Upregulation of selected HERVW loci in multiple sclerosis

Sofía Macías-Redondo¹, Mark Strunk², Alberto Cebollada-Solanas³, José-Ramón Ara⁴,⁵, Jesús Martín⁴,⁵ and Jon Schoorlemmer¹,⁶,⁷*

Abstract and Introduction
Human endogenous retrovirus (HERV) are the present day versions of retroviral germline infections that have occurred millions of years ago, which occupy about 8 % of the genome [1]. While they are mostly replication deficient, they are known to express RNA and protein [2] during particular developmental stages, or as a response to aging [3], inflammation and a wide range of pathologies [4]. A human retrovirus discovered in Multiple Sclerosis (MS) patients [5], turned out to be the prototype of a novel HERV family referred to as HERVW [6]. The HERV W family consists of 213 elements, 12 out of which are complete proviral copies with intact LTRs [7]. Increased expression of HERV in peripheral blood mononuclear cells (PBMCs) has been repeatedly associated with MS, and the presence of HERVW protein or elevated RNA transcription has been correlated with disease activity [8–10]. While a contribution of HERVW-encoded proteins to brain disease is suggested by their presence in MS-associated brain lesions, expression in peripheral organs may be involved in the disease process through cytokine-induced damage to the blood brain barrier and subsequent infiltration of monocytes. Alterations in peripheral expression may also serve as a useful and practical marker for the diagnostics of this CNS disease. Therefore, we quantified overall HERVW levels and identified individual HERVW loci actually transcribed in PBMCs. Analysis was carried out in patients diagnosed with Clinically Isolated Syndrome (CIS), a precursor to MS, defined by a single episode of neurologic symptoms lasting at least 24 h. CIS is an indicator of future development of MS, as 60 % of the people diagnosed with CIS develop MS [11]. These patients potentially represent the earliest stage of MS routinely available for clinical analysis. We undertook a Next Generation Sequencing (NGS)-based analysis of transcripts amplified from cDNA obtained from patients with CIS and samples from healthy controls. Data presented from this pilot experiment indicate that the relative frequency of specific HERVW copies is altered in PBMC of CIS patients, even in the absence of overall HERVW over-expression. Such altered frequency appears to be derived from less abundantly transcribed but potentially MS-related HERVW loci.

Methods

Patients (Table 1)
The local ethics committee (CEICA) approved the study protocol (CP - CI PI14/0021 dated 26/02/2014; modified on 25/10/2017), and patients provided written informed consent (protocolo y información para el paciente v2 de 29/12/2013). Blood samples were collected from MS patients and healthy controls from the Neurology Department of Miguel Servet University Hospital (Zaragoza, Spain). Whole fresh blood was drawn into vacutainer tubes (Becton Dickinson Vacutainer) containing EDTA. Within 24 h, PBMC were isolated as previously described [12, 13].

Expression analysis and PCR
RNA isolation and random-primed cDNA synthesis [14] was carried out as described before. HERVW ENV levels were determined by triplicate qPCR assays as described [14, 15]. For the identification and localization of transcribed HERVW loci, cDNA was amplified employing the external primers of an established PCR assay for HERVW ENV [15]. Products were purified and subjected to NGS analysis.

* Correspondence: jonas@araid.es
¹Instituto Aragonés de Ciencias de la Salud (IACS), c/Juan Bosco 13, 50009 Zaragoza, Spain
²ARAID Foundation, Avda. de Ranillas 1-D, 50018 Zaragoza, Spain
Full list of author information is available at the end of the article

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NGS analysis

Library preparation and sequencing was carried out using the Ion Torrent technology workflow on an Ion Torrent S5XL platform using an Ion 530 chip. Resulting reads were mapped to the human reference genome (version hg19) using strict criteria to maximize mapping differences between different HERVW copies. Relative frequencies were calculated as the number of reads mapping to an individual HERVW ENV element relative to the total number of reads. Details in Suppl. Methods.

Statistical analysis

SPSS software was used for all analyses and graphs (Version 15.0). Normality and statistical significance of differences were assessed using specific tests. Data were further analyzed using the DESeq2 package [16] to correct p values for multiple testing (False Discovery Rate < 0.05).

More detailed information is available in Suppl. M&M.

Table 1 Clinical features of MS patients included in this study

| Case | MS subtype | Age | Sex | Status | RRMS designation |
|------|------------|-----|-----|--------|------------------|
| RMS-49 | CIS | 25 | F | NA | NO |
| RMS-58 | CIS | 44 | F | A | YES |
| RMS-70 | CIS | 47 | F | A | YES |
| RMS-73 | CIS | 39 | M | NA | YES |
| RMS-103 | CIS | 53 | F | NA | YES |
| RMS-114 | CIS | 44 | M | NA | NO |

| No MS Controls | Age | Sex |
|----------------|-----|-----|
| RMS-3 | 36 | F |
| RMS-15 | 31 | M |
| RMS-23 | 46 | F |
| RMS-30 | 46 | M |
| RMS-42 | 39 | M |
| RMS-46 | 44 | M |
| RMS-62 | 29 | F |
| RMS-72 | 46 | M |
| RMS-80 | 48 | M |
| RMS-98 | 47 | M |
| RMS-99* | 17 | M |
| RMS-102* | 56 | F |
| RMS-104 | 44 | M |
| RMS-106* | 59 | M |
| RMS-109 | 45 | M |
| RMS-112 | 47 | F |
| RMS-113 | 28 | M |
| RMS-118 | 30 | F |

Clinical data of patients whose PBMCs were analyzed for HERVW expression. Median ages for both patients and controls groups were 44 years (mean and SEM are 42.0 ± 4.25 and 40.4 ± 1.94 for patient and control groups, respectively). A/NA status refers to active and non-active patients respectively. Posterior progression towards MS diagnosis (RRMS) is indicated for all CIS cases. Samples analyzed by NGS are marked in blue. Median ages in these groups are 44 years for patients and 47 years for controls. * indicates samples only analyzed by NGS.

Results

We carried out HERVW ENV expression analyses using an optimized assay described by Mameli et al. [15]. No significantly increased expression of HERVW was detected in a small cohort of CIS patients (n = 6) compared to age-matched controls (n = 15) (U-Mann-Whitney p = 0.267) (Fig. 1). Results were not skewed by the use of GAPDH as a reference gene (Fig. 1), as comparison with RPL19 and HSDA reference genes (Table S1 and Suppl Figure 1) showed that there is no statistical difference between the use of either GAPDH or the mean of the three genes (Welch’s t-test; p < 0.05).

In the absence of increased overall expression levels of HERVW in CIS samples, we wondered whether specific copies of HERVW (Table S2) might be differentially expressed. We performed NGS analysis to identify individual HERVW copies with altered expression in PBMC from CIS patients (n = 5) and controls (n = 5). Reads obtained (70,694 ± 24,812 per sample; 25,286–136,704) were mapped to the human genome. Once assigned to unique genomic locations, reads corresponding to 39 HERVW ENV loci were extracted (Table S3 and Table 2). As expected, > 99.85% of mapped reads correspond to the 39 loci analyzed (data not shown). The resulting data showed that reads obtained from CIS patients mapped to a significant higher number of different HERVW ENV loci (31 ± 13), compared to those obtained from controls (16 ± 5.5) (t-student; p = 0.018) (Fig. 2 A). Over 70% of the reads mapped to either of two loci: 19q13.2, Xq22.3. Extending the range, reads mapped with high frequency (> 3.6% of total reads/locus) to a limited number of loci, in particular to HERVW ENV copies located on chromosomes 19q13.2, Xq22.3, 8q21.11, 15q21.3, 12q23.3 and 4q21.22 (Fig. 2 B). We found no significant differences between CIS patients and controls in the relative frequency of reads mapping to these loci (Fig. 2 B; Table 2).

Lower numbers of reads mapped to the remaining 33 loci, with relative frequencies ranging from 0.01 to 1.66%. We found differences between CIS patients and controls in the relative frequency of reads mapping to several of these. Reads mapping to a subset of HERVW loci, including copy_chr3-1, copy_chr4-3, copy_chr5-1, copy_chr9-1, copy_chr9-2, copy_chr12-4 and copy_chr19-1 differed between CIS patients and controls (Table 3; Fig. 2 C-E). The relative frequency (ranging from 0.3 – 2.95%) of reads mapping to these loci showed a > 7 fold increase in CIS patients compared to controls (Table 3). When corrected for multi-locus analysis, significant differential expression (p < 0.05; FDR < 0.05) of five of these loci loci was evident (Table 3), with significant increases in expression of HERVW copies 3q11.21, 4q31.1, 9q31.3 and 19p12 and a significant decrease in 12q23.3.
Table 2 Percentages of reads mapped to individual HERVW loci

| Locus | CIS 58 | CIS 70 | CIS 49 | CIS 73 | CIS 114 | Control 102 | Control 104 | Control 106 | Control 99 | Control 98 |
|-------|--------|--------|--------|--------|---------|-------------|-------------|-------------|-------------|-------------|
|       | % of reads |
| chr1-1 | 0.01 | 0.03 | 0.06 |
| chr2-2 | 0.07 | 0.05 | 0.02 | 0.02 | 0.05 | 0.06 | 0.23 |
| chr2-3 | 0.03 | 0.02 | 0.01 |
| chr2-4 | 0.00 | 0.00 | 0.00 |
| chr2-5 | 0.04 | 0.01 | 0.02 |
| chr3-1 | 0.27 | 0.49 | 1.26 | 0.16 | 0.01 | 0.03 | 0.04 | 0.08 |
| chr3-2 | 0.01 | 0.00 | 0.05 | 0.02 |
| chr3-3 | 0.01 | 0.00 | 0.04 | 0.02 |
| chr3-4 | 0.68 | 0.60 | 2.31 | 0.34 | 0.15 | 0.34 | 0.32 | 0.75 | 0.19 | 0.05 |
| chr3-5 | 0.01 | 0.01 | 0.66 | 0.24 | 0.04 | 0.26 | 0.14 | 0.19 | 0.08 | 0.15 |
| chr4-2 | 0.00 | 0.00 | 0.01 | 0.00 | 0.04 |
| chr4-3 | 0.00 | 0.00 | 0.03 | 0.02 | 0.03 | 0.01 | 0.01 | 0.01 | 0.02 |
| chr5-1 | 0.35 | 0.23 | 0.02 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| chr5-2 | 0.47 | 0.50 | 0.19 | 11.25 | 9.15 | 0.32 | 0.25 | 0.26 | 0.50 | 41.29 |
| chr6-2 | 0.76 | 1.55 | 2.33 | 0.20 | 0.02 | 0.07 | 0.20 | 0.33 | 0.14 | 0.06 |
| chr6-3 | 0.03 | 0.03 | 0.03 |
| chr8-1 | 0.01 | 0.00 | 0.02 |
| chr9-1 | 0.00 | 0.00 | 0.00 | 0.03 |
| chr10-1 | 0.00 | 0.00 | 0.00 |
| chr10-2 | 0.00 | 0.00 | 0.00 | 0.00 |
| chr11-1 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| chr11-2 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| chr11-3 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| chr11-4 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| chr12-1 | 0.01 | 0.01 | 0.02 | 0.02 | 0.03 | 0.06 |
| chr12-2 | 0.01 | 0.00 | 0.04 |
| chr12-3 | 0.16 | 1.30 | 1.50 | 0.40 | 0.30 | 0.09 | 0.73 | 0.83 | 0.73 | 0.09 |
| chr12-4 | 0.43 | 0.32 | 0.59 | 0.09 | 0.39 | 0.72 | 0.39 | 0.72 | 0.72 | 0.72 |
| chr14-1 | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 |
| chr15-1 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| chr15-2 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| chr16-1 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 |
| chr16-2 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 |
| chr17-1 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| chr17-2 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| chr17-3 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 |
| chr18-1 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| chr18-2 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| chr19-1 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 |
| chr19-2 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 |
| chr20-1 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| chr20-2 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| chr21-1 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| chr21-2 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| chr22-1 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| chr22-2 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |

Table 2 Percentages of reads mapped to individual HERVW loci.

Mapped reads (Table S3), were recalculated as the number of reads mapping to an individual HERVW ENV element relative to the total number of reads, and represented as a percentage. The HERVW loci to which an increased number of reads mapped in CIS patients are indicated in blue.
Discussion
In contrast to the small group of CIS patients analyzed in this study, increased HERVW levels have been associated frequently with MS. Our inability to demonstrate a statistically significant increase of overall HERVW levels in PBMC of CIS patients may be explained by the selection of this particular group or more likely simply by small sample size. However, lack of increased expression is not unprecedented as it was previously reported in a cohort of South African MS patients, although different primers were used for this analysis [17].

We performed NGS analysis to identify individual HERVW copies that show altered expression in PBMC, comparing CIS patients \((n=5)\) to controls \((n=5)\). Although more definite answers require future analysis of more subjects, in the CIS patients analyzed more HERVW loci are expressed than in control subjects. A similar increase has been reported previously

Table 3 HERVW copies differentially expressed in CIS patients

| HERVW Copy | Repeatmasker name | Reads in CIS | Reads in Controls | Fold increase | \(P\) value |
|------------|-------------------|--------------|------------------|---------------|------------|
| chr 3-1    | 3q11.2            | 2221         | 124              | 14.76         | 0.0040     |
| chr 4-3    | 4q31.1            | 92           | 6                | 12.64         | 0.0333     |
| chr 5-1    | 5p12              | 3999         | 562              | 5.86          | 0.27       |
| chr 9-1    | 9q22.31           | 5678         | 614              | 9.2           | 0.12       |
| chr 9-2    | 9q31.3            | 81           | 1                | > 20          | 0.0333     |
| chr12-4    | 12q23.3           | 18962        | 20812            | -1.33         | 0.0015     |
| chr19-1    | 19p12             | 192          | 1                | > 20          | 0.0015     |

The table lists several HERVW copies, using references from Tables S2-S3. Names used in RepeatMasker are also indicated. The sum of mapped reads in samples from five CIS patients or five controls is listed, as well as the resulting fold change in CIS versus control. The \(P\) value for the difference between NDI and CIS samples was adjusted for multiple testing (FDR <0.05)
in MS brain [18]. While previous studies failed to identify MS-specific loci or expression [18, 19], in the CIS patients we found statistically significant overrepresentation of reads corresponding to specific loci (i.e. 3q11.2 and 19p12, see Table 3 for complete list). Locus-specific qPCR assays may first help confirm this finding in a larger patient cohort, and subsequently be evaluated as a potential prognostic assay.

These combined overrepresented loci produce only 1–3% of total transcripts (Fig. 2 C–E). The combined findings on low levels of overexpression, activation of more loci, and activation of low-expressing HERV W elements in CIS patients suggest that their potential contribution to the pathology may be unrelated to overall high expression levels. None of the copies identified encode full-length ENV protein, as the sequences corresponding to the ENV gene are truncated, lack ATG codons, and/or carry frame shifts and STOP codons (Suppl Figure 2). CIS-associated copies may produce proteins (either or not ENV-related) that are especially active in activation of TLR4 [20], or RNAs that trigger the native immune system through TLR3 [21, 22]. Although our analysis shows that upregulation of specific HERVW loci in PBMC is associated with CIS, the presence of these transcripts in MS brain is unknown at present. A potential role of these transcripts in proviral protein production and activation of either the peripheral immune system or CNS disease remains to be established.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s13100-021-00243-1.

Additional file 1: Table S1. The levels of GAPDH, RPL19 and HSDA were determined by qPCR in the samples indicated, as described in the legend to Suppl Fig. 1. The Table lists the Cts obtained for each gene (columns named accordingly), the mean of all three (column “mean”) and the difference between the Cts obtained using either GAPDH (G) or the mean of three reference genes (P) (Column P-G). The difference (mean 2.26; standard deviation 0.29) is statistically constant among samples (Welch’s t-test; p < 0.05).

Additional file 2: Table S2. List of HERV ENV loci according to the GRCh37.p5 version of the human genome database. Loci identified by unbiased read mapping were verified as HERV loci by comparison with the Repbase Update library of repeats from the Genetic Information Research Institute (GIRI) [23], using the the RepeatMasker program. Nomenclature used in Tables is indicated, the genomic location of each copy as well as alternative names used in the literature. Sequences corresponding to the coordinates listed were downloaded and compared to primer sequences using the “align” function in SerialCloner (version 2.6.1). Identity with primer sequence is indicated in black, mismatches in red.

Additional file 3: Table S3. Number of reads mapping to the HERW copies indicated in each of the five CIS or NDI (control) samples. The total number of reads per sample is indicated. The sum of reads in the CIS and NDI groups is indicated.

Additional file 4: Table S4. Primers used.

Additional file 5: Figure S1. Comparison of reference genes.
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