J. Neurovirol. (2017) 23:734–741
DOI 10.1007/s13365-017-0560-x

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JCPyV microRNA in plasma inversely correlates with JCPyV seropositivity among long-term natalizumab-treated relapsing-remitting multiple sclerosis patients

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Received: 5 April 2017 / Revised: 21 June 2017 / Accepted: 24 July 2017 / Published online: 22 August 2017
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Abstract Sensitive biomarkers are needed to better manage multiple sclerosis (MS) patients for natalizumab (NTZ)-associated risk of progressive multifocal leukoencephalopathy (PML). A currently used risk stratification algorithm, mainly based on JC polyomavirus (JCPyV) serology, has not led to a reduction of PML incidence. Therefore, this study was designed to evaluate the presence and prevalence of JCPyV miRNAs in plasma of NTZ-treated MS patients, and to explore their biomarker potential for NTZ-associated PML risk assessment. Altogether, 102 plasma samples from 49 NTZ-treated and 28 interferon-beta (IFN-β)-treated relapsing-remitting MS patients, and 25 healthy controls (HCs) were analyzed for jcv-miR-J1-5p (5p miRNA) and jcv-miR-J1-3p (3p miRNA) expression. The overall detection rate of 5p miRNA was 84% (41/49) among NTZ-treated patients, 75% (21/28) among IFN-β-treated patients, and 92% (23/25) in HCs. Relative 5p miRNA expression levels were lower in NTZ-treated patients as compared to patients treated with IFN-β (p = 0.027) but not to HCs. Moreover, 5p miRNA expression inversely correlated with anti-JCPyV antibody index among JCPyV seropositive long-term NTZ-treated patients (r = −0.756; p = 0.002). The overall detection rate of 3p miRNA was low. Our results suggest that JCPyV miRNA in plasma may be linked to the reactivation of persistent JCPyV, to enhanced virus replication, and eventually to the risk of developing PML among NTZ-treated MS patients. However, further study is warranted in a larger data set including samples from PML patients to confirm the clinical relevance of JCPyV miRNA as a sign of/in viral reactivation, and to identify its potential to predict developing PML risk.

Keywords Anti-JCPyV antibody index · Biomarker · IFN-β · JCPyV miRNA · Multiple sclerosis · Natalizumab · PML

Electronic supplementary material The online version of this article (doi:10.1007/s13365-017-0560-x) contains supplementary material, which is available to authorized users.

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Introduction

Natalizumab (NTZ, Tysabri®) is one of the most effective therapies for active relapsing-remitting multiple sclerosis (RRMS) (Polman et al. 2006). Regardless of its efficacy, long-term use (mostly more than 18 months) is associated with increased risk of progressive multifocal leukoencephalopathy (PML), caused by reactivation of JC polyomavirus (JCPyV) (Clifford et al. 2010). Primary JCPyV infection is encountered in childhood in 60–80% of the human population, and asymptomatic lifelong persistent infection is established (Ferenczy et al. 2012). PML is a rare consequence of reactivation of latent virus, followed by lytic infection of oligodendrocytes and astrocytes under immunosuppressive conditions (Khalili et al. 2007). Although the exact mechanisms of JCPyV reactivation leading to PML lack experimental evidence, two main conceptions prevail (Wollebo et al. 2015). Rearrangements may emerge in the genome of persistent archetype virus either in B cells where immunoglobulin gene rearrangement machinery can be exploited, or by homologous recombination events taking place during virus replication. According to the second conception, the proportions of existing

viral quasispecies in the JCPyV population of the individual may change as a response to alterations in immunological and other circumstances. In order to cause PML, the virus has to enter the brain and replicate under immunosuppressive conditions (Wollebo et al. 2015). Currently, the risk of PML among NTZ-treated MS patients is stratified using three parameters: anti-JCPyV-antibody (Ab) status, prior use of immunosuppressants, and duration of NTZ treatment (Bloomgren et al. 2012).

Presently, the measurement of serum or plasma anti-JCPyV Ab is widely used to establish past exposure to JCPyV infection, and Ab level expressed as JCPyV Ab index helps to stratify patients at PML risk (Plavina et al. 2014). The rationale behind JCPyV Ab determination is to identify individuals who have Ab as a sign of past exposure and who thus have persistent JCPyV in their organism, whereas JCPyV Ab-negative individuals are thought not to have encountered the virus. However, there has been no reduction in the NTZ-associated PML incidence concomitant with the utilization of JCPyV serology (Cutter and Stuve 2014), and thus it seems insufficient to evaluate complete risk. Moreover, as 60–70% of the MS patients are JCPyV Ab positive, further tools are needed to narrow down high PML risk among patients receiving NTZ (Kolasa et al. 2016; Olsson et al. 2013). Previously evaluated tools based on leukocyte cell membrane markers such as CD11a, CD49d, and CD62L (Basnyat et al. 2015; Jilek et al. 2013; Schwab et al. 2013), and quantification of JCPyV load in the blood or urine still lack applicability in a clinical setting (Chen et al. 2009; Jilek et al. 2010; Rudick et al. 2010). Specifically, quantification of JCPyV DNA from urine and plasma is not sufficiently sensitive to rule out PML risk (Rudick et al. 2010).

JCPyV belongs to human polyomaviruses, which are nonenveloped, double-stranded DNA viruses with genomes of approximately 5130 nucleotides. JCPyV encodes a pre-microRNA which is processed into two mature microRNAs (miRNAs), jcv-miR-J1-5p (5p miRNA) and jcv-miR-J1-3p (3p miRNA), that appear late in infection to autoregulate early gene expression (Seo et al. 2008). jcv-miR-J1-5p is unique for JCPyV, but jcv-miR-J1-3p shares identical sequence with BK polyomavirus (BKPyV)-encoded bkv-miR-B1-3p, and thus its origin cannot be differentiated (Seo et al. 2008). Polyomavirus encoded miRNAs are known to play a key role in controlling viral replication through downregulation of large T (tumor) antigen (LTAg) expression (Seo et al. 2008). They also modify host immune responses by controlling killer receptor NKG2D-mediated killing of virus-infected cells by natural killer (NK) cells through downregulation of the stress-induced ligand ULBP3 (Bauman et al. 2011).

The biomarker potential of circulating human miRNAs in blood has been studied for NTZ-associated PML (Munoz-Culla et al. 2014), but studies on JCPyV-specific miRNAs for their potential as biomarkers of PML risk in NTZ-treated patients are still lacking. Recent studies signify the potential of jcv-miR-J1-5p as a biomarker of past JCPyV infection as it can be frequently detected in plasma, urine, and CSF of both JCPyV seropositive and seronegative healthy individuals and immunosuppressed patients (Lagatie et al. 2014; Pietila et al. 2015). Therefore, jcv-miR-J1-5p could serve as a more sensitive biomarker of JCPyV infection than serology. JCPyV miRNA has been recently proposed as a potential biomarker for JCPyV infection in the gastrointestinal tract, and expression of these microRNAs has been further shown in brain tissues from PML patients (Link et al. 2014; Seo et al. 2008). In the present study, we characterized JCPyV miRNA expression in the plasma of NTZ-treated RRMS patients as well as in patients treated with interferon-beta (IFN-β), and in healthy controls (HCs) to evaluate the utility of these miRNAs in PML risk assessment.

Patients and methods

Patients

This cross-sectional study included a total of 102 subjects of whom 49 RRMS patients had been treated with NTZ, 28 patients with IFN-β (21 patients with IFN-β -1a sc 22 μg and 7 patients with IFN-β -1a sc 44 μg), and 25 subjects were HCs. MS patients were enrolled consecutively from four Finnish MS outpatient departments. Approximately 75% (58/77) of our MS patients were female, representative of the global gender distribution among MS patients (Kira 2014). The HCs were selected to have similar distribution. The clinical characteristics of the patients are shown in Table 1. All patients underwent clinical and neurological examinations before blood sampling. The diagnosis of MS was based on the revised McDonald Criteria (Polman et al. 2005), and the diagnosis was definite. Neurological disability was evaluated by the expanded disability status scale (EDSS) score (Kurtzke 1983). No cases of PML developed among the patients enrolled in this study. The study was approved by the Ethics Committee of Tampere University Hospital, and all subjects gave informed consent.

Determination of the anti-JCPyV antibody index

A confirmatory second generation ELISA (STRATIFY JCPyV™ DxSelect) was used to test sera of the MS patients for anti-JCPyV Ab at Unilabs, Copenhagen, Denmark. A screen index value of less than 0.2 was considered anti-JCPyV Ab negative, and of greater than 0.4 as anti-JCPyV Ab positive. The samples with a screen index between 0.2 and 0.4 were evaluated with a supplementary confirmatory inhibition test, and samples showing greater than 45%

Table 1 Clinical characteristics of MS patients and healthy controls

Characteristics	NTZ-treated $n = 49$	IFN-β-treated $n = 28$	HCs $n = 25$	p value
Sex (F/M)[a]	38/11	20/8	18/7	
Age (years)[b]	38.3 ± 7.6 (23–52)	35.6 ± 10 (20–53)	33.3 ± 11.3 (22–60)	NS
Disease duration (years)[b]	9.2 ± 5.3 (1.8–22.4)	4.3 ± 5.1 (0.2–18.1)	–	<0.001
EDSS[b]	2.6 ± 1.8 (0–6.5)	1.3 ± 1.3 (0–5.5)	–	0.001
Number of relapses[b,c]	2.0 ± 1.0 (0–4)	–	–	–
Duration of treatment (years)[b]	2.8 ± 1.4 (0.4–5.8)	2.5 ± 3.0 (0.1–13.1)	–	NS
Anti-JCPyV Ab seropositivity[a]	24 (49%)	12 (43%)	–	NS
Anti-JCPyV Ab index[d,e]	1.0 (0.3–3.1)	1.9 (0.7–2.9)	–	NS

IFN-β interferon-β, *HC* healthy controls, *EDSS* Expanded Disability Status Scale, *JCPyV* JC polyomavirus, *NS* not significant, − not applicable

[a] Number of patients

[b] Mean ± SD (range)

[c] Two years before starting natalizumab

[d] Median (range)

[e] Anti-JCPyV antibody index of seropositive patients

inhibition in blocking with specific antigen were classified as anti-JCPyV Ab positive (Lee et al. 2013). The anti-JCPyV-Ab index was only determined for the MS patients, not for HCs.

RNA extractions

Total RNA was extracted from 620 μl of plasma using the mirVana™ PARIS™ RNA and native Protein Purification Kit (Ambion, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Total RNA was eluted into 95 μl of elution solution and spiked with 5 μl of cel-39-3p miRNA (5 fmol/μl, Integrated DNA Technologies, Coralville, IA, USA). This synthetic *Caenorhabditis elegans* miRNA was used to control the success of reverse transcription and miRNA amplification, as well as for result normalization (see below). All RNA preparations were stored at −70 °C until analyzed.

TaqMan miRNA assays

For reverse transcription (RT) and miRNA detection, TaqMan miRNA assays (Thermo Fisher Scientific) were used. The specific targets were jcv-miR-J1-5p, bkv-miR-B1-3p/jcv-miR-J1-3p (identical sequences), and cel-miR-39-3p. Each 15 μl RT reaction mixture contained 1× RT buffer, 0.25 mM of each dNTP, 1× RT primer, 3.33 U/μl MultiScribe RT enzyme, 0.25 U/μl RNase inhibitor, and 10 ng of total RNA. RT reactions were incubated 30 min at 16 °C, 30 min at 42 °C, and 5 min at 85 °C. If real-time PCR was performed directly after RT, the tubes were cooled to 4 °C, but for longer storage, the reactions were placed in −20 °C as instructed by the manufacturer.

For PCR amplification, the Applied Biosystems® 7500 Real-Time PCR System (Thermo Fisher Scientific) was used. Each 10 μl RT reaction contained 1.3 μl of diluted (1:2) RT reaction, 1× TaqMan® assay mixture, and 1× TaqMan® Universal Master Mix II, no UNG (Thermo Fisher Scientific). All miRNA assays were performed in three replicate reactions in the following conditions: enzyme activation in 95 °C for 10 min, after which 40 cycles of 15 s denaturation in 95 °C and 1 min annealing and extension in 60 °C was performed. In each 96-microwell plate, three replicate no template controls (NTC) were run for each master mix. The functionality of the miRNA assays was confirmed using synthetic oligonucleotides (Integrated DNA Technologies) representing the target sequence of each specific miRNA assay as templates.

Baseline and threshold values were set manually for each PCR run according to instrument instructions. The same baseline values were applied in all runs, and threshold was set to the exponential phase of the amplification curves. The sample was interpreted as positive if a Ct cycle was obtained for two or three of the replicates, and then a mean Ct was calculated (Supplementary Table 1). Mean Ct was calculated independent of the amplitude of variation between replicates. If only one of the replicates gave a signal, the sample was not interpreted as true positive. Even though there is no consensus available for normalization of polyomavirus miRNA expression levels in plasma, we used Ct values of spiked synthetic *Caenorhabditis elegans* (cel-miR-39-3p) miRNA to normalize the JCPyV miRNA expression in each patient and HC (ΔCt). We calculated the relative miRNA expression of sick versus healthy individuals by using standard delta delta Ct ($2^{-\Delta\Delta Ct}$) method (Auvinen 2017). cel-miR-39-3p does not share homology with any known human miRNAs and it is widely used as spike-in control (Kroh et al. 2010).

Statistical analysis

Statistical analyses were performed using SPSS version 22.0 for Windows (SPSS Inc., Chicago, IL, USA). Differences in the detection rate of JCPyV miRNA between different groups were assessed using Fisher's exact test. A non-parametric, two-tailed Mann–Whitney U test was used to compare the differences between the clinical parameters and JCPyV miRNA expression levels in the different patient groups. Spearman's correlation coefficient was used to explore the association between the relative miRNA expression and clinical parameters. A p value less than 0.05 was considered statistically significant.

Results

Clinical data

NTZ-treated patients had a longer disease duration and higher EDSS scores than IFN-β-treated patients, but the age of the patients did not differ between the groups (Table 1). In the NTZ-treated group, 46 out of 49 patients had been previously treated with other immunomodulatory or immunosuppressive (IS) drugs: 15 patients with IFN-β only, 29 patients with IFN-β, and other immunomodulatory drugs and two with only IS drug. The remaining three patients did not have any previous treatment. In the IFN-β-treated group, no patients had any previous medication with any other immunomodulatory or IS drugs. Two years before initiating NTZ therapy, almost all (96%) patients had had relapses (mean 2.0 ± SD 1.0 (range 0–4, 17 patients had one relapse, 18 patients had two relapses, six patients had three relapses, and six patients had four relapses)). During therapy, relapses were observed in seven (14%) patients (four patients had one relapse each and three patients had two relapses each) among whom the NTZ treatment duration ranged from 1.7 to 5.8 years. The seroprevalence of anti-JCPyV Ab was 49% among NTZ-treated and 43% among IFN-β-treated patients.

Overall detection rate of JCPyV miRNA

The presence of JCPyV-encoded 5p and 3p miRNAs was studied in altogether 102 plasma samples obtained from 77 RRMS patients and 25 HCs. The results showed that the overall detection rate for 5p miRNA was 80.5% among MS patients and 92% in HCs. In groups based on current medication, the overall detection rate for 5p miRNA was 84% among NTZ-treated patients, and 75% among IFN-β-treated patients. However, the differences in detection rates were not found statistically significant ($p > 0.5$). Furthermore, the detection rate of 5p miRNA among JCPyV seropositive and seronegative patients receiving either NTZ or IFN-β was similar (Table 2). Surprisingly, the rate of 5p miRNA detection among JCPyV seronegative patients treated with NTZ was higher than among IFN-β-treated patients (Table 2), although the difference was not statistically significant ($p > 0.5$). The anti-JCPyV antibody assay was not available for HCs, and therefore we were not able to correlate miRNA detection rates to JCPyV antibody status among HCs.

We could not detect true positive 3p miRNA signals indisputably in any of the samples. Occasional amplification, with Ct cycle for just one replicate, was obtained in samples of four MS patients and four HCs. Only one sample from a HC gave positive signal for 3p miRNA with high Ct cycles for two replicates (Supplementary Table 1). Therefore, we were unable to assess 3p miRNA expression levels in different patient groups and in HCs.

Assessment of relative 5p miRNA expression levels in different patient cohorts

The 5p miRNA expression levels were found to be similar in the whole MS cohort compared to HCs ($p = 0.06$). In groups based on current medication, the expression levels were found to be lower in NTZ-treated patients as compared to patients treated with IFN-β ($p = 0.027$) but not as compared to HCs ($p = 0.454$). IFN-β-treated patients had considerably higher levels of 5p miRNA than NTZ or HCs as shown by fold change in Fig. 1 ($p = 0.001$). 5p miRNA expression levels between JCPyV seropositive and seronegative patients in the different MS cohorts, treated either with NTZ or IFN-β, were found to be similar ($p > 0.5$).

NTZ-treated patients were further stratified according to their anti-JCPyV Ab index into groups with high risk of developing PML (anti-JCPyV Ab index >1.5, $n = 9$) and low risk group of developing PML (anti-JCPyV Ab index ≤1.5, $n = 40$) (Plavina et al. 2014). The levels of 5p miRNA expression did

Table 2 jcv-miR-J1-5p detection rates in the different patient groups. The overall detection rate among all groups was 85/102 (83%)

	All MS patients	NTZ-treated	IFN-β-treated	HCs	p value
All	62/77 (80.5%)	41/49 (84%)	21/28 (75%)	23/25 (92%)	>0.5
JCPyV Ab$^+$	30/36 (83%)	20/24 (83%)	10/12 (83%)	NA	>0.5
JCPyV Ab$^-$	32/41 (78%)	21/25 (84%)	11/16 (69%)	NA	>0.5

JCPyV Ab$^+$ anti-JCPyV antibody positive, *JCPyV Ab*$^-$ anti-JCPyV antibody negative, *NA* not available

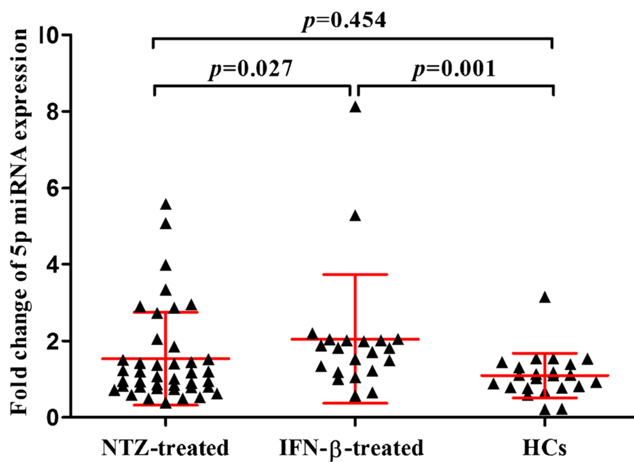

Fig. 1 Scatterplot of relative jcv-miR-J1-5p expression levels. *Bars* indicate mean ± standard deviation. The values on the *y*-axis reflect fold change of 5p miRNA expression among individual NTZ-treated patients and IFN-beta-treated patients as compared to HCs. The scatterplot of the HCs (on the *right*) presents the variation among individual HCs. Plasma levels of 5p miRNA were lower among NTZ-treated patients (n = 49) compared to IFN-beta-treated patients (n = 28), whereas the expression levels remained similar to those among HCs (n = 25). Significantly enhanced levels were observed among IFN-beta-treated patients as compared to HCs. In the calculation of relative expression levels or fold changes, the mean Ct value of HCs was subtracted from the values of each NTZ- or IFN-beta-treated patient, and thus the mean value among HCs is equal to 1

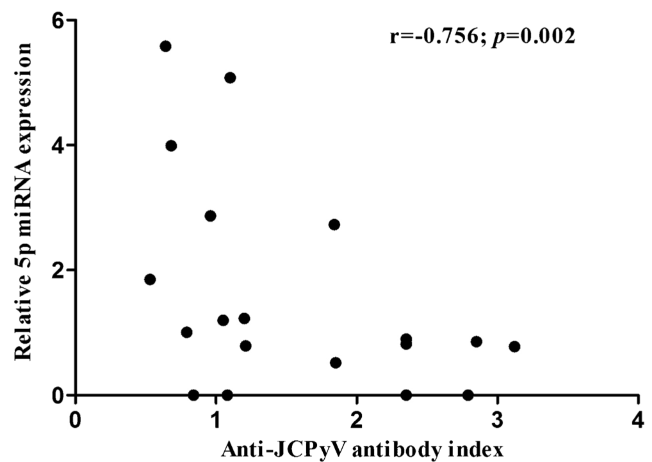

Fig. 2 Inverse correlation between jcv-miR-J1-5p expression and the anti-JCPyV Ab index among JCPyV seropositive patients treated long-term with NTZ

not differ between the high-risk group and low-risk group of developing PML (p = 0.38).

Relationship between JCPyV miRNA expression and anti-JCPyV antibody index

Association between plasma 5p miRNA levels and anti-JCPyV Ab index was further studied by correlation analyses along with other clinical characteristics of MS patients (age, disease duration, drug duration, and EDSS). There were no significant correlations found between 5p miRNA expression and clinical characteristics of patients. Among all NTZ-treated patients (n = 49), and patients who had been treated more than 18 months (n = 40), 5p miRNA expression did not correlate with anti-JCPyV Ab index. Interestingly, a significant inverse correlation between 5p miRNA expression and anti-JCPyV Ab index was established among those patients (n = 19) who had been treated long-term with NTZ and were JCPyV seropositive (r = −0.756, p = 0.002, Fig. 2). The mean duration of NTZ treatment among JCPyV seropositive MS patients was 3.0 years (SD ±1.3 years, range 1.6–5.3 years). Moreover, further analyses by linear regression model showed that the observed correlation between relative 5p miRNA expression levels and anti-JCPyV Ab indices was not affected when adjusted for age, sex, or for both (data not shown).

Discussion

When considering appropriate medication for MS patients, risk of PML has remained a major challenge for clinicians, because in addition to NTZ also other effective MS drugs such as fingolimod and dimethyl fumarate have been reported to increase PML risk in MS patients (Faulkner 2015). This has led to the urgent need of reliable and sensitive prognostic markers to better manage the patients for PML risk on an individual basis. Since polyomavirus microRNAs circulating in biological fluids currently hold potential as diagnostic markers of several diseases (Martelli and Giannecchini 2017), we analyzed the presence of JCPyV encoded miRNAs in plasma and explored their biomarker potential in NTZ-therapy-associated PML risk assessment in MS patients.

Altogether, 83% of plasma samples 5p miRNA could be detected, whereas credible signals for 3p miRNA were obtained only for one sample from a HC. Although previous studies have suggested that the 3p miRNA is expressed at lower rates as compared to the 5p miRNAs (Lagatie et al. 2013; Seo et al. 2008), such low detection rate was surprising. In our previous study, we showed high detection rates of the 3p miRNA in plasma, urine, and CSF samples of transplant and other immunosuppressed patients using similar assays (Pietila et al. 2015). We are not able to provide an exhaustive explanation to this discrepancy, except for the differences in the studied patient populations and different real-time PCR platforms used in the two studies. The functionality of all the assays was verified using synthetic oligonucleotide templates. The spike-in controls in all samples were valid. A LightCycler 480 platform (Roche Applied Science, Penzberg, Germany) was used in the previous study, which may partially explain the differences in detection rates and Ct cycles. It has been shown earlier that the results obtained using different real-time PCR platforms are not directly comparable (Lu et al. 2010;

Silvy et al. 2005). Furthermore, our preliminary data of BKPyV miRNA detection from renal transplant patients (Virtanen et al., unpublished), show high detection rates for bkv-miR-B1-3p/jcv-miR-J1-3p, suggesting clear differences in 3p detection rates between different patient populations. However, the low detection rate of 3p miRNA in this study does not interfere with our interpretation of 5p miRNA analyses.

Consistent with other studies, we found equal rates of 5p miRNA in JCPyV seronegative and seropositive patients indicating that a negative JCPyV Ab result does not necessarily mean the absence of JCPyV infection (Lagatie et al. 2014). Indeed some recent NTZ-related PML cases have been reported in MS patients in whom no anti-JCPyV Ab had been detected (Gagne Brosseau et al. 2016). Recent studies have reported higher levels of 5p miRNA in various clinical samples such as in tissue samples of patients with colonic neoplasia compared to healthy subjects (Link et al. 2014), as well as in PBMC and in exosomes obtained from plasma and urine of NTZ-treated MS patients as compared to untreated and healthy subjects (Giovannelli et al. 2015). Our present work is the first to show differential expression of JCPyV 5p miRNA in MS patients treated with NTZ or IFN-β, and to display the association of 5p miRNA with JCPyV seropositivity in NTZ-treated MS patients. Indeed, in a previous attempt, an association with developing PML risk in NTZ-treated patients positive for JCPyV DNA in blood or urine could not be established (Rudick et al. 2010).

It has been suggested that subclinical reactivation of JCPyV may occur in NTZ-treated patients with MS (Chen et al. 2009). Upon reactivation, increased viral replication may be accompanied by rearrangements of archetype JCPyV, involving putative alterations within the non-coding control region (NCCR). JCPyV-carrying archetype NCCR is mainly present in asymptomatic individuals whereas viral strains with rearranged NCCR forms are frequently found in patients with PML (Martelli and Giannecchini 2017). Increased transcription, DNA replication, and expression of viral gene products would allow better immune detection of infection by both innate and adaptive immune systems of the host. NTZ treatment has further been shown to increase cellular immune responses specific to virus and myelin proteins in peripheral blood (Jilek et al. 2010). On the other hand, considerable downregulation of archetype BKPyV DNA replication has been shown due to viral microRNA expression, which helps to maintain persistent virus in a healthy host despite a functional immune system (Broekema and Imperiale 2013). In analogy, downregulation of miRNA expression would release viral early gene transcription and DNA replication from negative regulation and, in the case of JCPyV, enable efficient replication and, consequently, putative emergence of rearranged neurotropic strains. Along these lines, our present study on plasma samples revealed lower levels of 5p miRNA in NTZ-treated patients as compared to IFN-β-treated patients, but the levels were similar to HCs. Although no PML cases were included in our patient population, the observed reduced levels of 5p miRNA among patients treated with NTZ suggest a possible involvement in support of viral reactivation. However, regulation of JCPyV miRNA expression is not well studied, although some very rare LTAg mutants have been reported which do not encode microRNAs, with putative effect on viral replication and host immune response (Chen et al. 2014).

Furthermore, growing evidence suggests that JCPyV miRNAs, similar to other viral miRNAs, can reside in exosomes, and that the exosomal miRNAs may have a role in viral persistence and reactivation (Martelli and Giannecchini 2017). Remarkably, these miRNAs have been detected in exosomes obtained from plasma and urine of both JCPyV-DNA positive and JCPyV-DNA negative MS patients (Giovannelli et al. 2015), suggesting that these miRNAs may provide new element in identifying patients at risk of PML.

Interestingly, we found higher levels of 5p miRNA in IFN-β-treated patients as compared to NTZ-treated and HCs. The different miRNA levels that we observed in these two groups of patients may either be a direct consequence of a particular treatment, or an off-target phenomenon, and it should be evaluated further. To date, there have been no reported cases of PML due to reactivation of JCPyV in either IFN-β or glatiramer acetate-treated MS patients. In addition to antiviral activity of IFN-β, it has been shown that the treatment leads to the reduction of JCPyV viral load in peripheral blood of MS patients (Delbue et al. 2007), which might be associated with the higher 5p miRNA levels found in the present study. However, further investigations are needed to explain the mechanism and impact of high 5p miRNA expression observed in our MS patients treated with IFN-β.

It is noteworthy that 5p miRNA levels correlated inversely with anti-JCPyV Ab index in JCPyV seropositive patients treated long-term with NTZ. At present, there are no further reports available on the association between anti-JCPyV Ab and JCPyV miRNA expression patterns in MS. However, two recent studies have reported inverse correlations of 5p miRNA expression: with JCPyV T-Ag expression in colorectal cancer tissues (Link et al. 2014) and with JCPyV DNA load in blood and CSF of HIV patients who are at risk of developing PML (Rocca et al. 2015), confirming that miRNA expression may indeed restrict virus replication in order to suppress host immune responses. Nevertheless, considering the observed correlation, further studies are needed to confirm the clinical relevance of the relationship between 5p miRNA and anti-JCPyV Ab index in identifying individual patients at risk of developing PML.

Our study has some limitations. The number of patients enrolled was relatively small. Further, the assessment of viral loads, and JCPyV antibodies among HCs, as performed in a previous study (Lagatie et al. 2014), would have enabled

additional analyses on the impact of our microRNA findings, but the antibody assay was only available for MS patients in the present study.

Conclusion

Taken together, our results suggest that the presence of JCPyV miRNA in plasma may indicate asymptomatic JCPyV infection in MS patients and therefore may hold potential as a biomarker for PML risk assessment. However, due to the lack of PML samples in our study, these preliminary findings should be evaluated further in a larger set of longitudinal samples including samples from NTZ-associated PML patients before and after the onset of the disease to better define the predictive value of JCPyV miRNA. Furthermore, this study confirms our previous observation that JCPyV miRNA can be determined in plasma of MS patients and healthy subjects (Pietila et al. 2015), and further suggests that JCPyV serostatus actually underestimates the true infection rates.

Acknowledgements The authors thank Keijo Koivisto (Seinäjoki Central Hospital, Department of Neurology, Seinäjoki, Finland), Auli Verkkoniemi-Ahola (Department of Clinical Neurosciences, Helsinki University Central Hospital, Helsinki, Finland), and Laura Airas (Department of Clinical Neurosciences, Turku University hospital, Turku, Finland) for providing samples and clinical information on patients from their centers. The authors also thank Mika Helminen (School of Health Sciences, University of Tampere) for his advice in statistical analyses. The study was financially supported by the Tampere University Hospital Medical Fund, Pirkanmaa Regional Fund of the Finnish Cultural Foundation, Finnish MS Foundation, and Alfred Kordelin Foundation of Finland.

Compliance with ethical standards

Conflict of interests The authors have no conflict of interests to declare.

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