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Original Article

The molecular markers of the highly pathogenic H5N1 influenza virus in children

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Abstract: This study aims to investigate the potential of microRNA126 (miRNA126) as a molecular marker for the early diagnosis of H5N1 influenza. This study included a total of 16 H5N1 virus-infected children. The patients were given an intravenous injection of cephalosporins (0.03-0.05 g/day) for 3 consecutive weeks. The serum interleukin-6 (IL-6) levels were measured to determine the effectiveness of the treatment. Sixteen healthy children were used as controls. The pre- and post-treatment levels of miRNA126 in the blood cells of the patient group were compared with the control group. The association between the miRNA126 levels in the blood cells and the severity of H5N1 influenza was analyzed using Pearson's correlation test. The pre-treatment miRNA126 levels in the H5N1 group were significantly higher compared with the levels in the control group ($P < 0.05$). The miRNA126 levels in the severe H5N1 group (defined as IL-6 > 32.1 ng/L) were significantly higher than the levels in the mild/moderate H5N1 group (defined as 11.8 ≤ IL-6 < 32.1 ng/L) before the treatment ($P < 0.05$). After the 3-week treatment, the miRNA126 levels in the patients were significantly reduced ($P < 0.05$). The miRNA126 level in blood cells was positively correlated with the severity of H5N1 influenza ($r = 0.94$, $P < 0.05$). The positive correlation between the miRNA126 level and the severity of H5N1 influenza suggests that the miRNA126 level in blood cells might be a molecular marker for evaluating the severity of pediatric H5N1 influenza and might be used in the early diagnosis of the disease.

Keywords: Pediatric highly pathogenic H5N1 influenza virus, miRNA126, diagnosis, IL-6, molecular biomarkers

Introduction

Influenza is a common childhood disease caused by an infection of the influenza virus. The highly pathogenic H5N1 influenza, a new subtype that has emerged recently, is an important cause of acute respiratory syndrome and organ failure in children [1, 2]. The causative pathogen, H5N1, is a subtype of the influenza A virus which can cause illness in humans and many other animal species [3, 4]. H5N1 is contagious among humans [5-7]. More importantly, vaccination with the H5N1 vaccine may not prevent the infection due to the mutation of the virus [8-10]. Therefore, the early detection of pediatric highly pathogenic H5N1 is essential for the treatment of the disease. An analysis of the viral RNA has been the gold standard for the clinical testing of the H5N1 virus. However, the method requires a high containment biological safety level 3 (BSL-3/P3) laboratory to ensure the safety of the testing personnel, so the method cannot be widely used for the diagnosis of H5N1 infection. Although interleukin-6 (IL-6) is known as an important indicator for highly pathogenic H5N1 infection in children, it often undergoes small changes during the course of treatment. Moreover, the measurement of IL-6 level requires a special, expensive, enzyme-linked immuno-sorbent assay (ELISA) kit, so it is not suitable for poor areas [11]. More importantly, the method is not suitable for all patients, especially those with weakened immunity [12]. Therefore, a reliable and sensitive biomarker is urgently needed for the effective diagnosis of H5N1 infection in children.

MicroRNAs are a class of small non-coding single-stranded RNA molecules that are involved in the regulation of a wide range of biological processes such as inflammation. MicroRNAs have been used as clinical molecular markers

for the diagnosis of several diseases including cancer [13, 14]. For instance, microRNA-18a is a biomarker for prostate cancer [15], microRNA 25, microRNA145, and microRNA210 for liver cancer [13], and microRNA-200c-141 for lung cancer [16]. While microRNA126 (miRNA126) is involved in the inflammation of the body [17], the occurrence of pediatric highly pathogenic H5N1 influenza is also closely associated with the inflammatory response [18], suggesting that a microRNA might also be a molecular marker for highly pathogenic H5N1 influenza in children. Considering the role of miRNA126 in the regulation of inflammation, whether it plays a role in the diagnosis of H5N1 influenza remains poorly understood. In this study, we aimed to investigate the potential of microRNA126 (miRNA126) as a molecular marker for the early diagnosis of H5N1 influenza, which might provide a theoretical basis for the clinical diagnosis and treatment of the disease.

Methods

Subjects

This study included a total of 16 H5N1 virus-infected children who were treated in The First Affiliated Hospital of Hubei University of Science and Technology between May 2015 and May 2017. The H5N1 infection was confirmed by quantitative reverse transcription PCR (qRT-PCR) as described previously [19, 20]. The patients were given an intravenous injection of cephalosporins (0.03-0.05 g/day) for 3 consecutive weeks. Their serum interleukin-6 (IL-6) levels were measured as described below to determine the treatment effectiveness. Sixteen healthy children were used as controls. The research was approved by the Research Ethics Committee of The First Affiliated Hospital of Hubei University of Science and Technology. All the participants' parents signed an informed consent form before enrollment in the study.

Samples collection

Fasting blood samples (6 mL) were collected from each child before and after the treatment and centrifuged at 800 rpm for 10 min to isolate blood cells and serum which were stored at -20°C until use.

Determination of the serum IL-6 levels

The serum IL-6 levels were determined using a human IL-6 ELISA kit (Neobioscience, Shenzhen, China). The normal range of IL-6 is below 11.8 ng/L. The patients were divided into two subgroups: the mild/moderate H5N1 subgroup, with an IL-6 level between 11.8 and 32.1 ng/L, and the severe H5N1 subgroup, with an IL-6 level above 32.1 ng/L.

PCR analyses

The total RNA in the blood cells was extracted using the Trizol reagent (Takara, Dalian, China). The RNA concentration was determined using a spectrometer by measuring the absorption value of a wavelength of 260 nm. Total RNA was reversely transcribed into cDNA using a reverse transcription kit (Dingguo Biotech., Beijing, China) according to the manual. The level of miRNA126 was measured by PCR using cDNA as a template and the following primers (synthesized by Beijing Sunbiotech Inc.): miRNA126 forward: 5'-GACAATGCTGCGCTATGTGG-3', reverse: 5'-GGCAGGAAAGTGGAAAGTGC-3'; actin forward: 5'-CGGCAACTCAACTGGTGGA-3' and reverse: 5'-GTGGAGTACTGGTGTCAGC-3'. The reaction mixture was prepared: 0.5 μl of cDNA, 1 μl of forward primer, 1 μl of reverse primer, 2 μl of dNTP mixture, 1 μl of reaction buffer, 0.25 μl of Taq enzymes. The reaction conditions were as follows: 95°C for 5 min, followed by 28 cycles of 95°C for 45 s, 57°C for 36 s, and 72°C for 36 s, and 72°C for 7 min. The experiment was performed in triplicate. The PCR products were analyzed using 0.8% agarose gel electrophoresis. The intensity of the bands was determined using the BDA imaging system (Boyue Instruments, China). The gray values of bands were analyzed by Image J 1.8 (National Institutes of Health, USA). The relative expression level of miRNA126 was calculated as its grey value to that of actin.

Statistical analyses

The numeric data was expressed as the mean ± standard deviation and subjected to normality tests. The statistical analysis was performed using SPSS 16.0 (IBM SPSS., Chicago, IL, USA). The differences among groups were compared using ANOVA. The pairwise comparisons were performed using Student's *t*-test. The severity of the influenza was calculated as the IL-6 level of a patient compared to that of the control (11.8 ng/L). The association between the level of miRNA126 and the severity of the H5N1 influenza was analyzed using Pearson's correla-

Table 1. Comparison of the basic information between the H5N1 and control groups (x ± s)

	H5N1 group	Control group	*P* value
Male/female	16/16	16/16	0.96
Age (years)	5.2±1.2	6.2±1.3	0.87
Pre-treatment IL-6 level	32.1±2.4 ng/L	11.8±3.5 ng/L	0.0041
Post-treatment IL-6 level	14.1±1.5 ng/L	10.7±2.6 ng/L	0.79

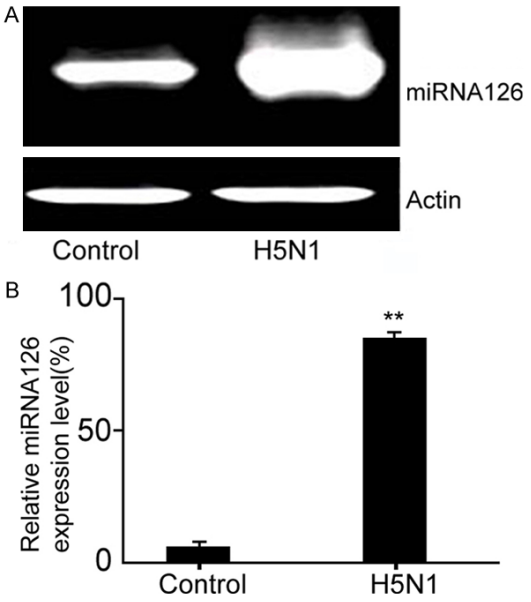

Figure 1. An RT-PCR analysis comparing the pre-treatment miRNA126 expressions in the blood cells between the H5N1 patients and the controls. **, P < 0.01 compared with the control group.

tion test. A multivariate logistic regression analysis was used to analyze the factors affecting the prognosis of the influenza virus. P < 0.05 was considered significantly different.

Results

Basic patient information

This study included a total of 16 H5N1-infected patients with a mean age of 5.2 ± 1.2 years. The mean age of the control group was 6.2 ± 1.3 years. The study subjects' basic information of is listed in **Table 1**. There were no significant differences in age or sex ratio between the two groups (P > 0.05). The pre-treatment IL-6 level in the H5N1 group (32.1 ± 2.4 ng/L) was significantly higher compared with the level in the control group (11.8 ± 3.5 ng/L, P < 0.05), but the post-treatment IL-6 level was similar to the level in the control group (P > 0.05).

Comparison of pre-treatment miRNA126 expressions

The miRNA126 level in the blood cells in the different groups was compared using RT-PCR. As shown in **Figure 1**, the pre-treatment the miRNA126 level in the H5N1 group was significantly higher than it was in the controls (P < 0.01, **Figure 1**). Moreover, the pre-treatment miRNA126 level in the severe H5N1 subgroup was significantly higher compared with the level in mild/moderate H5N1 subgroup (P < 0.05, **Figure 2**).

Comparison of pre- and post-treatment miRNA126 expressions

The treatment with cephalosporin significantly reduced the miRNA126 levels in the H5N1 patients (P < 0.05, **Figure 3**).

Correlation between the miRNA126 level and the severity of H5N1 influenza

The Pearson's correlation analyses showed that the miRNA126 levels in the blood cells were positively correlated with the severity of H5N1 influenza (r = 0.94, P < 0.05, **Figure 4**).

Multivariate regression analysis of the predictive role of miRNA126

A multivariate logistic regression analysis was used to analyze the independent factor (mean IL-6, severe pediatric hyperpathogenic H5N1, pediatric highly pathogenic H5N1 and miRNA126) affecting the prognosis of the influenza virus. The results showed that miRNA126 was an independent factor affecting the prognosis of the influenza virus (**Table 2**). An ROC curve analysis showed that the area under the curve of miRNA126 predicting the MACE of H5N1 (95% CI) was 0.876 (0.828~0.957), the sensitivity was 100.0%, and the specificity was 68.1% (**Figure 5**).

Discussion

The early diagnosis and treatment of pediatric highly pathogenic H5N1 influenza is extremely important [21]. Nevertheless, there is currently no reliable and sensitive biomarker for the effective diagnosis of H5N1 infection in children [22]. We herein evaluated the potential of

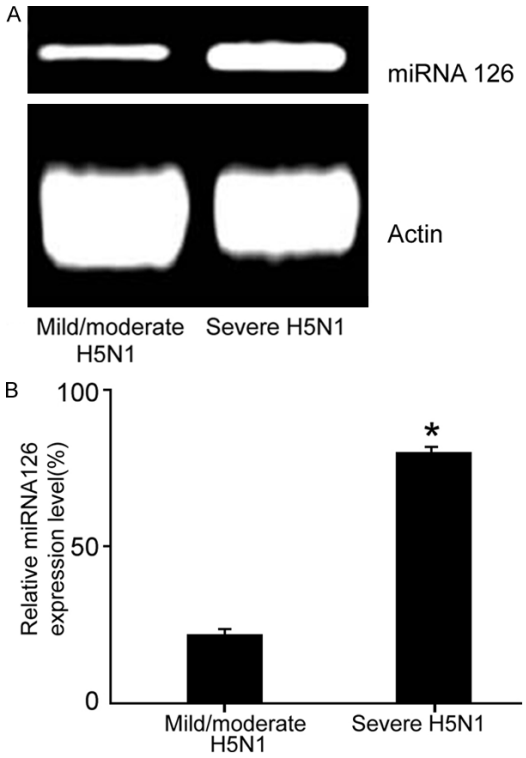

Figure 2. An RT-PCR analysis comparing the pre-treatment miRNA126 expressions in the blood cells between the mild/moderate and the severe H5N1 patients. *, P < 0.05 compared with the mild/moderate H5N1 group.

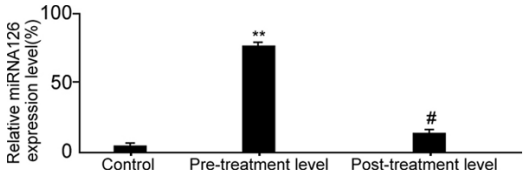

Figure 3. An RT-PCR analysis showing a significantly reduced miRNA126 expression in the H5N1 patients after a 3-week treatment of cephalosporins. **, P < 0.01 compared with the control group; #, P < 0.05 compared with the pre-treatment level.

miRNA126 as a diagnostic biomarker for H5N1 influenza in order to provide a theoretical basis for the clinical diagnosis and treatment of the disease.

Allen et al. have suggested that C-reactive protein (CRP), serum amyloid A (SAA), and procalcitonin (PCT) are potential molecular markers for influenza [22]. Recent studies have shown that microRNA-33a, microRNA-9 and microRNA-650 are closely associated with the occurrence and

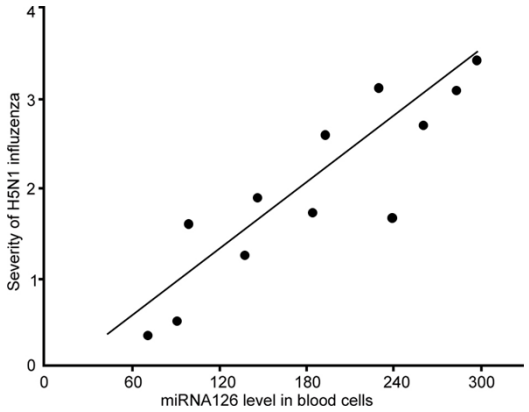

Figure 4. Pearson's correlation test showing a positive correlation between the miRNA126 level in the blood cells and the severity of osteoporosis.

development of type A influenza. The level of these microRNAs is correlated with the severity of the disease, indicating their potentials as biomarkers [23, 24]. In this study, we found that the miRNA126 level in the H5N1 group was significantly higher compared with the level in the control group, but it was significantly reduced after a 3-week treatment with cephalosporins. Moreover, the miRNA126 level in blood cells was positively correlated with the severity of H5N1 influenza, suggesting that miRNA126 might be a diagnostic marker for H5N1 influenza. Our results support previous studies which found that microRNAs are potential biomarkers for influenza.

The drugs for the treatment of H5N1 influenza mainly include benzene alkyl amines (such as verapamil) and neuraminidase inhibitors [25-27]. In the current study, the patients were treated with intravenous injections of cephalosporins for 3 weeks. All the patients were cured at the end of the treatment.

The current study has several limitations. For instance, our study included only 16 patients. No statistical analyses were conducted on the basic information (age, sex ratio, etc.) between the mild/moderate and the severe H5N1 subgroups due to the small sample size. Further studies should be performed on a larger number of cases to validate the diagnostic value of miRNA126 in H5N1 influenza. Additionally, in clinical practice, most pediatric H5N1 patients are treated with medications [28], so whether the miRNA126 level is affected by such treat-

Table 2. Multivariate logistic regression analysis

Parameters	Regression coefficients	SD	Wald	P	OR (95% CI)
miRNA126	0.047	0.018	6.231	0.014	1.067 (1.022-1.084)
Constant	-12.919	2.892	14.23	0.004	

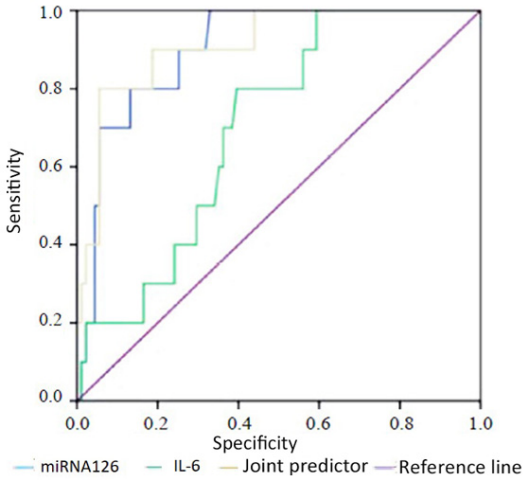

Figure 5. An ROC curve analysis of the specificity and sensitivity of miRNA-126 in the prediction of the MACE of H5N1.

ment must be investigated in the future. Furthermore, the potential of miRNA126 as a molecular target for H5N1 influenza gene therapy should be explored in animal models. Moreover, due to the small sample size in the present study, a large cohort clinical study is required to confirm our findings.

Conclusion

In conclusion, our study has shown a positive correlation between miRNA126 expression and the severity of pediatric H5N1 influenza, suggesting that miRNA126 might be a specific biomarker for the disease.

Disclosure of conflict of interest

None.

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References

[1] Moran J, Ramirez-Martinez G, Jimenez-Alvarez L, Cruz A, Perez-Patrigeon S, Hidalgo A, Orozco L, Martinez A, Padilla-Noriega L, Avila-Moreno F, Cabello C, Granados J, Ortiz-Quintero B, Ramirez-Venegas A, Ruiz-Palacios GM, Zlotnik A, Merino E and Zuniga J. Circulating levels of miR-150 are associated with poorer outcomes of A/H1N1 infection. Exp Mol Pathol 2015; 99: 253-61.

[2] Vijay R, Hua X, Meyerholz DK, Miki Y, Yamamoto K, Gelb M, Murakami M and Perlman S. Critical role of phospholipase A2 group IID in age-related susceptibility to severe acute respiratory syndrome-CoV infection. J Exp Med 2015; 212: 1851-68.

[3] Wu Z, Hao R, Li P, Zhang X, Liu N, Qiu S, Wang L, Wang Y, Xue W, Liu K, Yang G, Cui J, Zhang C and Song H. MicroRNA expression profile of mouse lung infected with 2009 pandemic H1N1 influenza virus. PLoS One 2013; 8: e74190.

[4] Li Y, Chan EY, Li J, Ni C, Peng X, Rosenzweig E, Tumpey TM and Katze MG. MicroRNA expression and virulence in pandemic influenza virus-infected mice. J Virol 2010; 84: 3023-32.

[5] Park KJ, Song MS, Kim EH, Kwon HI, Baek YH, Choi EH, Park SJ, Kim SM, Kim YI, Choi WS, Yoo DW, Kim CJ and Choi YK. Molecular characterization of mammalian-adapted Korean-type avian H9N2 virus and evaluation of its virulence in mice. J Microbiol 2015; 53: 570-7.

[6] Guo J, Huang F, Liu J, Chen Y, Wang W, Cao B, Zou Z, Liu S, Pan J, Bao C, Zeng M, Xiao H, Gao H, Yang S, Zhao Y, Liu Q, Zhou H, Zhu J, Liu X, Liang W, Yang Y, Zheng S, Yang J, Diao H, Su K, Shao L, Cao H, Wu Y, Zhao M, Tan S, Li H, Xu X, Wang C, Zhang J, Wang L, Wang J, Xu J, Li D, Zhong N, Cao X, Gao GF, Li L and Jiang C. The serum profile of hypercytokinemia factors identified in H7N9-infected patients can predict fatal outcomes. Sci Rep 2015; 5: 10942.

[7] Wang Z, Wan Y, Qiu C, Quinones-Parra S, Zhu Z, Loh L, Tian D, Ren Y, Hu Y, Zhang X, Thomas PG, Inouye M, Doherty PC, Kedzierska K and Xu J. Recovery from severe H7N9 disease is associated with diverse response mechanisms dominated by CD8(+) T cells. Nat Commun 2015; 6: 6833.

[8] Li C, Jiao S, Wang G, Gao Y, Liu C, He X, Zhang C, Xiao J, Li W, Zhang G, Wei B, Chen H and Wang H. The immune adaptor ADAP regulates reciprocal TGF-beta1-integrin crosstalk to protect from influenza virus infection. PLoS Pathog 2015; 11: e1004824.

[9] Lin SJ, Lo M, Kuo RL, Shih SR, Ojcius DM, Lu J, Lee CK, Chen HC, Lin MY, Leu CM, Lin CN and

Tsai CH. The pathological effects of CCR2+ inflammatory monocytes are amplified by an IFNAR1-triggered chemokine feedback loop in highly pathogenic influenza infection. J Biomed Sci 2014; 21: 99.
[10] Yang P, Gu H, Zhao Z, Wang W, Cao B, Lai C, Yang X, Zhang L, Duan Y, Zhang S, Chen W, Zhen W, Cai M, Penninger JM, Jiang C and Wang X. Angiotensin-converting enzyme 2 (ACE2) mediates influenza H7N9 virus-induced acute lung injury. Sci Rep 2014; 4: 7027.
[11] Liu B, Zhang X, Deng W, Liu J, Li H, Wen M, Bao L, Qu J, Liu Y, Li F, An Y, Qin C, Cao B and Wang C. Severe influenza A(H1N1)pdm09 infection induces thymic atrophy through activating innate CD8(+)CD44(hi) T cells by upregulating IFN-gamma. Cell Death Dis 2014; 5: e1440.
[12] Cerbulo-Vazquez A, Figueroa-Damian R, Arriaga-Pizano LA, Hernandez-Andrade E, Mancilla-Herrera I, Flores-Mejia LA, Arteaga-Troncoso G, Lopez-Macias C, Isibasi A and Mancilla-Ramirez J. Pregnant women infected with pandemic H1N1pdm2009 influenza virus displayed overproduction of peripheral blood CD69+ lymphocytes and increased levels of serum cytokines. PLoS One 2014; 9: e107900.
[13] Shi SB, Wang M, Tian J, Li R, Chang CX and Qi JL. MicroRNA 25, microRNA 145, and microRNA 210 as biomarkers for predicting the efficacy of maintenance treatment with pemetrexed in lung adenocarcinoma patients who are negative for epidermal growth factor receptor mutations or anaplastic lymphoma kinase translocations. Transl Res 2016; 170: 1-7.
[14] Makkoch J, Poomipak W, Saengchoowong S, Khongnomnan K, Praianantathavorn K, Jinato T, Poovorawan Y and Payungporn S. Human microRNAs profiling in response to influenza A viruses (subtypes pH1N1, H3N2, and H5N1). Exp Biol Med (Maywood) 2016; 241: 409-20.
[15] Al-Kafaji G, Al-Naieb ZT and Bakhiet M. Increased oncogenic microRNA-18a expression in the peripheral blood of patients with prostate cancer: a potential novel non-invasive biomarker. Oncol Lett 2016; 11: 1201-1206.
[16] Li XY, Li H, Bu J, Xiong L, Guo HB, Liu LH and Xiao T. Prognostic role of microRNA-200c-141 cluster in various human solid malignant neoplasms. Dis Markers 2015; 2015: 935626.
[17] Sun C, Alkhoury K, Wang YI, Foster GA, Radecke CE, Tam K, Edwards CM, Facciotti MT, Armstrong EJ, Knowlton AA, Newman JW, Passerini AG and Simon SI. IRF-1 and miRNA126 modulate VCAM-1 expression in response to a high-fat meal. Circ Res 2012; 111: 1054-64.
[18] Schneider C, Nobs SP, Heer AK, Kurrer M, Klinke G, van Rooijen N, Vogel J and Kopf M. Alveolar macrophages are essential for protection from respiratory failure and associated morbidity following influenza virus infection. PLoS Pathog 2014; 10: e1004053.
[19] Yehia N, Arafa AS, Abd El Wahed A, El-Sanousi AA, Weidmann M and Shalaby MA. Development of reverse transcription recombinase polymerase amplification assay for avian influenza H5N1 HA gene detection. J Virol Methods 2015; 223: 45-9.
[20] Zhu Z, Fan H, Qi X, Qi Y, Shi Z, Wang H, Cui L and Zhou M. Development and evaluation of a SYBR green-based real time RT-PCR assay for detection of the emerging avian influenza A (H7N9) virus. PLoS One 2013; 8: e80028.
[21] Rodrigue-Gervais IG, Labbe K, Dagenais M, Dupaul-Chicoine J, Champagne C, Morizot A, Skeldon A, Brincks EL, Vidal SM, Griffith TS and Saleh M. Cellular inhibitor of apoptosis protein cIAP2 protects against pulmonary tissue necrosis during influenza virus infection to promote host survival. Cell Host Microbe 2014; 15: 23-35.
[22] Allen JE, Gardner SN, Vitalis EA and Slezak TR. Conserved amino acid markers from past influenza pandemic strains. BMC Microbiol 2009; 9: 77.
[23] Hu Y, Jiang L, Lai W, Qin Y, Zhang T, Wang S and Ye X. MicroRNA-33a disturbs influenza A virus replication by targeting ARCN1 and inhibiting viral ribonucleoprotein activity. J Gen Virol 2016; 97: 27-38.
[24] Pichulik T, Khatamzas E, Liu X, Brain O, Delmiro Garcia M, Leslie A, Danis B, Mayer A, Baban D, Ragoussis J, Weber AN and Simmons A. Pattern recognition receptor mediated downregulation of microRNA-650 fine-tunes MxA expression in dendritic cells infected with influenza A virus. Eur J Immunol 2016; 46: 167-77.
[25] Sriwilaijaroen N, Suzuki K, Takashita E, Hiramatsu H, Kanie O and Suzuki Y. 6SLN-lipo PGA specifically catches (coats) human influenza virus and synergizes neuraminidase-targeting drugs for human influenza therapeutic potential. J Antimicrob Chemother 2015; 70: 2797-809.
[26] Balannik V, Wang J, Ohigashi Y, Jing X, Magavern E, Lamb RA, Degrado WF and Pinto LH. Design and pharmacological characterization of inhibitors of amantadine-resistant mutants of the M2 ion channel of influenza A virus. Biochemistry 2009; 48: 11872-82.
[27] Gasparini R, Amicizia D, Lai PL, Bragazzi NL and Panatto D. Compounds with anti-influenza activity: present and future of strategies for the optimal treatment and management of influenza. Part II: future compounds against influenza virus. J Prev Med Hyg 2014; 55: 109-29.
[28] Van Zandt MC, Jagdmann GE, Whitehouse D, Ji MK, Savoy J, Potapova O, Cousido-Siah A, Mitschler A, Howard EI, Pyle AM and Podjarny AD. Discovery of N-substituted 3-amino-4-(3-boronopropyl)pyrrolidine-3-carboxylic acids as highly potent third generation inhibitors of human arginase I and II. J Med Chem 2019; 62: 8164-8177.