified transcripts with uncorrected statistical significance between MS phenotypic groups and fold change $> = 2$. As part of our mitigation strategy in case no transcripts were statistically significant, we selected candidates that were closest to statistical significance for testing in the validation cohort. A heat map visualizing expression levels of selected candidate genes as well as an unsupervised Euclidean hierarchical clustering was performed using the *pheatmap* R package.

Digital droplet PCR (ddPCR) validation

Reverse transcription was performed with iScript cDNA Synthesis Kit (Bio-Rad) and random hexamers. We used custom primers, with ddPCR EvaGreen Supermix (Bio-Rad), as per manufacturer's protocol on a Bio-Rad QX200 digital droplet qPCR system (ddPCR). These ddPCR assays were used over conventional qPCR, as they provide absolute quantification and are able to detect isoforms at very low abundances (Campomenosi et al., 2016; Hindson et al., 2011). lncRNA expression was normalized to *HPRT1* and *B2M* expression. We first used ddPCR to validate the most differentially expressed candidates from RNAseq data, and those that were consistent between RNAseq and ddPCR were selected for ddPCR validation in the confirmation cohort of 44 participants. Significance of differential expression between mild and severe subgroups was tested using a one-tailed Mann-Whitney U test. GraphPad Prism 8.0 was used for these analyses. To determine the specificity and sensitivity of significantly differentially expressed lncRNAs to identify patients with severe phenotype, we performed a random forest analysis. This was conducted with 100 decision trees, 10-fold cross-validation, and we defined mild phenotype as “negative” and severe phenotype as “positive”.

Results

Our included participants had highly significant differences in

Table 1
Clinical characteristics of included participants.

	Mild phenotype	Severe phenotype	*P* Value*
Discovery Cohort			
Number of participants	*N* = 10	*N* = 10	
Patient age at onset (range)	33.9 (16.6–56.2)	32.2 (14.6–47.3)	0.0929
Patient age at consent (range)	57.8 (45.3–70.1)	54.6 (34.4–67.1)	0.4480
Sex (percent F)	7 (70%)	10 (100%)	0.2105
Patient ARMSSS (range)	1.864 (0.225–3.017)	6.518 (5.804–7.891)	< 0.0001
Patient EDSS (range)	2.1 (0.0–6.0)	5.2 (1.0–7.0)	< 0.0001
Confirmation Cohort			
Number of participants	*N* = 33	*N* = 11	
Patient age at onset (range)	35.6 (20.9–46.2)	28.7 (14.8–46.1)	0.7649
Patient age at consent (range)	53.4 (32.4–67.4)	52.1 (39.6–59.5)	0.7133
Sex (percent F)	20 (60.6%)	11 (100%)	0.0189
Patient ARMSSS (range)	1.177 (0.060–2.194)	7.255 (6.187–9.401)	< 0.0001
Patient EDSS (range)	1.2 (0.0–3.5)	5.3 (2.5–8.0)	< 0.0001

* Significant values.

ARMSS and EDSS scores, which was expected based on our inclusion criteria (Table 1). Other variables such as age at disease onset or age at recruitment did not differ significantly between groups. There were no men in the severe phenotype groups of either cohort but the proportion in the mild phenotype groups reflected typical MS populations. A majority of the participants were receiving one of the MS disease modifying therapies, and this is represented in **Supplemental Table 1**. The therapies received did not significantly differ between mild and severe groups.

Whole transcriptome sequencing was completed for 20 samples in the discovery cohort. Bioinformatic analysis identified 1344 lncRNAs with detectable expression levels, with 16 of these meeting significance criteria (unadjusted $p < 0.05$ and fold change ≥ 2). We selected eight of these candidates for further analysis and validation (Table 2; the remaining candidates are available in **Supplemental Table 2**). Unsupervised hierarchical clustering of mild and severe patients based on expression of these eight candidate lncRNAs revealed moderate segregation of mild and severe phenotype MS patients (Fig. 1). We proceeded with validation using ddPCR in the validation cohort using custom-designed ddPCR assays as described above. This identified four lncRNA transcripts which were significantly differentially expressed between mild and severe participant groups, which are designated as ENSG00000260302, ENSG00000270972, ENSG00000272512 and ENSG00000223387 (Table 2 and Fig. 2).

To assess ideal sample sizes needed to perform future validation studies, a prospective power analysis was conducted assuming an acceptable Type I error rate of 5%. Expression data were modelled following a Gaussian distribution with mean and standard deviation for each group estimated from the corresponding sample statistics in the discovery cohort. Assuming that the observed numbers of mild to severe phenotype patients in a larger validation cohort will follow the same 3:1 mild:severe ratio observed in the validation cohort at hand, and assuming that validation testing would be performed separately at each candidate locus, our simulations ($n = 1000$) suggested that 80% power can be achieved in an ideal sample of 415 individuals. This value is largely determined by the candidate from the discovery cohort with the weakest effect size (ENSG00000213373). If excluded, a sample size of 152 individuals is predicted sufficient to achieve at least 80% power at all remaining 7 loci. If we similarly excluded the locus with the smallest remaining effect, a sample size of 90 would be sufficient to achieve at least 80% power at the remaining 6.

Finally, in order to optimistically evaluate the best possible performance of a classifier based on a combination of these four lncRNAs, a random forest classifier was trained and evaluated by 10-fold cross

Table 2
Candidate lncRNA Transcripts and associated discovery and confirmation cohort results. *P* values for differential expression between mild and severe subgroups are presented for the 8 candidate lncRNA transcripts. Data are shown for targeted transcriptome sequencing, ddPCR assays conducted for the discovery cohort, and ddPCR assays conducted for the confirmation cohort. Targeted transcriptome sequencing data was analyzed using the DESeq2 R package. DdPCR count data for discovery and confirmation cohorts was analyzed using Graphpad Prism 8.0 (one-tailed Mann Whitney U test). Asterisk (*) indicates significance ($p < 0.05$) and double asterisk (**) indicates a high level of significance ($p < 0.01$). An additional lncRNA approached significance († indicating $p < 0.1$).

	Discovery cohort, RNAseq (lncRNAs only)		Confirmation cohort ddPCR Validation
lncRNA candidates	Mean (standard deviation)	*P*-value (differential transcript levels)	*P*-value normalized to *HPRT1* and *B2M*
ENSG00000260302	Mild: 5.9 (5.9) Severe: 15.5 (8.9)	0.00061	**0.0012** **
ENSG00000272512	Mild: 2.8 (2.2) Severe: 10.7 (12.5)	0.00078	**0.0368** *
ENSG00000249790	Mild: 314.1 (279.7) Severe: 1090.1 (881.7)	0.00236	0.3492
ENSG00000224177	Mild: 9.4 (5.9) Severe: 20.9 (11.1)	0.00256	0.0810 †
ENSG00000223387	Mild: 7.3 (7.9) Severe: 25.4 (30.8)	0.00265	**0.0427** *
ENSG00000260979	Mild: 4.1 (3.5) Severe: 9.1 (4.8)	0.00522	0.1403
ENSG00000270972	Mild: 32.1 (15.5) Severe: 55.5 (54.0)	0.03411	**0.0200** *
ENSG00000213373	Mild: 21.6 (8.6) Severe: 34.4 (48.6)	0.0423	0.3077

* Significant values.

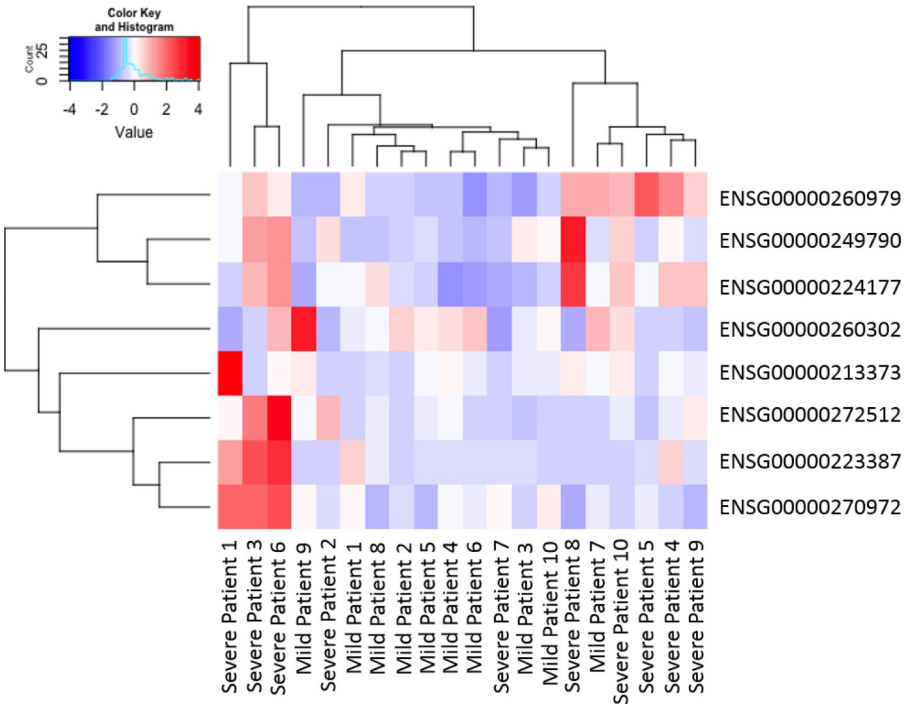

Fig. 1. Clustering of expression profiles for eight candidate lncRNA markers in the discovery cohort. Heat map depicts expression levels for each candidate lncRNA (rows) for individual patients (columns). Red and blue squares indicate higher and lower expression levels of candidate genes respectively. Unsupervised hierarchical clustering indicates that most severe phenotype patients (excluding severe patients 2 and 7) cluster into two groups, which collectively contain only a single mild phenotype patient (patient 7).

validation on a combination of the discovery and validation cohorts. Random forests is a widely-used machine learning algorithm based on an ensemble of decision trees, where each tree is fit to a different, randomly selected set of features. This approach is superior to conducting univariate tests at each locus, because it is able to effectively exploit joint information across features that can be discriminating between groups. This analysis revealed a best case specificity of 86.4% but a sensitivity of merely 45.4% for the identification of the severe MS phenotype.

Discussion

This study investigated the relative expression profiles of lncRNA transcripts between mild and severe phenotype MS, in order to identify potential biomarkers able to differentiate between these subgroups. To our knowledge, this is the first study in MS participants to attempt to identify a prognostic biomarker using unbiased investigation of lncRNAs. Of the candidates examined, ENSG00000260302, ENSG00000270972, ENSG00000272512 and ENSG00000223387 were consistently differentially expressed between the target subgroups in both the discovery and confirmation cohorts, suggesting they may have some utility as candidate prognostic biomarkers. The direction of the effect was the same in the discovery and confirmation cohorts, with an increase in transcript levels appearing in the severe group for each lncRNA.

The most significantly differentially expressed lncRNA, ENSG00000260302, is highly expressed in double-positive CD4+/CD8 + thymocytes (EMBL-EBI Expression Atlas, BLUEPRINT Epigenome project, accessed Jan 25, 2019). Interestingly, this T-cell population has received some limited prior study in MS. One preliminary study suggested that this population of cells was over-represented in MS patients compared with controls (Munschauer et al., 1993), however, a subsequent study was not able to validate this finding (Waschbisch et al., 2014). As pointed out by the authors, the follow-up study may have failed to detect a difference because it included patients with mild or early-onset disease (Waschbisch et al., 2014). Taken together, these results and our findings may be consistent with a role for double-positive T-cells in the etiology of severe MS. Future studies should directly investigate the dual-positive T-cell population in a similar cohort, which would allow the potential relationship with lncRNA expression to be further interrogated.

ENSG00000270972 is most highly expressed in mature neutrophils, among other immune cells such as CD-8 positive αβ-thymocytes (EMBL-EBI Expression Atlas, BLUEPRINT Epigenome project). This finding is of great interest because of the prior description of elevated neutrophil to lymphocyte ratios in prior studies of MS (Bisgaard et al., 2017), that appear to correlate with clinical relapses (Demirci et al., 2016), greater disease severity (Hasselbalch et al., 2018), in addition to being associated with other features that affect overall disease severity (Al-Hussain et al., 2017). Neutrophils are described to be more numerous and in a relatively more activated state in MS (Naegele et al., 2012), which is also consistent with this finding of our study.

ENSG00000272512 is most highly expressed in mature eosinophils, but is also produced from CD4+/CD8 + thymocytes (EMBL-EBI Expression Atlas, BLUEPRINT Epigenome project). This association with eosinophils is of interest because this cell type can be upregulated with certain types of disease modifying therapy, specifically natalizumab (Abbas et al., 2011). None of the participants in our cohort received this agent. Eosinophils are also characteristically present in lesions of people with neuromyelitis optica spectrum disease; they are not typically present in people with MS except in fulminant cases (Lucchinetti et al., 2002). This lncRNA may therefore have some biological plausibility given its relative higher levels in severe phenotype participants.

ENSG00000223387 has received limited study and was not clearly correlated with a particular cell type, except for very low expression in megakaryocytes (EMBL-EBI Expression Atlas, BLUEPRINT Epigenome project). The association of this lncRNA with severe phenotype in our study is of unknown significance.

Based on the findings in our ddPCR assays, the differences between mild and severe phenotype MS participants are not substantial enough to act as biomarkers on their own, and there is significant overlap between groups (Fig. 1A, B, E and G). Based on our simple random forest analysis, the four identified lncRNAs also appear insufficient to act as a biomarker when considered together. However, our ability to evaluate the utility of joint expression signatures is limited by our relatively small sample size. The clinical utility of expression differences at these loci must therefore be evaluated in future, larger studies, potentially in

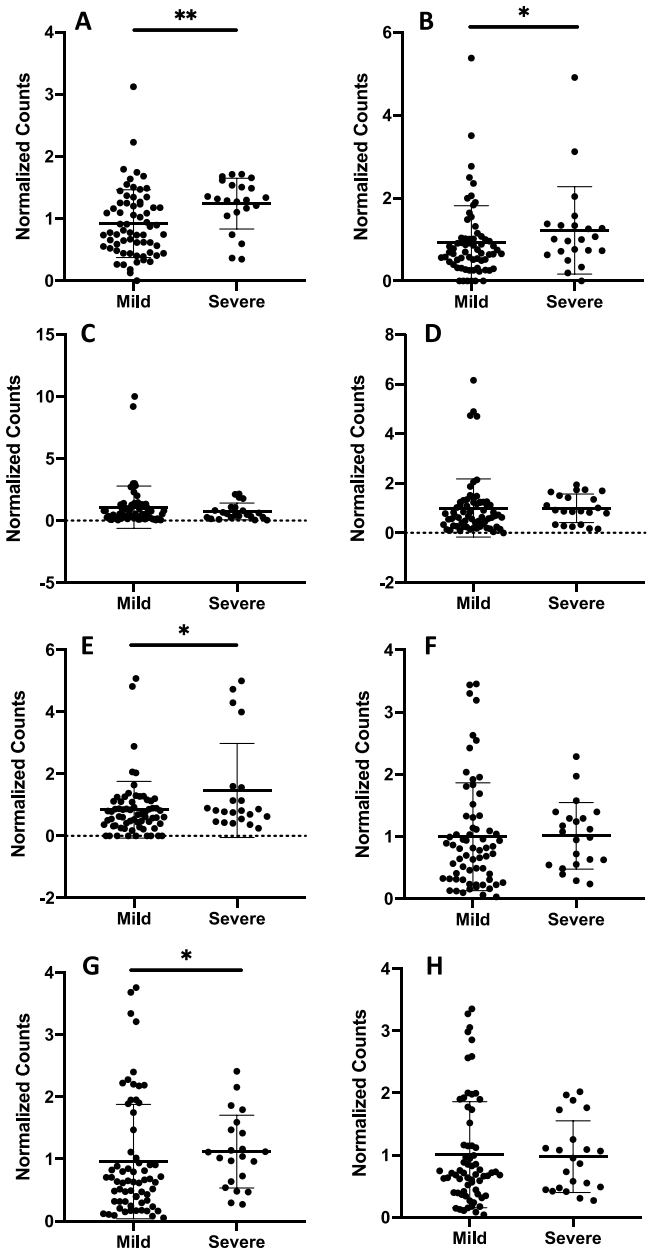

Fig. 2. Scatter plots presenting ddPCR data from the validation cohort counts were normalized to B2M and HPRT1 and are presented in the same order as in Table 2, (a) ENSG00000260302, (b) ENSG00000272512, (c) ENSG00000249790, (d) ENSG00000224177, (e) ENSG00000223387, (f) ENSG00000260979, (g) ENSG00000270972, (h) ENSG00000213373. The lncRNAs ENSG00000260302, ENSG00000270972, ENSG00000272512 and ENSG00000223387 were significantly differentially expressed in the validation cohort (panels 1A, 1B, 1E, and 1G). Mean and standard deviation is indicated.

combination with other types of informative data.

In addition to their potential for predicting MS phenotype, based on what is known about the four differentially validated lncRNAs, these may be indicating potential biological importance in the pathogenesis of MS which merits future study. The role of these candidate biomarkers will need to be validated in a separate cohort, and future study may consider assaying RNA isolated from different blood compounds (WBC fractions, serum, exosomes, as examples) to obtain better resolution regarding this finding. Future studies using *in vitro* or animal models would aid in determining whether these lncRNA isoforms have a role in the severity of MS.

There are several limitations to this study. The lack of men in the severe participant groups could have affected the results, although when analysing women only, three of the four identified lncRNA candidates remained significant (**Supplemental Table 3**). Information about HLA types and EBV status may be helpful to include in future study. The number of participants in the severe disease group was also smaller than the mild phenotype group which could have affected our ability to detect significant differences. This study was limited by a relatively small cohort size and was cross sectional in nature. The use of RNA isolated from whole blood has advantages as well as disadvantages—the approach benefits from stability of the RNA and uniformity in collection and processing. However, disadvantages of whole blood include the fact that its contents may differ widely depending on numerous factors such as coincident medical conditions or viral infections. Another disadvantage of this study is that we did not record white blood cell counts at the time of recruitment, which would have been a valuable observation given that three of the identified lncRNAs are highly expressed in leucocytes. Despite these limitations, this study identified three candidates with a biologically plausible role in MS phenotypic severity and if validated in future study, would be of substantial clinical value.

Future functional analysis will be crucial in order to confirm and understand the nature of the involvement of these lncRNAs in MS pathogenesis. One example of methodology that may be useful in further investigation would include the investigation of these lncRNAs in T-cells, of which examples exist in the literature (Zhang et al., 2016; Zhang et al., 2017; Zhang et al., 2018).

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

Acknowledgements

This work was funded by a Seed Grant from the Alberta Multiple Sclerosis Collaboration and Multiple Sclerosis Society of Canada. Mehul Gupta is the recipient of a Markin Undergraduate Studentship (2018). Gerald Pfeffer is the recipient of funding from the University of Calgary, Cumming School of Medicine, Hotchkiss Brain Institute, and Canada Foundation for Innovation. Sequencing was performed at the Centre for Health Genomics and Informatics (CHGI), which is supported by the University of Calgary.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.msard.2019.101407.

References

Abbas, M., Lalive, P.H., Chofflon, M., Simon, H.U., Chizzolini, C., Ribi, C, 2011. Hypereosinophilia in patients with multiple sclerosis treated with natalizumab. Neurology 77 (16), 1561–1564.

Al-Hussain, F., Alfallaj, M.M., Alahmari, A.N., Almazyad, A.N., Alsaeed, T.K., Abdurrahman, A.A., et al., 2017. Relationship between neutrophil-to-lymphocyte ratio and stress in multiple sclerosis patients. J. Clin. Diagn. Res. 11 (5), CC01–CCC4.

Beck, C.A., Metz, L.M., Svenson, L.W., Patten, S.B, 2005. Regional variation of multiple sclerosis prevalence in Canada. Mult. Scler. 11 (5), 516–519.

Bina, P., Pahlevan Kakhki, M., Sahraian, M.A., Behmanesh, M., 2017. The expression of lnc-IL-7R long non-coding RNA dramatically correlated with soluble and membrane-bound isoforms of IL-7Ra gene in multiple sclerosis patients. Neurosci. Lett. 642, 174–178.

Bisgaard, A.K., Pihl-Jensen, G., Frederiksen, J.L, 2017. The neutrophil-to-lymphocyte ratio as disease actvity marker in multiple sclerosis and optic neuritis. Mult. Scler. Relat. Disord. 18, 213–217.

Campomenosi, P., Gini, E., Noonan, D.M., Poli, A., D'Antona, P., Rotolo, N., et al., 2016. A comparison between quantitative PCR and droplet digital PCR technologies for circulating microRNA quantification in human lung cancer. BMC Biotechnol. 16 (1), 60.

Dastmalchi, R., Ghafouri-Fard, S., Omrani, M.D., Mazdeh, M., Sayad, A., Taheri, M., 2018. Dysregulation of long non-coding RNA profile in peripheral blood of multiple sclerosis patients. Mult Scler Relat Disord 25, 219–226.

Demirci, S., Demirci, S., Kutluhan, S., Koyuncuoglu, H.R., Yurekli, V.A, 2016. The clinical

significance of the neutrophil-to-lymphocyte ratio in multiple sclerosis. Int. J. Neurosci. 126 (8), 700–706.
Duan, C., Liu, Y., Li, Y., Chen, H., Liu, X., Chen, X., et al., 2018. Sulfasalazine alters microglia phenotype by competing endogenous RNA effect of miR-136-5p and long non-coding RNA HOTAIR in cuprizone-induced demyelination. Biochem. Pharmacol. 155, 110–123.
Eftekharian, M.M., Ghafouri-Fard, S., Soudyab, M., Omrani, M.D., Rahimi, M., Sayad, A., et al., 2017. Expression analysis of long non-coding RNAs in the blood of multiple sclerosis patients. J. Mol. Neurosci. 63 (3–4), 333–341.
Ganji, M., Sayad, A., Omrani, M.D., Arsang-Jang, S., Mazdeh, M., Taheri, M., 2019. Expression analysis of long non-coding RNAs and their target genes in multiple sclerosis patients. Neurol. Sci. 40 (4), 801–811.
Gharesouran, J., Taheri, M., Sayad, A., Ghafouri-Fard, S., Mazdeh, M., Omrani, M.D., 2018. The growth arrest-specific transcript 5 (GAS5) and nuclear receptor subfamily 3 group C member 1 (NR3C1): novel markers involved in multiple sclerosis. Int. J. Mol. Cell. Med. 7 (2), 102–110.
Gharesouran, J., Taheri, M., Sayad, A., Ghafouri-Fard, S., Mazdeh, M., Omrani, M.D., 2019. A novel regulatory function of long non-coding RNAs at different levels of gene expression in multiple sclerosis. J. Mol. Neurosci. 67 (3), 434–440.
Gharzi, V., Gangi, M., Sayad, A., Mazdeh, M., Arsang-Jang, S., Taheri, M, 2018. Expression analysis of BDNF gene and BDNF-AS long noncoding RNA in whole blood samples of multiple sclerosis patients: not always a negative correlation between them. Iran. J. Allergy. Asthma. Immunol. 17 (6), 548–556.
Guo, W., Lei, W., Yu, D., Ge, Y., Chen, Y., Xue, W., et al., 2017. Involvement of lncRNA-1700040D17Rik in Th17 cell differentiation and the pathogenesis of EAE. Int. Immunopharmacol. 47, 141–149.
Hasselbalch, I.C., Sondergaard, H.B., Koch-Henriksen, N., Olsson, A., Ullum, H., Sellebjerg, F., et al., 2018. The neutrophil-to-lymphocyte ratio is associated with multiple sclerosis. Mult. Scler. J. Exp. Transl. Clin. 4 (4), 2055217318813183.
Hindson, B.J., Ness, K.D., Masquelier, D.A., Belgrader, P., Heredia, N.J., Makarewicz, A.J., et al., 2011. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Anal. Chem. 83 (22), 8604–8610.
Li, Z., Gong, P., Wang, W., Qu, S., Wang, X., He, Z., et al., 2018. Functional network analysis reveals biological roles of lncRNAs and mRNAs in MOG35-55 specific CD4(+)T helper cells. Genomics 110 (6), 337–346.
Liu, X., Zhang, Q., Wang, W., Zuo, D., Wang, J., Zhou, F., et al., 2018. Analysis of long noncoding RNA and mRNA expression profiles in IL-9-Activated astrocytes and EAE Mice. Cell. Physiol. Biochem. 45 (5), 1986–1998.
Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome. Biol. 15 (12), 550.
Lucchinetti, C.F., Mandler, R.N., McGavern, D., Bruck, W., Gleich, G., Ransohoff, R.M., et al., 2002. A role for humoral mechanisms in the pathogenesis of Devic's neuromyelitis optica. Brain 125 (Pt 7), 1450–1461.
Mahdi Eftekharian, M., Noroozi, R., Komaki, A., Mazdeh, M., Taheri, M., Ghafouri-Fard, S., 2019. GAS5 genomic variants and risk of multiple sclerosis. Neurosci. Lett. 701, 54–57.
Manouchehrinia, A., Westerlind, H., Kingwell, E., Zhu, F., Carruthers, R., Ramanujam, R., et al., 2017. Age related multiple sclerosis severity score: disability ranked by age. Mult. Scler. 23 (14), 1938–1946.
Masoumi, F., Ghorbani, S., Talebi, F., Branton, W.G., Rajaei, S., Power, C., et al., 2019. Malat1 long noncoding RNA regulates inflammation and leukocyte differentiation in experimental autoimmune encephalomyelitis. J. Neuroimmunol. 328, 50–59.
Munschauer, F.E., Stewart, C., Jacobs, L., Kaba, S., Ghorishi, Z., Greenberg, S.J., et al., 1993. Circulating CD3+ CD4+ CD8+ T lymphocytes in multiple sclerosis. J Clin Immunol 13 (2), 113–118.
Naegele, M., Tillack, K., Reinhardt, S., Schippling, S., Martin, R., Sospedra, M., 2012. Neutrophils in multiple sclerosis are characterized by a primed phenotype. J. Neuroimmunol. 242 (1–2), 60–71.
Pahlevan Kakhki, M., Nikravesh, A., Shirvani Farsani, Z., Sahraian, M.A., Behmanesh, M., 2018. HOTAIR but not ANRIL long non-coding RNA contributes to the pathogenesis of multiple sclerosis. Immunology 153 (4), 479–487.
Rezazadeh, M., Gharesouran, J., Moradi, M., Noroozi, R., Omrani, M.D., Taheri, M., et al., 2018. Association study of ANRIL genetic variants and multiple sclerosis. J. Mol. Neurosci. 65 (1), 54–59.
Santoro, M., Nociti, V., Lucchini, M., De Fino, C., Losavio, F.A., Mirabella, M., 2016. Expression profile of long non-coding RNAs in serum of patients with multiple sclerosis. J. Mol. Neurosci. 59 (1), 18–23.
Scolding, N., Barnes, D., Cader, S., Chataway, J., Chaudhuri, A., Coles, A., et al., 2015. Association of British neurologists: revised (2015) guidelines for prescribing disease-modifying treatments in multiple sclerosis. Pract. Neurol. 15 (4), 273–279.
Sun, D., Yu, Z., Fang, X., Liu, M., Pu, Y., Shao, Q., et al., 2017. LncRNA GAS5 inhibits microglial M2 polarization and exacerbates demyelination. EMBO Rep. 18 (10), 1801–1816.
Waschbisch, A., Sammet, L., Schroder, S., Lee, D.H., Barrantes-Freer, A., Stadelmann, C., et al., 2014. Analysis of CD4+ CD8+ double-positive T cells in blood, cerebrospinal fluid and multiple sclerosis lesions. Clin Exp Immunol 177 (2), 404–411.
Zhang, F., Gao, C., Ma, X.F., Peng, X.L., Zhang, R.X., Kong, D.X., et al., 2016. Expression profile of long noncoding RNAs in peripheral blood mononuclear cells from multiple sclerosis patients. CNS Neurosci. Ther. 22 (4), 298–305.
Zhang, F., Liu, G., Li, D., Wei, C., Hao, J, 2018. DDIT4 and associated lncDDIT4 modulate Th17 differentiation through the DDIT4/TSC/mTOR pathway. J. Immunol. 200 (5), 1618–1626.
Zhang, F., Liu, G., Wei, C., Gao, C., Hao, J, 2017. Linc-MAF-4 regulates Th1/Th2 differentiation and is associated with the pathogenesis of multiple sclerosis by targeting MAF. FASEB J. 31 (2), 519–525.