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Short communication

Polyomavirus JC microRNA expression after infection in vitro

Irene Giovannelli[a], Valeria Clausi[a], Souichi Nukuzuma[b], Nunzia Della Malva[a], Daniele Nosi[a], Simone Giannecchini[a,*]

[a] Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy
[b] Department of Infectious Diseases, Kobe Institute of Health, 4-6-5 Minatojima-Nakamachi, Chuo-ku, Kobe, Japan

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ABSTRACT

The in vitro expression of the Polyomavirus JC (JCPyV) microRNAs, JC-miRNA-3p and -5p, at early time points post-infection was investigated. The expression of the JCPyV microRNAs was monitored in hematopoietic progenitor KG-1 cells and in kidney fibroblast-like COS-7 cells transformed with SV40 after infection with a JCPyV CY archetype viral clone. The JCPyV DNA viral load was low in KG-1 cells compared with that in COS-7 cells, which showed productive viral replication. The expression of the JCPyV microRNAs was observed from 12 h after the viral infection of both cell types and in the exosomes present in their cell supernatant. Additionally, this study verified that the JCPyV microRNAs in the exosomes present in the supernatants produced by the infected cells might be carried into uninfected cells. These findings suggest that additional investigations of the expression of JCPyV microRNAs and their presence in exosomes are necessary to shed light on their regulatory role during viral reactivation.

Polyomavirus JC (JCPyV) is the etiological agent of progressive multifocal leukoencephalopathy (PML), a rare subacute demyelinating disease of the brain caused by a viral lytic infection of oligodendrocytes in an immunocompromised setting (Ferenczy et al., 2012). Over the past few years, PML has been increasingly diagnosed in patients treated with immunomodulatory therapy, such as natalizumab in multiple sclerosis patients (Bloomgren et al., 2012). Although JCPyV has been extensively studied, the risk factors of JCPyV reactivation remain elusive (Bloomgren et al., 2012). In this context, the activation of JCPyV replication in different cell compartments is associated with the presence of several host transcription factors (Marshall and Major, 2010). Moreover, JCPyV replication in brain tissue is dependent on the arrangements of the noncoding control region (NCCR) of the viral genome, which increase the number of binding sites for cellular transcription factors (Marshall and Major, 2010). Recently, it was demonstrated that JCPyV encodes two mature microRNAs (miRNAs), JC-miRNA-3p and -5p, that can down-regulate early viral expression and are likely involved in the regulation of viral persistence (Lagatie et al., 2013; Bauman and Mandelboim, 2011). The expression of these JCPyV-encoded miRNAs has been identified in PML tissues (Seo et al., 2008), in multiple sclerosis patients undergoing natalizumab treatment (Giovannelli et al., 2015), in HIV-infected patients (Rocca et al., 2015), in cancer patients (Link et al., 2014) and also in healthy people (Lagatie et al., 2014). In this scenario, in the absence of a rearranged NCCR (NCCR archetype), the expression of viral miRNAs may be involved in the control of JCPyV replication, which is required for viral asymptomatic persistence in the blood (Lagatie et al., 2014; Giovannelli et al., 2015). Additional evidence suggests that the reduction of JC-miRNA expression can be associated with viral reactivation (Rocca et al., 2015). The objective of the present study was to assess the relationship between the replication of JCPyV DNA carrying a NCCR archetype structure and the expression of viral miRNAs in cells and exosomes present in their cell supernatants at the early time post-infection. This time was selected because both viral miRNAs are expressed in cell substrate beginning at 12 h post infection (Seo et al., 2008). Here, the replication of the CY molecular clone of JCPyV possessing the archetype non-pathogenic NCCR structure was investigated in the hematopoietic CD34 KG-1 cell line propagated in RPMI 1640 with 2% fetal bovine serum (FBS). The JCPyV CY clone was selected because the objective of the study was to investigate the early events of viral replication in a JCPyV strain that is representative of the virus that asymptomatically persists in the host cells, preceding the pathogenic virus PML-type JCPyV generated by NCCR rearrangements (Ferenczy et al., 2012). The KG-1 cell line was selected because it is susceptible to the JCPyV infection (Monaco et al., 1996) and representative of hematopoietic stem cells that are mobilized into blood circulation from the bone marrow

* Corresponding author at: Dipartimento di Medicina Sperimentale e Clinica, Università di Firenze, Viale Morgagni 48, I-50134 Firenze, Italy.
E-mail address: simone.giannecchini@unifi.it (S. Giannecchini).

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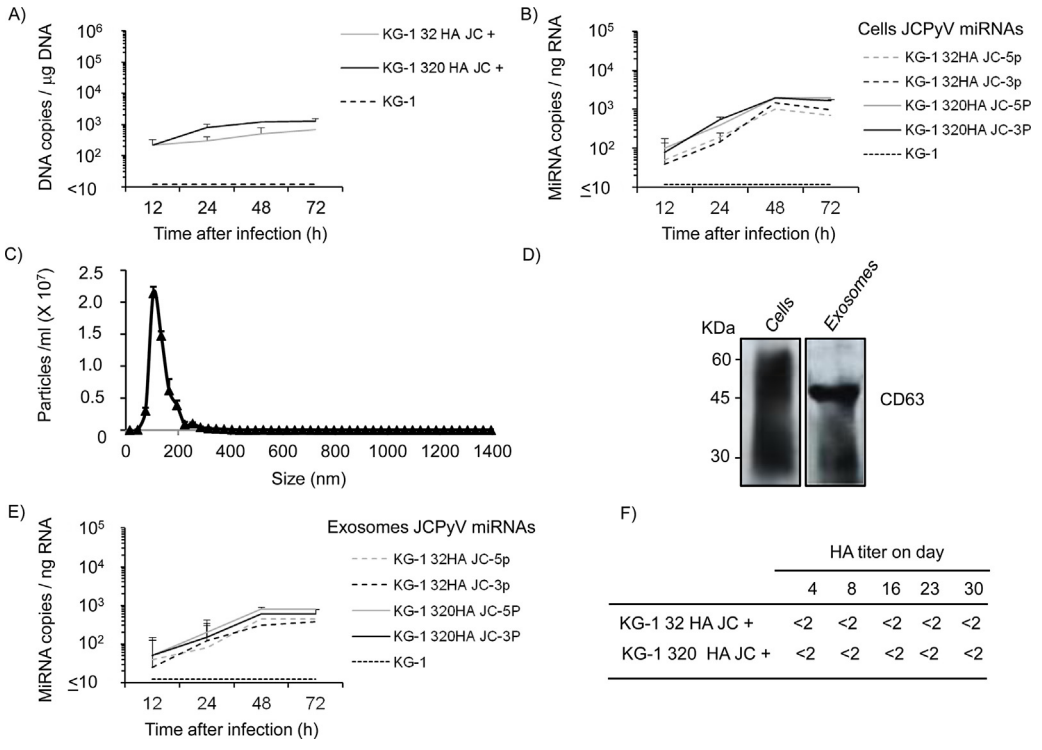
during the natalizumab treatment that is potentially involved in the increased JCPyV DNA viremia (Frohman et al., 2014). The JCPyV virus was produced using the genomic DNA (CY clone) by transfection as described previously (Nukuzuma et al., 2009). Briefly, 1.5 μg of viral DNA excised from a recombinant plasmid was transfected into COS-7 cells using Lipofectamine (Invitrogen, Carlsbad, CA, USA) and passaged at a split ratio of 1:3 every 3 or 4 days in Minimal Essential Medium (MEM) supplemented with 2% FBS. The JCPyV virus was obtained on day 32, and the HA titer was determined as described previously (Nukuzuma et al., 2009). KG-1 cells were exposed to 100 μl of the JCPyV virus (32 and 320HA) for 2 h at 37 °C. After a trypsinization at 37 °C for 30 min and a wash step to remove the excess inoculum, the DNA viral replication in the cultures was analyzed at 12 h, 24 h, 48 h, and 72 h post-infection using a sensitive real time PCR assay (Ryschkewitsch et al., 2013). Briefly, the JCPyV infection was assessed by extracting DNA from 2.0×10^5 KG-1 cells using the QIAamp DNA Mini Kit (Qiagen) and amplifying 50 ng of total DNA in each reaction in triplicate using a real-time PCR assay with primer and probes targeting the large T antigen (JCT-3F forward primer 5′-AGTGTTGGGATCCTGTGTTTTCA-3′, JCT-4R reverse primer 5′-GTGGGATGAAGACCTGTTTTGC-3′ and TaqMan MGB JCT-1.2 probe Fam-5′ CATCACTGGCAAACAT 3′) and noncoding control region (JRR-1F forward primer 5′-GGAGCCCTGGCTGCAT-3′, JRR-2R reverse primer 5′-TGTGATTAAGGACTATGGGAGG-3′ and TaqMan MGB JRR-1.1 probe VIC-5′ CTGGCAGTTATAGTGAAACC-3′). Each reaction was performed with negative controls (no template) and DNA standards (diluted to contain 10–10^6 copies) of a plasmid containing the JCPyV molecular clone. The lower limit of detection of the assay was 10 copy of JCPyV for reaction. As shown in Fig. 1A, the JCPyV DNA viral load in KG-1 cells showed a slow replicative kinetics indicating a non-productive infection. In all experiments, non-infected KG-1 cells gave negative results for the viral DNA. The JCPyV miRNA expression in the cells and exosomes present in the cell supernatant were then assessed. We investigated the viral miRNA expression in exosomes because these vesicles are involved in intercellular communication and deliver miRNA molecules from an infected cell to a recipient cell (Pegtel et al., 2010). Therefore, similar to other viruses, it is hypothesized that during JCPyV infection, the exosomes containing viral miRNAs delivered to uninfected cells could be a potential viral counteracting mechanism involved in the repression of viral replication to maintain JCPyV asymptomatically in the host (Giovannelli et al., 2015; Pegtel et al., 2010). Total RNA was isolated from 2.0×10^6 KG-1 cells using the mirVana miRNA isolation Kit (Ambion) and from exosomes contained in 250 μl of cell-free supernatant using an exosome-specific extraction kit (Norgen). The miRNA expression was measured and quantified with the JCPyV JC-miRNA-3p and -5p quantitative stem-loop RT-PCR MiRNA assay whose primers were designed on the specific miRNA region of the JCPyV CY clone used (Life Technologies, Foster City, CA) according to the manufacturer's protocol. Each reaction was performed in triplicate using 10 ng of extracted RNA, including negative controls (no template) and synthesized oligonucleotides as standards (diluted to contain 10^1–10^6 copies). The lower limit of detection of the assay was 10 copies/ng of RNA. The assay was specific and reproducible, as demonstrated in preliminary experiments using a JCPyV JC-miRNA oligonucleotide standard (with <0.5 Ct value inter-assay variation) and observing no amplification of unrelated oligonucleotide targets. Fig. 1B shows that although in the presence of modest viral DNA replication producing low copy numbers, both JCPyV miRNAs



	HA titer on day				
	4	8	16	23	30
KG-1 32 HA JC +	<2	<2	<2	<2	<2
KG-1 320 HA JC +	<2	<2	<2	<2	<2

Fig. 1. KG-1 cells infected with JCPyV showed JC-miRNA expression in the presence of non-productive DNA replication. A total of 3×10^6 KG-1 cells were infected with 32 and 320HA units of JCPyV (CY clone) for 2 h, and aliquots of 2.5×10^5 cells were harvested at 12 h, 24 h, 48 h, and 72 h after infection. (A) DNA was extracted from 10^5 cells and amplified and quantified by qPCR. JCPyV DNA genome copies became undetectable after 30 days of infection. (B) MiRNAs were extracted from 10^5 cells and retrotranscribed and quantified using a JC-miRNAs stem-loop qPCR. (C) The exosomes purified from the cell-free supernatants of infected cells were analyzed with the NanoSight NS300 Nanoparticle analysis system. (D) Western blot profiles of CD63 from KG-1 JCPyV infected cell lysates and lysates from their purified exosomes electrophoresed on 10% SDS-PAGE and then probed with anti-CD63 monoclonal antibody antibody (CD63 under nonreducing conditions) followed by peroxidase-conjugated anti-mouse IgG polyclonal antibody. (E) MiRNAs were extracted from exosomes derived from 250 μl of cell-free of infected cell supernatants and retrotranscribed and quantified by JC-miRNA stem-loop qPCR. (F) HA titer obtained at the indicated time post infection. A–C and E) The values shown are the means ± standard deviations of 3 independent experiments.