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Repressing PU.1 by miR-29a* in NK cells of HCV patients, diminishes its cytolytic effect on HCV infected cell models

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ABSTRACT

Objectives: Natural killer cells are immune safeguards against HCV infection. PU.1 is a pivotal transcription factor in the development of NK cells. This study aimed at studying the regulatory effect of miRNAs on both development and function of NK cells isolated from HCV patients.
Methods: NK cells were isolated from 17 chronic HCV patients and 12 healthy controls; after which miRNA and mRNA were quantified using qRT-PCR. Manipulating miRNA expression using mimics and antagomirs, was performed followed by investigating downstream targets as well as viral abundance.
Results: PU.1 expression levels were upregulated in NK cells of HCV patients. In silico analysis revealed PU.1 to be a potential downstream target of miR-29a*, where miR-29a* overexpression in NK cells caused a significant downregulation in PU.1 mRNA. Forcing miR-29a* caused a downregulation of the cytotoxicity determinant NK activating receptor (NKG2D) via upregulation of miR-155. Moreover, perforin-1 mRNA was found to be downregulated upon forcing the expression of miR-29a* in NK cells of HCV patients. This decrease in NK cytolytic function was accompanied by an 80% viral load increase in cocultured HCVcc cell models.
Conclusions: This study showed that HCV infection might abrogate NK cytotoxic potential through altering PU.1, NKG2D receptor and perforin molecules.

1. Introduction

Natural killer cells (NK cells) are large granular lymphocytes that possess a crucial role in the interplay between innate and adaptive immune systems. They are immune effector cells that are highly efficient in recognizing and killing virally infected cells [1–3]. Their function is basically either performing direct spontaneous cytotoxic effect by the release of granules containing perforin and granzymes, or by being able to secrete antiviral cytokines such as IFN γ or TNF α [4–6]. The function of NK cells is not a simple "on–off" switch; it is rather a balance between inhibitory and activating receptors [7]. Inhibitory receptors help maintain immunological homeostasis as they abort NK cell function towards normal healthy cells such as killer immunoglobulin receptors (KIRs), while activating receptors such as NK cytotoxicity

Abbreviations: B2m, beta-2-microglobulin; CO_2, carbon dioxide; CT, cycle threshold; DMEM, Dulbecco's modified eagle's medium; EDTA, ethylenediamine tetra acetic acid; Ets, Ezb transformation-specific sequence; FBS, fetal bovine serum; HCV, hepatitis C virus; HCVcc, cell culture derived HCV particles; hsa, homosapiens; HSC, hematopoietic stem cells; IFN γ, interferon gamma; KIR, killer cell immunoglobulin-like receptor; KLRK1, killer cell lectin-like receptor subfamily K, member 1; MACS, magnetic-activated cell sorting; miRNA/miR, microRNA; ml, milliliter; mRNA, messenger RNA; NCR, Natural Cytotoxicity Receptor; NK, natural killer; NKG2D, natural killer cell receptor G2D; NKPs, natural killer precursors; PBMCs, Peripheral Blood Mononuclear Cells; PCR, polymerase chain reaction; Prf1, perforin; PU.1, purine rich box-1; qRT-PCR, quantitative real-time reverse transcription PCR; RNA, ribonucleic acid; RPMI, Roswell park memorial institute medium; RQ, relative quantification; Spi1, spleen focus-forming virus integration site-1; TGF-β, transforming growth factor β; TNF α, tumor necrosis factor α; UTR, untranslated region; μm, micrometer.

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receptors (NCRs) and NKG2D receptor recognize specific ligands that are expressed on stressed, transformed or infected cells [8,9].

Purine-rich box 1 (PU.1), a member of the Ets family of transcription factors, has a vital role in the commitment of hematopoietic stem cells (HSCs) to become the first precursor for NK cells [10,11]. PU.1 affects all lymphocyte lineages as well as certain myeloid cells [12]. The lymphoid defect in PU.1-deficient mice can be traced to deficiencies in expression of the IL-7Rα chain and of the Flt3 receptor [13,14], which are crucial for B and T cell development [15]. PU.1-deficient HSC were reported to generate a reduction in the numbers of natural killer precursors (NKPs) and consequently a diminished peripheral NK cell pool, although the phenotype and cytotoxic activity of residual PU.1-deficient NK cells was largely conserved [16].

On the other hand, miRNAs were reported to regulate NK cell function [17,18]. MicroRNAs (miRNAs), are small 22 nucleotides noncoding RNA molecules that play a pivotal role in regulating expression of various genes thus affecting several physiological and pathological conditions [19–22]. The role of miRNAs in regulating NK cell function has been previously investigated, where miR-150 was reported to cause a significant reduction in the number of peripheral, mature NK cells in mice, via a developmental blockage in NK cell maturation [23]. Moreover, miR-181 was suggested to play a critical role in NK cell development by inhibiting Notch signaling [24–26]. miR-27a* (miR-27a-5p) was found to target the 3′UTRs of both GzmB and Prf1, key players of NK cell cytotoxicity. In addition, miR-29a/b/c family has been reported to regulate the IFN-γ pathway [27–29].

However, the impact of PU.1 and microRNAs on NK cells during the course of HCV infection has never been investigated before. Hence, this study aimed at studying the regulatory effect of miRNAs on the development of NK cells through targeting PU.1 and its sequential impact on the functional status of NK cells of HCV patients.

2. Materials and methods

2.1. Peripheral Blood Mononuclear Cells (PBMCs) and NK cell isolation

8 ml of peripheral venous blood were withdrawn from 17 chronic HCV patients and 12 healthy controls. All patients were negative for the hepatitis B surface antigen and treatment naïve. Both patients and controls included in this study gave their written informed consent. All experiments were performed in compliance with the guidelines of the Institutional Review Board of Kasr El Aini Medical School in Cairo University and in accordance with the ethical standards of the declaration of Helsinki.

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from whole blood samples using Ficoll density gradient centrifugation method. 15×10^6 PBMCs were used as a starting count for negative untouched NK cell selection using MACS human NK cell isolation kit (Miltenyi Biotec, USA).

2.2. Culture, transfection, total RNA extraction and quantification using qRT-PCR

NK cells were cultured in complete RPMI medium supplemented with L-glutamine (Lonza, Belgium), containing 5% fetal bovine serum (FBS; Lonza, Belgium) and 1% Penicillin/Streptomycin. Transfection of NK cells was performed using HiPerfect Transfection Reagent (Qiagen, Germany) with miR-29a* and miR-155 mimics (MSY0004503 and MSY0000646, respectively) and inhibitors (MIN0004503 and MIN0000646, respectively) (Qiagen, Germany). 48 h post transfection, total RNA was extracted using Biozol (BIOR, China), then reversely transcribed and quantified using Real time PCR (StepOne, Applied Biosystems, USA). Beta-2-microglobulin (B2m) was used as housekeeping gene for relative quantitation for mRNA while RNU6B (Catalog # 4427975) was used as housekeeping for relative quantitation for miRNA. The expression of gene or miRNA in patients was normalized to the average expression of those in healthy controls.

2.3. Huh7 HCVcc cell models

Huh7 cell lines were grown at 37 °C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium supplemented with L-glutamine (DMEM; Lonza, Belgium), containing 10% fetal bovine serum (FBS; Lonza, Belgium) and 1% Penicillin/Streptomycin. The HCV construct chimeric ED43/JFH1 full length genome was generously provided by Prof. Jens Buch, where it was first linearized using XbaI restriction enzyme (Thermo-scientific, USA), and then purified using phenol/chloroform extraction. The linearized DNA vector was then subjected to in vitro transcription using T7 MEGAscript Kit (Ambion-Life Technologies, USA) to obtain RNA. Huh7 cells were transfected with 10 μg of viral RNA using SuperFect Transfection Reagent (Qiagen, Germany). 3 days post transfection, the supernatant was collected and filtered using 0.45 μm filter and was used for further infection.

2.4. Coculture of NK cells with HCV-infected Huh7 cell lines

1×10^4 ED43/JFH-1 chimeric HCV harboring-Huh7 cells were seeded the day before co-culture in a 96-well plate in complete DMEM medium and incubated under normal growth conditions (37 °C, 5% CO_2). NK cells either untransfected, or miR-29a* mimicked or miR-29a* antagonized, were pelleted after 48 h of transfection and then resuspended in complete RPMI medium, free from any transfection complex. These NK cells were then added on HCVcc cells, after removal of the complete DMEM placed the day before. The number of NK cells to be added depends on the effector-to-target ratio (E:T), where 1:1 and 4:1 ratios were implemented. After 24 h of coculture, HCV viral RNA was extracted and quantified.

2.5. HCV RNA extraction and quantification

HCV viral RNA was extracted using HCV RNA extraction Kit (Invisorb Spin Virus RNA Mini kit, Germany) according to manufacturer's protocol. Viral Titers were quantified using HCV-Genesig Onstep Quantification kit (Primer design Ltd., UK). Four Standards of known HCV quantity were added to each run to quantify copy number in each sample.

2.6. Luciferase reporter assay

To confirm the binding of miR-29a* to PU.1 3′UTR and miR-155 to NKG2D 3′UTR, firefly luciferase reporter construct was used (pmirGLO) (Promega, USA). pmirGLO was double digested using XbaI and SacI enzymes (Thermoscientific, USA), after which the wild or mutant insert types were ligated using T4 Ligase (Takara Shuzo Co., Ltd, Japan). Forward and reverse primers' sequences for each target (Table 1) were designed, where the type of mutation applied was deletion of the full binding site. Huh7 cells were transfected with empty pmirGLO vector, pmirGLO harboring wild type or pmirGLO harboring mutant target region, using Superfect transfection reagent (Qiagen, Germany). After 24 h, cells were either kept untransfected or transfected with miR-29a* or miR-155 mimics using Hiperfect transfection reagent (Qiagen, Germany). Luciferase activity was measured 48 h post-

Table 1
Designed primers for confirmation of miR-29a*/miR-155 to their respective targets.

	miR-29a* – PU.1	miR-155 – NKG2D
Wild type primers	Forward: 5′ CTGGGA GTCTCAAGTC CGTA TGTAAATCAG ATCTCCCCT 3′ Reverse: 5′ CTAGAGGGGAGATCTG ATTT ACATACG GACTT GAGACTCCC AG AGCT 3′	Forward: 5′ CGTGG GC CATGAT TATCTTA AAGG CA TTAT TCTC CAGT 3′ Reverse: 5′ CTAGACTGGAG AATAATGCCTTT AAGATAATCATGGCCCACGAGCT 3′
Mutant type primers	Forward: 5′ CTGGGA GTCTCAA GTCC GTA TGTATCTCCCCT 3′ Reverse: 5′ CTAGAG GGGAGATA CATACGGA CTTGAGACTCCCAGAGCT 3′	Forward: 5′ CGTGGG CCATGATTT AAAGTTCTCCAGT 3′ Reverse: 5′ CTAGACT GGAGAAC TTTAAATCA TGGCC CACGAGCT 3′

Forward and reverse designed primers for luciferase reporter assay for target sites of miR-29a* on PU.1 3′UTR and miR-155 on NKG2D 3′UTR, to be used in binding confirmation experiments.

transfection using the Steady-Glo luciferase reporter assay kit (Promega, USA).

2.7. Statistical analysis

The relative quantification of the results obtained by real-time qRT-PCR was performed using $2^{-\Delta\Delta CT}$. miRNA/mRNA expression were compared using the Mann–Whitney and firefly luciferase readings were compared using Student's unpaired *t*-test. A *p*-value less than 0.05 was considered statistically significant. Analysis was performed using the GraphPad Prism 5.00 software.

3. Results

3.1. PU.1 expression profiling in NK cells of chronic HCV patients

In order to explore the effect of HCV on the PU.1 transcription factor, PU.1 mRNA expression was assessed in NK cells of HCV-infected patients as well as healthy controls, where it was found be significantly upregulated in NK cells of HCV patients ($p = 0.0028$) (Fig. 1A).

3.2. Expression profiling of miR-29a*, potential upstream miRNA to PU.1

In silico analysis was carried out to predict potential miRNAs that might target PU.1 mRNA, using the miRanda software (www.microrna.org). hsa-miR-29a* (hsa-miR-29a-5p) was selected due to its high binding score to PU.1 gene (or so called SPI1). miR-29a* expression was assessed in NK cells of HCV-infected patients as well as healthy controls, where it was found to be significantly upregulated in NK cells of HCV patients ($p = 0.0357$) (Fig. 1B).

3.3. Impact of miR-29a* on PU.1 and NKG2D mRNA expression in NK cells of chronic HCV patients

To further characterize the impact of miR-29a* on PU.1 mRNA expression, NK cells from HCV patients were transfected with miR-29a* mimics and antagomirs. 48 h post transfection; total RNA was extracted and quantified using real time qRT-PCR. First, transfection efficiency was checked after each experiment, where NK cells transfected with miR-29a* mimics showed 9900 fold increase in the expression of miR-29a* compared to untransfected NK cells ($p < 0.001$) (Fig. 2A).

PU.1 expression was assessed in miR-29a* mimicked and antagonized NK cells of HCV-infected patients, where a significant downregulation in PU.1 expression was observed in miR-29a* mimicked compared to untransfected NK cells ($p = 0.0312$). Additionally, miR-29a* antagonized NK cells exhibited a significant upregulation in the PU.1 mRNA expression ($p = 0.0161$) (Fig. 2B).

In an attempt to investigate the impact of miR-29a* on NKG2D expression, NKG2D expression was assessed in miR-29a* mimicked and antagonized NK cells of HCV-infected patients. It was found that miR-29a* mimics significantly decreased NKG2D mRNA expression compared to untransfected cells ($p = 0.0337$). On the other hand, miR-29a* antagonized cells showed a significant upregulation in NKG2D mRNA expression ($p = 0.0112$) (Fig. 2C). However, in silico analysis revealed that miR-29a* does not bind to the 3′UTR of NKG2D gene (KLRK1), hence this alteration in expression is due to an indirect messenger.

3.4. In silico analysis of miR-155 and NKG2D

During the search whether miR-29a* might bind to the NKG2D mRNA using several bioinformatic softwares (miRWalk, miRanda), miR-155 was suggested to bind to NKG2D gene (KLRK1).

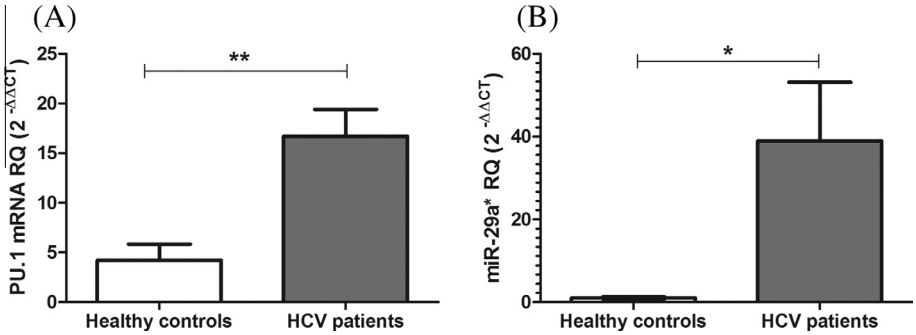

Fig. 1. Expression profiling of miR-29a* and its downstream target PU.1 in NK cells of HCV patients. (A) A significant upregulation was observed in PU.1 expression levels in NK cells of HCV patients ($p = 0.0028$). (B) miR-29* was found to be significantly upregulated in the expression in NK cells of HCV patients ($p = 0.0357$).

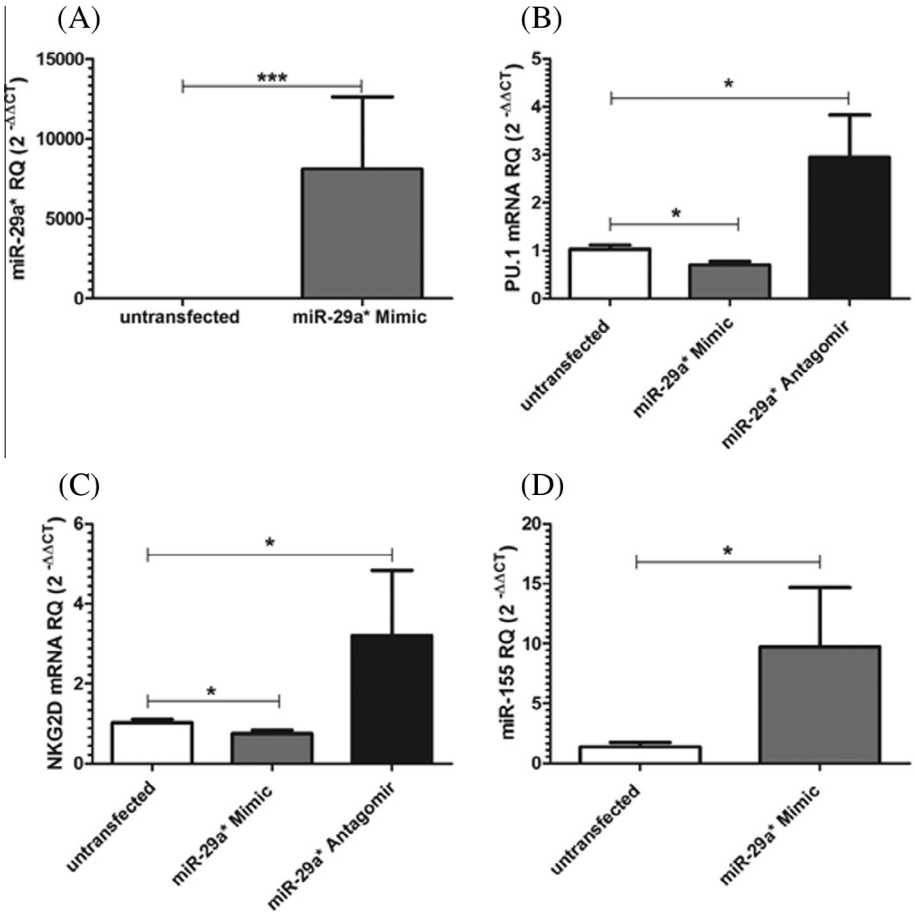

Fig. 2. Impact of miR-29a* on PU.1, NKG2D mRNA and miR-155 expression in NK cells of HCV patients. (A) To confirm transfection, 9900 fold increase in the expression level of miR-29a* was observed upon mimicking with miR-29a* compared to untransfected NK cells. (B) A significant downregulation in the PU.1 expression was observed in miR-29a* mimicked NK cells compared to the untransfected NK cells ($p = 0.0312$). miR-29a* antagonized NK cells exhibited a significant upregulation in the PU.1 mRNA expression levels ($p = 0.0161$). (C) A significant downregulation in the expression level of NKG2D was observed in miR-29a* mimicked NK cells compared to the untransfected NK cells ($p = 0.0337$). Additionally, miR-29a* antagonized NK cells exhibited a significant upregulation in the NKG2D mRNA expression levels ($p = 0.0112$). (D) Upon forcing the expression of miR-29a*, miR-155 expression was significantly upregulated compared to untransfected NK cells ($p = 0.0281$).

Additionally, according to previous studies, PU.1 was validated to act as transcriptional activator for miR-155 [30,31]. Hence, miR-155 might be the intermediate between PU.1 and NKG2D.

3.5. Impact of forcing miR-29a* on miR-155 expression in NK cells of chronic HCV patients

As a first attempt to investigate if miR-155 is the messenger between PU.1 and NKG2D, miR-155 was assessed upon forcing the expression of miR-29a* and thus manipulating PU.1. Interestingly, upon forcing the expression of miR-29a* in NK cells of HCV-infected patients, miR-155 expression showed a significant upregulation compared to untransfected cells ($p = 0.0281$) (Fig. 2D).

3.6. miR-155 and NKG2D expression in NK cells of chronic HCV patients

miR-155 baseline expression level was quantified in NK cells of HCV-infected patients as well as healthy controls. A significant upregulation was observed in NK cells of HCV-infected patients compared to healthy controls ($p = 0.0089$) (Fig. 3A). On the contrary, a significant downregulation was observed in NKG2D expression in NK cells of HCV patients compared to healthy controls ($p = 0.0081$) (Fig. 3B).

3.7. Impact of miR-155 on NKG2D mRNA expression in NK cells of chronic HCV patients

As suggested by bioinformatic analysis, NKG2D is a potential downstream target to miR-155. Therefore, NK cells of HCV-infected patients were transfected with miR-155 mimics and antagomirs. miR-155 mimicked NK cells showed a significantly downregulation in NKG2D mRNA compared to untransfected cells ($p = 0.0411$). On the other hand, miR-155 antagonized NK cells showed a significant upregulation in NKG2D mRNA expression ($p = 0.0476$) (Fig. 3C).

3.8. Luciferase reporter assay for 3′UTR of PU.1 gene (SPI1) and 3′UTR of NKG2D gene (KLRK1)

To confirm the direct effect of miR-29a* on PU.1, luciferase reporter assay was performed using pmirGlo vector. As shown in Fig. 4A, miR-29a* mimics led to 94% reduction in firefly luciferase activity in Huh7 cells transfected with pmirGLO harboring wild type PU.1 3'UTR when compared to untransfected cells ($p = 0.0073$). However, this percentage reduction was not observed in miR-29a* mimicked cells transfected with pmirGLO harboring mutant PU.1 3′UTR ($p = 0.2770$) (Fig. 4A). Regarding miR-155 possible binding to NKG2D, miR-155 mimics led to 70% reduction in firefly luciferase activity in Huh7 cells transfected with pmirGLO harboring wild type NKG2D 3'UTR when compared to untrans-

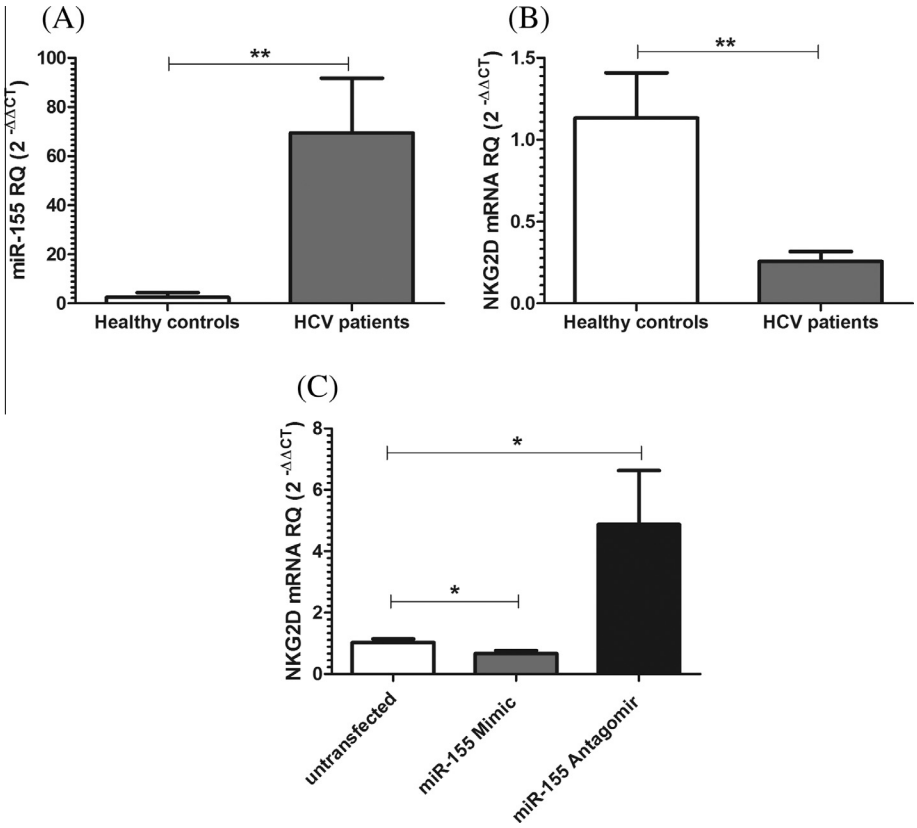

Fig. 3. Expression profiling of miR-155 and its downstream target NKG2D and its impact on NKG2D mRNA expression in NK cells of HCV patients. (A) The levels of miR-155 were significantly upregulated in NK cells of HCV-infected patients ($p = 0.0089$). (B) A significant downregulation in the expression level of NKG2D mRNA was observed in NK cells of HCV patients compared to healthy controls ($p = 0.0081$). (C) A significant downregulation in the expression level of NKG2D was observed in miR-155 mimicked NK cells compared to the untransfected NK cells ($p = 0.0411$). Additionally, miR-155 antagonized NK cells exhibited a significant upregulation in the NKG2D mRNA expression levels ($p = 0.0476$).

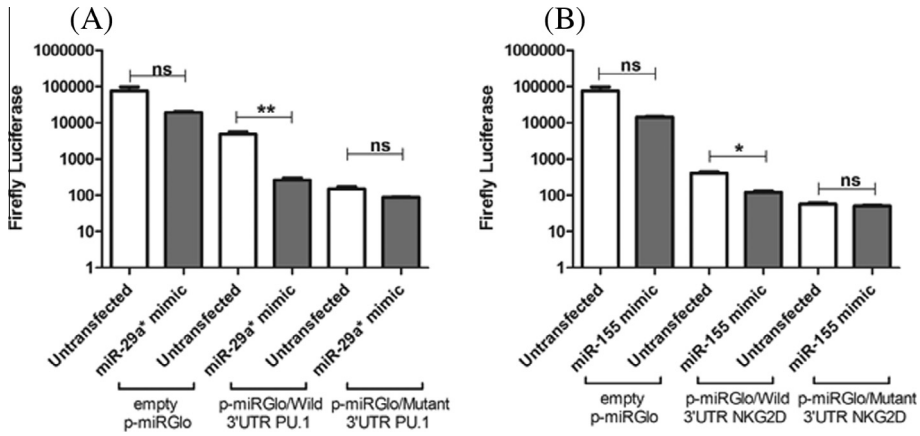

Fig. 4. Luciferase reporter assay for binding confirmation of miR-29a* to PU.1 3′UTR and of miR-155 to NKG2D 3′UTR. (A) Upon co-transfecting Huh7 cells with miR-29a* mimics and pmirGLO harboring wild type target site of miR-29a* on PU.1 3′untranslated region (3′UTR), the luciferase activity decreased by 94% compared to untransfected Huh7 cells. This percentage reduction was not shown in cells transfected with mutant type pmirGLO vector and miR-29a* mimics. (B) Upon co-transfecting Huh7 cells with miR-155 mimics and pmirGLO harboring wild type target site of miR-155 on NKG2D 3′untranslated region (3′UTR), the luciferase activity decreased by 70% compared to untransfected Huh7 cells. This percentage reduction was not shown in cells transfected with mutant type pmirGLO vector and miR-155 mimics.

fected cells ($p = 0.0269$). This percentage reduction was also not observed in miR-29a* mimicked cells transfected with pmirGLO harboring mutant NKG2D 3′UTR ($p = 0.2666$) (Fig. 4B).

3.9. Impact of miR-29a* on perforin (Prf-1) mRNA in NK cells of HCV patients

In order to assess whether miR-29a* affects cytotoxicity of NK cells, the pore-forming protein perforin (Prf-1) mRNA expression was assessed in miR-29a* mimicked and antagonized NK cells, where Prf-1 mRNA expression was found to be significantly downregulated in miR-29a* mimicked NK cells compared to untransfected NK cells ($p = 0.0262$) (Fig. 5A).

3.10. Impact of miR-29a* transfection on NK cell cytotoxicity

It was crucial to investigate the impact of the previous manipulations on NK cell cytolytic activity. NK cells were cocultured with

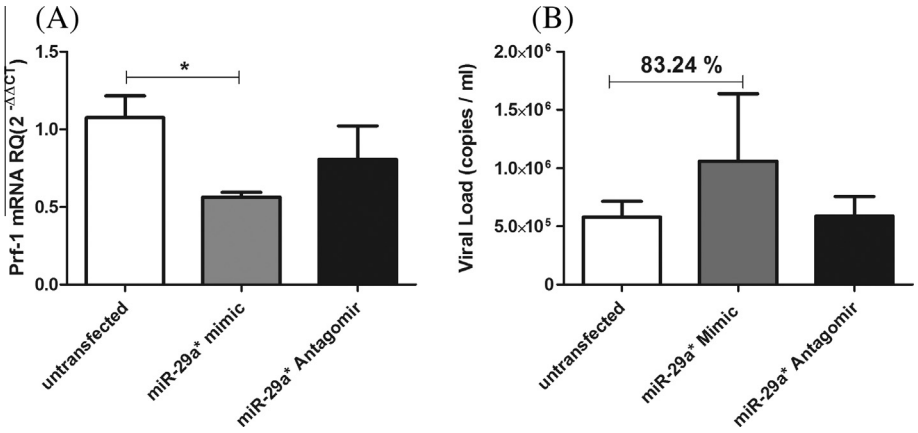

Fig. 5. Impact of manipulating the expression of miR-29a* on NK cell cytolytic effect on target cells. (A) A significant downregulation in the expression level of Prf-1 was observed in miR-29a* mimicked NK cells compared to the untransfected NK cells ($p = 0.0262$). (B) After forcing the expression of miR-29a* in NK cells of HCV patients, NK cells were cocultured with HCVcc Huh7 cell lines. After 24 h, viral RNA was extracted and quantified using qRT-PCR. Cytotoxic effect of NK cells that overexpress miR-29a* was reduced as seen by an 83% increase in the viral load in HCVcc cells, irrespective of E:T ratio.

ED43/JFH-1 chimeric HCVcc Huh7 cell lines, where after 24 h of coculture HCV viral RNA was quantified to test the cytotoxic effect of NK cells on these infected cells. Viral load represented by copies ml^{-1} showed 83% fold increase in infected Huh7 cells cocultured with miR-29a* mimicked NK cells compared to those cocultured with untransfected NK cells, irrespective of E:T ratio (Fig. 5B).

4. Discussion

Hepatitis C virus infection is considered as a major burden that affects approximately 160–170 million people (2–3% of the population) across the globe [32,33], with a high prevalence in Egypt up to 22% [34]. NK cells are crucial players in the line of defense against viral infection; however a line of evidence exists regarding its dysregulated function during the course of HCV infection [35]. This study aimed at exploring status of NK cells in HCV infection. PU.1 is a vital transcriptional factor in development of NK cells and other lymphoid cells [10–12]. PU.1 expression was investigated in this study by obtaining NK cells from HCV infected patients and its screening revealed its expression to be significantly upregulated in HCV patients compared to healthy controls (Fig. 1A). This is the first reported expression profiling of PU.1 in NK cells of HCV patients. PU.1 was previously reported to be upregulated in liver biopsies of HCV patients [36] but never reported before in NK cells. PU.1 has been previously shown to act mainly at natural killer precursor (NKP) stage, which is the first step of the developmental process of a NK cell [16,37]. The dysregulation of PU.1 in NK cells of HCV patients might suggest that HCV infection could affect development of NK cells through manipulating transcription factors and hence might alter their potential to perform any cytolytic activity. miRNAs are chief regulators of gene expression, in addition to being previously proven to regulate NK cell development and function [17–19]. Thus, in an attempt to correct the expression of PU.1 in HCV, in silico analysis was performed to propose a miRNA that could target PU.1 mRNA. miR-29a* was suggested to bind to the PU.1 3′UTR with high binding score. Since miR-29a* was never investigated before in HCV infection, it was crucial to assess its expression in NK cells of HCV patients. miR-29a* was found to be upregulated in NK cells of HCV patients compared to healthy controls (Fig. 1B). Furthermore, upon forcing the expression of miR-29a*, PU.1 mRNA expression was found to be significantly downregulated compared to untransfected mock cells (Fig. 2B). This was further confirmed by luciferase assay reporter where miR-29a* was proven to bind to PU.1 3′UTR (Fig. 4A). This might suggest that the upregulation of PU.1 mRNA expression in NK cells of HCV patients is a compensatory mechanism to counteract the impact of miR-29a* on PU.1 expression.

To assess the NK cell cytolytic function, NKG2D activating receptor was reported to play a vital role in the regulation of NK cell cytotoxicity [38–40]. NKG2D expression profiling in HCV infection was subjected to controversy in previous studies. Thus, it was important to investigate its expression in NK cells of chronic HCV patients; where it was found to be significantly downregulated compared to healthy controls (Fig. 3B). This goes in agreement with several studies which showed a similar downregulation in NKG2D expression of chronic HCV patients [41–43], while it goes in conflict with another study by Amadei et al.; where an upregulation in NKG2D expression was reported. This contradicting expression pattern compared to our results might owe to the nature of the patients studied; where the latter study focused on studying NKG2D expression in acute HCV infected patients [44], while this study was conducted on chronic HCV patients.

Accordingly, miR-29a* was investigated whether it might have any regulatory effect on NKG2D mRNA expression. Forcing the expression of miR-29a* caused a downregulation of NKG2D mRNA expression, while antagonizing miR-29a* resulted in an upregulation in NKG2D mRNA expression (Fig. 2C). However, we could not predict any in silico binding between miR-29a* and NKG2D by several softwares (miRWalk and miRanda), which might suggest that the mechanism of miR-29a* could be indirect, via an intermediate messenger.

Among the suggested upstream miRNAs by bioinformatic analysis that could bind to NKG2D mRNA is miR-155. miR-155 has been previously validated as a downstream target for PU.1, where PU.1 was shown to be a transcriptional activator of miR-155 [31]. miR-155 expression was never investigated in NK cells of HCV patients, and hence its expression was assessed where it showed significant upregulation compared to healthy controls (Fig. 3A). This drove our interest to investigate whether miR-155 is the intermediate in the pathway between miR-29a* and NKG2D mRNA. Therefore, forcing the expression of miR-29a* was performed, after which miR-155 expression levels were assessed, where it was found to be upregulated compared to untransfected cells (Fig. 2D), and hence this suggests that miR-29a* affects miR-155 expression and thus might alter NKG2D mRNA expression.

In turn, forcing miR-155 expression in NK cells of HCV patients caused a downregulation in NKG2D mRNA expression, while

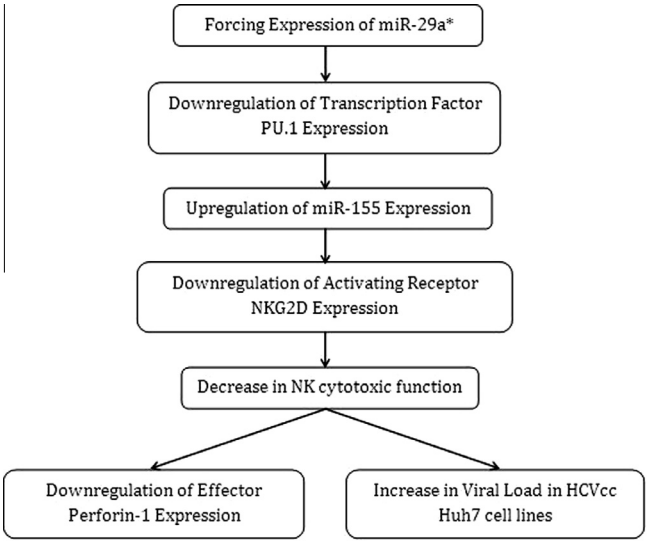

Fig. 6. miR-29a* affects PU.1, NKG2D mRNA through miR-155, deteriorating NK cytotoxicity function.

antagonizing miR-155 caused an upregulation of NKG2D mRNA expression (Fig. 3C). This pattern goes in line with the impact of miR-29a* on NKG2D which might predict miR-155 to be the intermediate between miR-29a*-PU.1 and NKG2D (Fig. 6). Using luciferase reporter assay, it was proven that miR-155 binds to the 3′UTR of NKG2D mRNA and hence alters its expression (Fig. 4B).

To evaluate the cytolytic effect of NK cells and since miR-29a* affected the expression of NKG2D mRNA; it was intriguing to investigate its impact on perforin (Prf-1) mRNA expression. It was found that upon forcing the expression of miR-29a* in NK cells of HCV patients, Prf-1 mRNA was found to be downregulated compared to untransfected cells (Fig. 5A). To see whether the cytolytic activity of NK cells is affected by miR-29a*, coculture of NK cells with ED43/JFH-1 chimeric HCVcc Huh7 cells was performed, after which viral load was quantified in HCVcc infected Huh7 cells 24 h post-coculture with the manipulated NK cells. NK cells overexpressing miR-29a* caused 83% increase in viral load of HCVcc infected Huh7 cells, again suggesting a determinate role of miR-29a* on NK cell cytolytic activity (Fig. 5B).

This study concludes that miR-29a* alters the expression of the transcription factor PU.1, which in turn manipulates the expression of miR-155 and its downstream target activating receptor NKG2D as well as the cytolytic effector Prf-1 and hence causes a reduction in cytolytic effect of NK cells on HCV infected cell models (Fig. 6). This study provides a promising approach to evade HCV through manipulating the expression of miRNAs in NK cells.

Declaration of interest

The authors fully declare no financial or potential conflict of interest.

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