Short Communication

Endogenous human retrovirus-K is not increased in the affected tissues of Japanese ALS patients

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ABSTRACT

Activation of human endogenous retrovirus-K (HERV-K) is one of the proposed risk factors for amyotrophic lateral sclerosis (ALS). The HERV-K envelope protein has been reported to show neurotoxicity, and development of therapy with reverse transcriptase inhibitors is being investigated. On the other hand, some reports have failed to show HERV-K activation in ALS. In this study, we analyzed the expression of HERV-K mRNA in the motor cortex and spinal cord of 15 Japanese patients with sporadic ALS and 19 controls using reverse transcriptase droplet digital PCR. This revealed no significant increase of HERV-K expression in ALS-affected tissues, suggesting that the association between ALS and HERV-K remains questionable.

Amotrophic lateral sclerosis (ALS) is a fatal motor neuron disease. Approximately 10 % of ALS cases are familial whereas 90 % are sporadic (Cirulli et al., 2015). More than 30 causal ALS genes have been identified; comprehensive analysis can identify causal gene mutations in the majority of familial cases but in only 10–20 % of sporadic cases (Cirulli et al., 2015; Nakamura et al., 2016; Dols-Icardo et al., 2018; Naruse et al., 2019). Identification of new genetic factors is helping to clarify the pathological mechanism involved and to develop new therapeutic approaches.

Activation of the human endogenous retrovirus-K (HERV-K) is one of the proposed risk factors for ALS, but its role remains somewhat controversial (Li et al., 2015; Kury et al., 2018; Garson et al., 2019). HERV-K is a retrotransposon integrated at multiple sites in the human genome. Some HERV-K integration sites retain retrovirus-like activity and have open reading frames for three proteins – gag (capsid), pol (reverse transcriptase), and env (envelope proteins) – without canonical disruptions (Kury et al., 2018). An association between ALS and retrovirus-like activity has been indicated by the presence of reverse transcriptase activity in some patients with non-HIV ALS (Steele et al., 2005; McCormick et al., 2008). The most significant study of the relationship between HERV-K and ALS was conducted by Li et al. in an ALS cohort in the USA (Li et al., 2015). They found high expression of HERV-K mRNA in post-mortem brain tissues of 11 ALS patients and 16 healthy controls using quantitative real-time reverse transcription PCR (RT-qPCR), and also demonstrated neuronal toxicity of the HERV-K env protein both in vitro and in a murine model.

However, subsequent studies were unable to confirm any significant increase of HERV-K expression in ALS-affected tissues. Mayer et al. examined the levels of HERV-K mRNA transcript in the spinal cord (15 ALS, 15 control), motor cortex (23 ALS, 12 control) and occipital lobe (14 ALS, 5 control) using RT-qPCR, but no significant inter-group differences were evident (Mayer et al., 2018). Garson et al. also used RT-qPCR to examine the premotor cortex in 34 ALS cases and 23 controls, but found no inter-group differences in the expression levels of HERV-K gag, pol or env mRNA (Garson et al., 2019). Thus, conflicting results have been reported from the USA (Li et al., 2015; Mayer et al., 2018) and the UK (Garson et al., 2019). To clarify whether HERV-K is indeed a fundamental risk factor for ALS, it is necessary to investigate HERV-K expression in ALS cases in other ethnic groups. Here, we examined the levels of HERV-K mRNA expression in brain tissues from...
Japanese ALS patients.

We analyzed clinical data and post-mortem tissues of 15 Japanese patients with sporadic ALS (mean age, 69.7 years; SD, 8.5 years) and 19 Japanese controls (mean age, 67.4 years; SD, 11.2 years) (Supplementary Table 1). Frozen samples of the motor cortex and spinal cord were obtained at our institution. Each of the ALS cases was diagnosed on the basis of clinical and pathological features, and all demonstrated TDP-43-positive inclusion bodies in the remaining motor neurons.

We investigated the ALS causative genes in all cases, and excluded cases with pathological mutations, or nonsynonymous or truncating variants with a minor allele frequency of <0.001. Samples of genomic DNA were collected from autopsied central nervous system (occipital lobe, motor cortex, and cerebellum) tissue using a DNA extraction kit (QIAamp DNA Mini Kit, Cat. No.51304, Qiagen, Hilden, Germany), and DNA quality checks were performed using an Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, USA). Variants of the known ALS causative genes (TARDBP, OPTN, FUS, SOD1, TBK1, SQSTM1, MATR3, TUBA4A, NEK1, HNRNPA2B1, VCP, ELF3, SETX, HNRNPA1, CCNF, VAPB, C21orf2, CHCHD10, NEFH, ANG, DCTN1, CHMP2B, UBQLN2, FIG4, PFN1, ARHGEF28, EWSR1, TAF15, ANXA11, DAO, ERBB4, TIA1, GLE1, PRPH, ALS2, SPG11, SIGMAR1, KIF5A, and DNAJC7) were analyzed by exome sequencing. Outsourced exome analysis (Takara Bio. Shiga, Japan) was performed using an Illumina NovaSeq 6000. C9orf72 intron1 GGGGCC repeats were measured separately using a previously reported method (Konno et al., 2013).

We prepared total RNA from 50–100-mg samples of frozen tissue from ALS patients and controls (Supplementary Table1). Total RNA was extracted using a mirVana™ miRNA isolation kit (Thermo Fisher Scientific, Massachusetts, USA) and contaminating DNA was removed using rDNase set (Cat. No.740963, Macherey Nagel, Düren, Germany) and NucleoSpin® RNA clean-up XS (Cat. No.740903.250, Takara Bio, Shiga, Japan). The cDNA was generated using the SuperScript™ VILO™ Master Mix kit (Cat. No. 11755250, Thermo Fisher Scientific, Massachusetts, USA). We determined the RNA integrity number equivalent (RINe) using the Agilent 2200 TapeStation system (Agilent Technologies, California, USA) and the mean RINe was 6.9 (all samples had an RINe >5) (Supplementary Table1). We prepared 29 samples from motor cortex tissue (13 ALS; 16 controls) and 15 samples from spinal cord

Fig. 1. HERV-K (env, pol, and gag) mRNA expression in the motor cortex (A) and spinal cord (B). The average HERV-K mRNA expression in the controls is expressed as 1. Statistical analysis was performed using the Mann-Whitney U test (A, B). All p values are >0.05, NS. (C) Correlation between HERV-K (env, pol, and gag) mRNA expression in the motor cortices of the patients and controls. HERV-K mRNA expression was adjusted according to the expression of RPLP1 mRNA (A-C). Abbreviations: ALS, amyotrophic lateral sclerosis. Blue circles indicate the controls and red circles indicate the ALS cases.
neural tissue (6 ALS; 9 controls).

We carried out reverse transcription droplet digital PCR (RT-ddPCR) using the QX200 system (Bio-Rad, California, USA). For quantification of mRNA expression of three HERV-K genes (env, pol, gag), we used Evagreen Supermix (Cat. No.186-4035, Bio-Rad Laboratories Inc. California, USA) with 50 nM PCR primers for each system. The HERV-K primer sequences were the same as previous report (Li et al., 2015). For quantification of mRNA expression of TARDBP and RPLP1, we used ddPCR™ Supermix for Probes (No dUTP) (Cat. No.186-3025, Bio-Rad Laboratories Inc. California, USA). The RPLP1 and TARDBP PCR primer and probe sequences were the same as previous report (Koike et al., 2021). RPLP1 mRNA expression was selected as the endogenous control (Ishihara et al., 2013). The ddPCR cycling parameters employed were: enzyme activation for 10 min at 95 °C, 40 cycles of 30 s at 94 °C (denaturation) and 2 min at 55 °C (annealing and extension), and enzyme deactivation for 10 min at 98 °C. The study was performed in accordance with the digital MIQE guidelines (Supplementary Table 2) (The dMIQE Group and Huggett, 2020).

The statistical significance of differences between groups was assessed by Mann-Whitney U test Pearson correlation coefficient was calculated for the correlation of each factor. Differences at p < 0.05 were considered significant.

We measured the levels of expression of mRNAs for HERV-K env, pol, gag, and TARDBP using RT-ddPCR. All of the ddPCR data were adjusted to the endogenous control, i.e. the level of RPLP1 mRNA. We assayed 29 samples from motor cortex tissue (13 ALS; 16 controls) (Fig. 1A) and 15 samples from spinal cord tissue (6 ALS; 9 controls) (Fig. 1B). In both the ALS patients and controls, there was substantial expression of HERV-K mRNA in central nervous system tissues. However, there was no significant increase of HERV-K mRNA expression in ALS-affected motor cortex (Fig. 1A) and spinal cord (Fig. 1B) tissues. There was a good correlation between the mRNA expressions of env, pol, and gag (Fig. 1C). The expression of pol mRNA was about twice that of gag and env, being consistent with a previous report (Li et al., 2015).

Previous reports have indicated that the mRNA and protein expression levels of TARDBP, which is the key ALS molecule, affected HERV-K expression (Douville et al., 2011; Li et al., 2015). In our present study, there was no correlation between HERV-K and TARDBP mRNA expression in motor cortex tissue (13 ALS; 16 controls) (Fig. 2A). We examined the relationship between HERV-K expression and age at onset, age at death, and disease duration. There was no correlation between age at onset and age at death (Fig. 2B–C); however, there was a mild positive correlation between disease duration and HERV-K expression, but no significant difference was observed (p = 0.06) (Fig. 2D).

Herein, we demonstrated that there was expression of HERV-K mRNA in central nervous system tissues (Figs. 1C, 2A). But there was no significant difference between ALS and control cases. The human genome sequence contains thousands of HERV proviruses, accounting for up to 8% of the human genome (Kury et al., 2018). Most HERVs contain nonsense mutations or severe deletions and are inactive for the most part (Kury et al., 2018). However, some HERV-K families have been active since the divergence of humans and chimpanzees (Belshaw et al., 2005; Kury et al., 2018). Here we found HERV-K mRNA expression in the human central nervous system. The mean level of mRNA expression for each HERV-K was 5–16% that of the mRNA expression for RPLP1, which was used as the endogenous control (Fig. 1C).

We did not find any increase of HERV-K mRNA expression in ALS-affected tissues (Fig. 1A–B), consistent with the reports of Mayer et al. and Garson et al. (Mayer et al., 2018; Garson et al., 2019). Furthermore, there was no correlation between the levels of HERV-K and TARDBP mRNA expression and age at onset or age at death (Fig. 2A–C). There was a mild positive correlation, although not significant (p = 0.06), between ALS disease duration and the level of HERV-K expression, indicating that the latter may not exacerbate the progression of ALS disease (Fig. 2D).

Several points need to be considered in the context of our experimental results. First, we need to discuss the effectiveness of the method used for quantification. In this study, we adopted the ddPCR method, which provides more accurate quantification than ordinary quantitative PCR (Huggett et al., 2015). The primer sequence is also important for selective amplification of the target. The HERV-K primers used in this study were those used in previous studies (Li et al., 2015). Furthermore,

![Fig. 2. Correlation between HERV-K and TARDBP mRNA expression in the motor cortices of the ALS patients and controls (A). Correlation between HERV-K env mRNA expression in the motor cortex and age at onset (B), age at death (C), and ALS disease duration (D). HERV-K and TARDBP mRNA expression were adjusted according to RPLP1 mRNA expression (A–D). Abbreviations: ALS, amyotrophic lateral sclerosis. Blue circles indicate the controls and red circles indicate the ALS cases.](image-url)
quantitative PCR methods including ddPCR require an appropriate endogenous control to correct the expression level of each sample. We have confirmed the expression levels of multiple endogenous control candidates in each target tissue and selected RPLP1 mRNA using a dedicated program (Ishihara et al., 2013). There was a good correlation among the HERV-K mRNA expression levels of env, pol, and gag (Fig. 1C), and the fact that pol expression was almost twice as high as that of gag or env was consistent with a previous report (Fig. 1C) (Li et al., 2015). These findings confirm the quantitative validity of our method.

Next, it is necessary to consider the eligibility of the target cases. In ALS cases, the accuracy of diagnosis becomes an issue. In this study, all ALS cases were pathologically confirmed. Another issue is the presence of cancer cases in the control group. Since increased expression of HERV-K has been reported in cancer tissues (Subramanian et al., 2011), if a control group contains many cancer cases, HERV-K expression in the ALS group may be relatively underestimated. In the present study, the number of cancer cases in the motor cortex control group was 2/16 (12.5 %) (lung cancer and T cell leukemia) and that in the spinal cord group was 1/9 (11.1 %) (T cell leukemia), being lower than in the previously reported studies (25 % (Garson et al., 2015) and 47 % (Li et al., 2015)). Therefore, the inclusion of cancer patients had only a small influence in the present study.

Sample size may also have affected the statistical power. In our motor cortex group, 13 cases of ALS and 16 controls were compared, which was the same size as the group previously reported by Li et al. (11 cases of ALS, 16 controls) and for which a clear significant difference (P < 0.001) was evident (Li et al., 2015). Therefore, it is unlikely that the sample size used in our study was too small for this analysis.

We were unable to confirm any increase in the expression of HERV-K in Japanese ALS cases. However, reports from a number of countries, including the USA (Mayer et al., 2018), UK (Garson et al., 2019) and Japan (this report), suggest that the role of HERV-K in ALS might be small if it exists at all. Li et al. have demonstrated the neurotoxicity of the HERV-K envelope protein in an animal model (Li et al., 2015), which may be important when considering potential treatment strategies for ALS, as such reverse transcriptases inhibit. However, our results suggest that HERV-K overexpression is unlikely to be related to ALS, and therefore more careful investigations will be necessary before suppression of HERV-K activation can be considered as a therapy for ALS.

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Author contributions
TI, AK, AK and OO designed the research project. TI, AK, TK, YK, YH and OO designed and performed the molecular experiments. RT, MT and AK performed the pathological analysis. TI, AK, YH managed the statistical analyses. TI, AK, AK and OO discussed the results and drafted the manuscript for intellectual content.

Ethical approval
The present study was approved by the Ethics Committee of Niigata University (G2015-0781, G2020-0031). Written informed consent for autopsy including the use of tissues for research purposes was obtained from the patients’ families.

Consent for publication
All family members have consented to publication.

Data availability
Data will be made available on request.
The data that has been used is confidential.

Declaration of Competing Interest
The authors report no declarations of interest.

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Not applicable.

Appendix A. Supplementary data
Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neures.2022.01.009.

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