Journal of Microbiology, Immunology and Infection (2019) 52, 667—671

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.e-jmii.com

Brief Communication

Lower expression of plasma-derived exosome miR-21 levels in HIV-1 elite controllers with decreasing CD4 T cell count

María J. Ruiz-de-León [a,2], María A. Jiménez-Sousa [b,2], Santiago Moreno [a], Marcial García [c,d], Mónica Gutiérrez-Rivas [b], Agathe León [e], Marta Montero-Alonso [f], Juan González-García [g], Salvador Resino [b], Norma Rallón [c,d,**,3], José M. Benito [c,d,3], Alejandro Vallejo [a,*,3] On behalf of the ECRIS Network integrated in the Spanish AIDS Research Network[1]

[a] Laboratory of Molecular Immunology, Department of Infectious Diseases, Instituto Ramón y Cajal Health Research Institute (IRyCIS), Ramón y Cajal University Hospital, Madrid, Spain
[b] Viral Infection and Immunity Unit, National Center of Microbiology, Carlos III Health Institute, Majadahonda, Spain
[c] IIS-Jiménez Díaz Foundation, UAM, Madrid, Spain
[d] Rey Juan Carlos University Hospital, Móstoles, Madrid, Spain
[e] Clinic Hospital-IDIBAPS, HIVACAT, Barcelona University, Barcelona, Spain
[f] Infectious Diseases Unit, La Fe University Hospital, Valencia, Spain
[g] Internal Medicine Department, La Paz University Hospital, Madrid, Spain

Received 2 April 2018; received in revised form 11 June 2018; accepted 13 July 2018
Available online 24 August 2018

KEYWORDS
Exosomes;
miRNAs;
HIV-1;

Abstract Exosome-derived miR-21 was independently associated with CD4 T cell decline in HIV-1-infected elite controllers (OR 0.369, 95% CI 0.137—0.994, p = 0.049). Also, a negative correlation between miR-21 expression and MCP-1 level was found (r = −0.649, p = 0.020), while no correlation between soluble biomarkers or cellular immune activation was found.

* Corresponding author. Laboratory of Molecular Immunovirology, Department of Infectious Diseases, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Hospital Universitario Ramón y Cajal, Ctra Colmenar Km 9, 28034, Madrid, Spain.
** Corresponding author. IIS-Jiménez Díaz Foundation, UAM, Av. Reyes Católicos, 2, Madrid, 28040, Spain. Fax: +34 91 550 48 49.
E-mail addresses: norma.rallon@fjd.es (N. Rallón), alejandro.vallejo@salud.madrid.org (A. Vallejo).
[1] The Clinical Centers and Research Groups that contribute to ECRIS Network are shown in Appendix.
[2] MJRL and MAJS contributed equally to this work.
[3] NR, JMB and AV contributed equally to this work.

https://doi.org/10.1016/j.jmii.2018.07.007

Elite controllers;
Immune activation;
Soluble biomarkers

Introduction

A small group of HIV-1-infected individuals (5–15%) control disease progression for several years in the absence of any antiretroviral therapy. Among this group, elite controllers (EC) spontaneously control HIV-1 replication (below 50 HIV-1 RNA copies/ml).[1]

Homeostatic factors contribute to maintain a stable pool of T cells in this situation where T cell apoptosis is enhanced. This situation promotes the release of microvesicles from cells, such as exosomes.[2] One of the active molecules carried by exosomes are miRNAs, small non-coding RNA capable of recognizing specific mRNA and inhibiting its translation into proteins. These molecules may thus promote hematopoietic stem cells and regulate the immune system and inflammatory processes that could influence the homeostasis cell equilibrium and a number of immunomodulatory processes.[2] HIV could interfere with the exosomal pathway, enhancing or inhibiting the release of exosomes or modifying their content, including host-derived miRNA cargo.[3]

Previous studies have showed the relevance of miRNAs in HIV pathogenesis. Whereas PBMCs-derived miR-150, miR-29a and miR-31 have been reported to positively correlate with CD4 counts, miR-181b has been associated negatively comparing EC with viremic progressors.[4] Other study observed plasma miRNAs that correlated with CD4 count in a group of EC and viremic progressors.[5] We conducted this cross-sectional study to analyze a set of exosome-derived miRNAs that could predict the decay of CD4 levels among an interesting group of patients who spontaneously control HIV replication (elite controllers); EC with stable CD4 count versus EC with significant decreased CD4 count. We also investigated the association of plasma-derived exosome miRNA levels with both soluble cytokine levels and cellular immune activation.

Material and methods

This retrospective cross-sectional study included HIV-1-infected elite controllers belonging to the multicentre Spanish Elite Controllers (EC) Cohort of the Spanish AIDS Research Network (ECRIS cohort), initiated in 2013.[6] To be included, patients had to be asymptomatic chronic HIV-1-infected patients with at least three consecutive plasma HIV-1 RNA loads below 50 HIV-RNA copies/mL during at least 12 months in the absence of any antiretroviral therapy. Elite controllers were classified in two groups; those with stable or increasing CD4 T cell count during minimum three years, named as stable CD4 T cell count elite controllers (SEC, N = 21); and those with significant decreased CD4 T cell count, name as decreasing CD4 T cell count elite controllers (DEC, N = 11).

Plasma and peripheral blood mononuclear cell samples, provided by the Spanish HIV Biobank,[7] were analyzed before the classification into SEC and DEC groups. The first plasma samples available after the inclusion of each patient in the cohort were used. Clinical and epidemiological data were provided by the ECRIS Cohort. All procedures were performed in accordance with the ethical standards of the Institutional Review Boards of the participating Hospitals, which complied with the stipulations of the Declaration of Helsinki. Informed consent was obtained from all individual participants included in the study.

EDTA-plasma was thawed and sequentially centrifuged at 300 g for 10 min at room temperature, 2.000 g for 30 min at 4 °C, and finally at 10.000 g for 45 min at 4 °C to remove cells and cell debris. Thrombine and DNase treatments were used to prevent platelet and DNA contamination, respectively. Plasmas were then filtered through 0.2 μm nylon syringe filter to eliminate larger vesicles such as large extracellular vesicles and apoptotic bodies. Exosomes were precipitated using miRCURY Exosome isolation kit (Exiqon A/S, Vedbaek, Denmark). The ExoELISA-ULTRA CD63 assay (SBI System Bioscience, Mountain View, CA, USA) was used to quantitate exosome content into the samples.

RNA extraction from isolated exosome-enriched pellet was performed using miRCURY RNA isolation kit-Biofluids (Exiqon A/S). UniSp2-4-5 RNA templates were used as internal control. Eluted RNA was assayed for concentration and purity (NanoDrop instrument, Thermo Scientific). All RNA samples with 260/280 ratio between 1.8 and 2 and 260/230 ratio near 2 were considered suitable for further analyses. Ten nanograms of RNA was reverse transcribed in 15 μl reactions (mirCURY LNA Universal RT microRNA PCR, Exiqon A/S), including UniSp6 RNA spike-in template reaction control. cDNA product was used for PCR reaction in triplicate using ExiLENT SYBR Green Master Mix (Exiqon A/S). LNA-based primers for hsa-miR-16-5p, hsa-miR-21-5p, hsa-miR-29a-3p, hsa-miR-146a-5p, hsa-miR-221-3p and hsa-miR-223-3p were used for individual qPCR assays using LyghtCycler 480 II instrument (Roche, Basel, Switzerland). Besides, miR-451a and miR-23a were tested to detect levels of hemolysis.

Thermocycler conditions were as follows: 95 °C hot start for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 45 s. Amplification curves were analyzed using Roche LightCycler 480 version 1.5.1.62 software. Reaction specificity was ascertained by performing the melt curve procedure. Ct values above 35 were considered negative and excluded from the analysis. If the standard deviation between triplicates was above 0.3 Ct, the data point was considered unreliable and excluded from further analysis. MiR-103a-3p, miR-425-5p, and miR-93-5p were used as reference for the calculation of ΔCt (Ct target miRNA - mean references Ct) as normalization method.[8] Expression levels of the individual miRNAs relative to the mean reference expression of miRNAs were calculated as $2^{-\Delta Ct}$. Relative concentration of miRNAs was shown as log_2 values.

Continuous variables were expressed as median and interquartile range (IQ_{25-75}), and categorical ones were described by proportions. The Mann–Whitney *U* test was used to compare continuous variables and contingency tables for categorical variables. Uni and multivariate logistic regression analysis was assessed using all independent variables studied. Statistical analysis was performed using SPSS software 16.0 (Chicago, Illinois, USA).

Results

Characteristics of the patients at the moment of the study are shown in Table 1. Median abundance of exosomes in SEC group was of 3.2×10^9 [$2.2 \times 10^9 - 4.1 \times 10^9$] and in DEC group was 2.8×10^9 [$2.1 \times 10^9 - 3.7 \times 10^9$], with no statistic difference ($p = 0.675$). The level of normalization miRNAs 103a-3p, 425-5p, and 93-5p were similar between both groups of patients ($p = 0.920$, $p = 0.923$, and $p = 0.875$, respectively).

Table 1 Characteristics of the patients at study point.

	SEC patients	DEC patients	p value
N	21	11	
Age (years)	41.5 [34.0–47.7]	41.5 [38.2–48.2]	0.650
Gender (male)	6 (30%)	7 (70%)	0.056
Rout of transmission			
Injecting drug user, n	14 (78%)	5 (50%)	**0.010**
Heterosexual and MSM, n	4 (22%)	3 (30%)	0.648
Other, n	0 (0%)	2 (20%)	0.073
Time maintaining EC status (months)	105 [83–122]	85 [81–131]	0.696
CD4 T cell count at baseline (cells/mm^3)	819 [639–1113]	1053 [850–1153]	0.067
CD4 T cell line at the end of follow up (cells/mm^3)	856 [669–1161]	633 [503–978]	0.042
CD4 T cell slope (cells/mm^3/year)	7.8 [−1.6–25.0]	−46.7 [−86.5 to −30.2]	<0.001
CD4 T cell count at study point (cells/mm^3)	912 [800–1157]	669 [491–1404]	0.764
HCV infection			
Antibodies anti-HCV positive, n	15 (71.4%)	7 (63.6%)	0.651
HCV PCR positive, n	11 (73.3%)	4 (57.1%)	0.447
Natural HIV suppressive factors[a]			
MIP-1α (pg/mL)	8.31 [5.60–13.28]	10.67 [5.45–13.12]	0.933
MIP-1β (pg/mL)	112 [81–119]	98 [86–114]	0.350
RANTES (pg/mL)	275 [211–349]	293 [190–320]	0.735
Chemoattractant chemokines[a]			
Eotaxina (pg/mL)	19.2 [17.78–29.97]	29.89 [23.49–54.93]	**0.042**
GROα (pg/mL)	6.72 [3.15–11.32]	6.19 [4.47–7.72]	0.611
MCP-1 (pg/mL)	17.60 [11.32–27.42]	17.60 [8.59–35.52]	0.767
SDF1α (pg/mL)	212 [189–284]	193 [169–237]	0.287
IL8 (pg/mL)	6.46 [4.91–9.37]	5.19 [4.27–9.33]	0.582
IP10 (pg/mL)	22.98 [14.63–34.44]	30.52 [18.78–41.58]	0.268
Inflammation interleukins and proteins[a]			
IL10 (pg/mL)	4.24 [4.08–4.36]	4.26 [4.08–4.43]	0.986
IL18 (pg/mL)	118 [54–247]	258 [105–525]	0.200
IL6 (pg/mL)	23.40 [23.17–23.74]	23.50 [23.25–23.63]	0.983
TNFα (pg/mL)	17.30 [16.63–18.58]	17.67 [16.14–17.85]	0.800
sTNFRI (pg/mL)	450 [277–1263]	1500 [555–2060]	**0.018**
Apoptosis inducing proteins[a]			
TRAIL (pg/mL)	9.09 [5.39–14.19]	4.39 [2.26–13.37]	0.287
FasL (pg/mL)	5.05 [3.97–6.60]	4.33 [3.49–5.41]	0.171
Immune activation[b]			
$CD4^+CD38^+HLA\text{-}DR^+$ T cells (%)	0.34 [0.24–0.42]	0.28 [0.19–0.52]	0.667
$CD8^+CD38^+HLA\text{-}DR^+$ T cells (%)	0.13 [0.09–0.25]	0.13 [0.08–0.26]	0.832

[a] The following ELISA kits (Affymetrix eBioscience, San Diego, CA, California, USA) were performed using Luminex 100™ analyzer (Austin, TX, USA): ProcartaPlex Human Chemokine 9plex panel Immunoassay for Eotaxin, GRO-α, IL-8, IP-10, MCP-1, SDF-1α, MIP-1α, MIP-1β, and RANTES; ProcartaPlex Simplex for IL-18, FasL, TRAIL, and sTNF-RI; ProcartaPlex High Sensitivity for IL-6, IL-10, and TNF-α.

[b] Co-expression of CD38 and HLA-DR in CD4 and CD8 T cells (antibodies from Sony Biotechnology, Surrey, UK) measured by flow cytometry was used as markers of cellular immune activation. Sample acquisition was performed on an SP6800 Spectral flow cytometer (Sony Biothecnology) and a minimum of 10^5 CD3 T cells were acquired for further analysis.

Mann–Whitney *U* test. SEC, stable CD4 T cell count elite controller; DEC, decreasing CD4 T cell count elite controllers; EC, elite controller; HCV, hepatitis C virus; MSM, men who have sex with men.

Significant when $p < 0.05$ in bold.

As shown in Fig. 1, significant lower expression of miR-16 and miR-21 was found in DEC patients compared to SEC patients ($p = 0.034$ and $p < 0.001$, for miR-16 and miR-21, respectively), while significant higher levels of miR-221 was found in DEC patients ($p = 0.025$). The level of miR-29a, miR-146 and miR-223 were similar between DEC and SEC patients. Multivariable logistic regression model performed with dependent categorical variable group (DEC versus SEC) and significant miRs in univariate logistic regression model (miR-16, miR-21 and miR-221) as independent variables, showed that only miR-21 was independently associated to CD4 T cell decline (OR 0.369, 95% CI 0.137—0.994, $p = 0.049$). Significant negative correlation between miR-21 and MCP-1 was found among the EC patients ($r = -0.532$, $p = 0.042$), but was evident only among DEC patients ($r = -0.649$, $p = 0.020$). No other correlations between miR-21 and natural HIV suppressive factors, inflammation interleukins/proteins, apoptosis or T cell activation were found.

Discussion

Plasma exosome-derived miR-16 and miR-21 were downregulated in DEC patients in comparison with SEC patients with stable CD4 T cell count along time. On the other hand, miR-221 was upregulated in DEC patients compared to SEC patients. Multivariate logistic regression model performed to analyze whether miR-16, miR-21 and miR-221 were independently associated to CD4 evolution in EC patients (SEC versus DEC patients), showed that only miR-21 was independently associated. While TNFR1 and eotaxin levels were significantly higher in DEC patients, unfortunately, none of the miRs analyzed correlated with the levels of these soluble markers.

Despite there are many published works investigating the role of miRNAs in the HIV-1 pathogenesis and disease progression, only a few works have focused on the differentially miRNA profile in HIV elite controllers (EC). Two of them showed that some PBMC-derived miRNAs are differentially expressed in EC. One showed that miR-146a and miR-16 were not differentially expressed in EC compared to viremic progressors or patients under ART, although miR-221 was significantly upregulated in EC.[9] The other work found that miR-29a was upregulated in EC in comparison with viremic progressors.[4] Only one work reported that plasma miR-146a was upregulated in EC in comparison with chronic HIV infected patients.[5]

MiR-21 has been reported to provide a link between inflammation and cancer. It is strongly involved in apoptosis

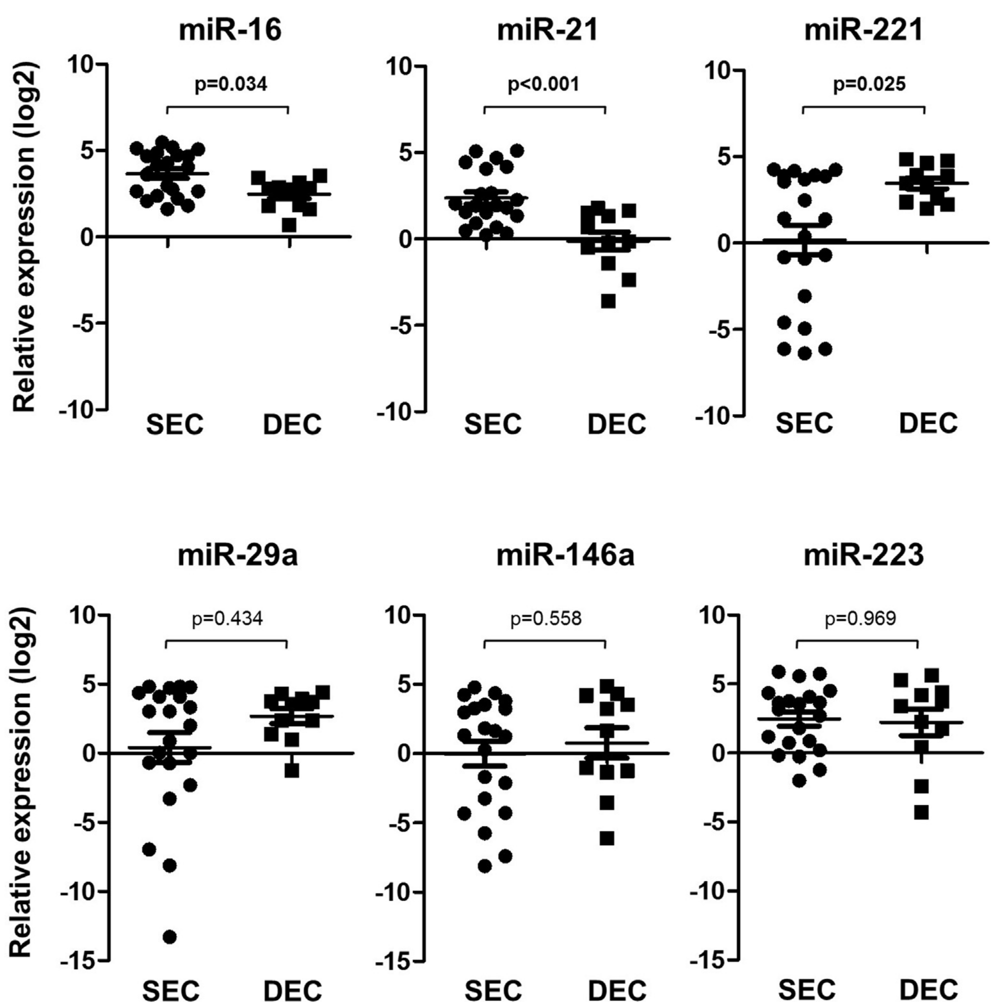

Figure 1. Relative expression of extracellular vesicle-derived miRNAs of HIV-1 elite controllers with stable CD4 T cell count (SEC patients) and decreasing CD4 T cell count (DEC patients) during the follow up. Mann—Whitney *U* test. Significant when $p < 0.05$.

and cell proliferation and several targets have been validated with experimental data.[10] Targets include B-cell translocation gene 2, a tumor suppressor gene,[11] the PI3K/Akt signaling pathway, inhibiting apoptosis,[12] or SMAD7, TGF-β negative regulator, inhibiting cell proliferation,[13] among others.

DEC patients showed negative CD4 T cell slope which is consistent with a decrease of cell proliferation, but these patients did not show different levels in apoptosis-related proteins such as FasL or TRAIL. Moreover, no association between miR-21 with immune activation markers was found.

Only the monocyte chemoattractant protein 1 (MCP-1) was negatively associated to the expression of miR-21. This could increase local inflammation leading to a decrease of cell proliferation. Interestingly, this negative correlation was only evident among DEC patients, suggesting that the decrease in the level of miR-21 could start increasing the levels of MCP-1 which could be a predictive value for the future decrease of CD4 count. Unfortunately, at the time of this study the levels of MCP-1 were similar between SEC and DEC patients. It is not described whether MCP-1 gene could be the target for miR-21.

One limitation of this study was the low number of patients analyzed, despite the intrinsic difficulty of sample availability of this kind of patients. Another limitation is that no other time point was available to perform a prospective analysis.

Overall, exosome-derived miR-21 might be used as a valuable predictive soluble biomarker to define HIV-1 elite controllers who will show significant decay in their CD4 T cell counts throughout time. Before the translation of the role of miR-21 to the clinical practice it is necessary to relate the expression of this miR with a specific molecular change within the cell.

Conflicts of interest

The authors declare that they have no conflict of interest.

Funding

This work has been partially funded by the Spanish Health Institute Carlos III (ISCIII) and European Regional Development Fund, Spanish AIDS Research Network (RIS) with grant numbers RD16/0025/0001, RD12/0017/0031, RD16/0025/0013, and RD16/0002/0002. Research Grant Project, Spanish Health Research Fund (FIS, ISCIII) number PI14/00011. Miguel Servet Research Program, Spanish Health Institute Carlos III (ISCIII) CP14/00198 to NR. Spanish Health Research Fund (FIS, ISCIII) and IIS-FJD Intramural Research Scholarship co-funded grant number CP14/00198 to MG. Spanish Health Institute Carlos III (ISCIII) grant CD13/00013 to MAJS. Grant Research, Madrid Education, Youth and Sport Counseling number PEJ15/bio/tl-0064 to MJRL.

Appendix

We want to thank all the patients for their participation. This study would not have been possible without the collaboration of medical, nursery staff and data managers who have taken part in the project. The members of the ECRIS Network are: IIS-Fundación Jimenez Díaz, UAM, Madrid: JM Benito, N Rallón, C Restrepo, N Rodríguez, M García, A Cabello, M Gorgolas. Instituto Salud Carlos III, Madrid: S Resino, V Briz, MA Jiménez, MS Vázquez, A Fernández, P García. Hospital Gregorio Marañón, Madrid: MA Muñoz, J Sánchez, JL Jiménez, D Sepúlveda, I García, I Consuegra. Hospital Clinic, Barcelona: A León, M Arnedo, M Plana, N Climent, F García. Hospital Virgen del Rocio, Sevilla: E Ruiz-Mateos, B Domínguez, L Tarancón, M Rafii-El-Idrissi, MJ Polaino, M Genebat, P Viciana, M Leal. Hospital Joan XXIII, Tarragona: F Vidal, E Rodríguez, C Viladés, J Peraire. Centro Sandoval, Madrid: J Romero, C Rodríguez, M Vera. Fundacion IRSI CAIXA, Badalona: J Esté, E Ballana, MA Martínez, S Franco, M Nevot. Hospital Ramón y Cajal, Madrid: A Vallejo, S Moreno. Instituto Salud Carlos III, Madrid: M Pernas, C Casado, C López. Instituto Salud Carlos III, Madrid: L Capa, M Pérez, J Alcami. Universidad de Valencia: R Sanjuán, JM Cuevas. Hospital 12 de Octubre, Madrid: R Delgado, O Sierra. Universidad de la Laguna, Sta. Cruz Tenerife: A Valenzuela. The clinical centers that contributed to ECRIS are listed in Supplementary file.

References

1. Migueles SA, Connors M. Long-term nonprogressive disease among untreated HIV-infected individuals: clinical implications of understanding immune control of HIV. *JAMA* 2010;**304**:194—201.
2. Robbins PD, Morelli AE. Regulation of immune responses by extracellular vesicles. *Nat Rev Immunol* 2014;**14**:195—208.
3. Ellwanger JH, Veit TD, Chies JAB. Exosomes in HIV infection: a review and critical look. *Infect Genet Evol* 2017;**53**:146—54.
4. Witwer KW, Watson AK, Blankson JN, Clements JE. Relationships of PBMC microRNA expression, plasma viral load, and CD4+ T-cell count in HIV-1-infected elite suppressors and viremic patients. *Retrovirology* 2012;**9**:5.
5. Reynoso R, Laufer N, Hackl M, Skalicky S, Monteforte R, Turk G, et al. MicroRNAs differentially present in the plasma of HIV elite controllers reduce HIV infection in vitro. *Sci Rep* 2014;**4**:5915.
6. Leon A, Perez I, Ruiz-Mateos E, Benito JM, Leal M, Lopez-Galindez C, et al. Rate and predictors of progression in elite and viremic HIV-1 controllers. *AIDS* 2016;**30**:1209—20.
7. Garcia-Merino I, De Las Cuevas N, Jimenez JL, Gallego J, Gómez C, Prieto C, et al. The Spanish HIV Biobank: a model of cooperative HIV research. *Retrovirology* 2009;**6**:27.
8. Livak KJ, Schmifgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-[Delta][Delta] CT method. *Methods* 2001;**25**:402—8.
9. Egaña-Gorroño L, Escribà T, Boulanger N, Guardo AC, Leon A, Bargallo ME, et al. HIV Controllers Consortium of the AIDS Spanish Network. Differential microRNA expression profile between stimulated PBMCs from HIV-1 infected elite controllers and viremic progressors. *PLoS One* 2014;**9**:e106360.
10. Buscaglia LE, Li Y. Apoptosis and the target genes of microRNA-21. *Chin J Cancer* 2011;**30**:371—80.
11. Mao B, Xiao H, Zhang Z, Wang D, Wang G. MicroRNA-21 regulates the expression of BTG2 in HepG2 liver cancer cells. *Mol Med Rep* 2015;**12**:4917—24.
12. Wang T, Cai Z, Hong G, Zheng G, Huang Y, Zhang S, et al. MicroRNA-21 increases cell viability and suppresses cellular apoptosis in non-small cell lung cancer by regulating the PI3K/Akt signaling pathway. *Mol Med Rep* 2017;**16**:6506—11.
13. Lin L, Gan H, Zhang H, Tang W, Sun Y, Tang X, et al. MicroRNA-21 inhibits SMAD7 expression through a target sequence in the 3' untranslated region and inhibits proliferation of renal tubular epithelial cells. *Mol Med Rep* 2014;**10**:707—12.