

# Coordinated increase of miRNA-155 and miRNA-196b expression correlates with the detection of the antigenomic strand of hepatitis C virus in peripheral blood mononuclear cells

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**Abstract.** A tight relationship has been revealed between cellular microRNAs (miRNAs) and the course of hepatitis C virus (HCV) replication in human hepatoma cells. Although the detection of the antigenomic HCV RNA strand in peripheral blood mononuclear cells (PBMCs) has provided evidence for viral replication in PBMCs, no reports have shown how miRNAs are affected upon HCV RNA synthesis in PBMCs. The aim of the present study was to assess if and how the expression levels of miRNA-155 and miRNA-196b in PBMCs are related to HCV replication in PBMCs of chronic hepatitis C (CHC) patients. Supporting analyses were performed to evaluate the expression of precursor pri-miR-155 (BIC) and Dicer protein. The genomic and antigenomic HCV RNA strands in PBMCs were detected by strand-specific qRT-PCR. The expression levels of miRNAs, BIC RNA and Dicer protein were assayed on PBMCs by qRT-PCR and Western blotting, respectively. miRNA-155 and miRNA-196b were detected in all studied PBMC samples, but their levels varied according to the presence of the antigenomic HCV RNA strand in PBMCs. Increased expression levels of miRNA-155 and miRNA-196b were associated with the presence of the antigenomic HCV RNA strand in PBMCs. In this group of patients higher frequency of BIC RNA and Dicer protein detection was also found. This study demon-

strates that HCV RNA replication in PBMCs of CHC patients is connected with the increased and coordinated expression of miRNA-155 and miRNA-196b.

## Introduction

microRNAs (miRNAs) are small, single-stranded, non-coding RNA molecules that are generated by the sequential processing of a long hairpin RNA transcript by enzymes, called Drosha and Dicer. Dicer is an endoribonuclease in the RNase III family that cleaves pre-miRNA, yielding the miRNA:miRNA\* duplexes of about 22 nucleotides length. Most miRNAs are expressed in a tissue-specific manner, contributing to distinctive gene expression patterns and specific tissue functions. Therefore, different expression patterns of miRNAs have been found in peripheral blood mononuclear cells (PBMCs) and in the liver tissue (1).

The replication of the positive (genomic) sense RNA strand of hepatitis C virus (HCV) involves antigenomic strand synthesis and most commonly occurs in the liver (2). However, several studies reported that both genomic and antigenomic HCV RNA strands were detected in PBMCs from chronically infected [chronic hepatitis C (CHC)] patients (3,4), giving the evidence that an *in vivo* virus can replicate in extrahepatic sites, like PBMCs.

Many recent findings have revealed a tight relationship between host miRNAs and viral infections (5-7). Liver-specific miRNA, miRNA-122, has been identified *in vitro* as the host factor responsible for positive regulation of HCV replication in the human hepatoma cell line (Huh-7) (8). Other miRNAs like miRNA-196, miRNA-1, miRNA-296, miRNA-351 and miRNA-448 were demonstrated as the negative regulators of HCV replication in Huh-7 cells (9). In addition, miRNA-196 was shown to directly target the NS5A region of the HCV JFH1 genome in Huh-7 cells (9) and interact with the 3'-UTR of Bach1 mRNA in 9-13 cells (10), leading to the down-regulation of the HCV RNA level. A link between HCV replication and expression of miRNAs in PBMCs has not yet been established. However, some observations indicate that miRNA-155 participates in the regulation of a human antiviral immune response in PBMCs (11,12). Moreover, an inducible

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**Abbreviations:** HCV, hepatitis C virus; miRNA, microRNA; PBMCs, peripheral blood mononuclear cells; poly I:C, polyriboinosinic polyribocytidylic acid; Huh-7, human hepatoma cell line; CHC, chronic hepatitis C; RT-PCR, reverse transcription polymerase chain reaction; snRNA U6, small nuclear RNA U6.

**Key words:** HCV, microRNA, PBMCs, BIC RNA, Dicer

expression of miRNA-155 and its primary precursor (pri-miRNA-155), called BIC RNA, was detected *in vitro* after exposure of macrophages to polyriboinosinic-polyribocytidylic acid (poly I:C) and cytokines like IFN $\alpha/\beta$  (12).

The major objective of the present study was to assess the relationship between the expression of miRNA-155, miRNA-196b and HCV RNA replication in PBMCs originating from CHC patients that were positive for the genomic HCV RNA strand. The analysis was supplemented with the evaluation of the expression of the miRNA-155 precursor, BIC, and the endoribonuclease Dicer, the enzyme crucial for the maturation of the precursor of miRNA sequences in human cells.

The results of the present study not only confirmed that HCV RNA replication is accompanied by significant increase of both miR-155 and miR-196 expression but also demonstrated a strong correlation between these two miRNA sequences. In addition, presence of the virus RNA antigenomic strand was associated with a higher frequency of BIC RNA and Dicer protein detection in PBMCs.

## Materials and methods

**Patients.** Blood samples were collected from 47 untreated, chronic hepatitis C patients (34 males, 13 females: ages 18-35) to identify the presence of HCV RNA in serum and genomic/antigenomic HCV RNA strands in PBMCs. The study was accepted by the Bioethical Committee of the Medical University of Lodz (RNN/93/07/KB); blood samples were collected after informed consent had been obtained.

**Preparation of PBMCs and sera.** PBMCs and sera were isolated by blood centrifugation on density gradient (Biocoll 1.077, Biochrom). PBMCs were washed two times with phosphate-buffered saline.

**RNA extraction.** Two fractions of RNA: the first containing RNA >200 nt and the second enriched in small RNA <200 nt, were extracted from PBMCs, using the mirVana™ miRNA Isolation kit (Ambion), according to the manufacturer's instructions.

**Detection of the genomic and the antigenomic HCV RNA strand in PBMCs.** The relative levels of the genomic and antigenomic HCV RNA strands in PBMCs were estimated, according to the Ct values, by strand specific qRT-PCR, described by Carreño *et al* (3). For the detection of the genomic strand, 40 ng of RNA (>200 nt) was reverse transcribed using 1.25 units of MasterAMP™ Tth DNA polymerase (Epicentre® Biotechnologies) and 100 nM of antisense primer (UTRLC2). For the detection of the antigenomic strand, cDNA was synthesized with sense primer (UTRLC1) under the same conditions. The amplification of each strand was performed on 5  $\mu$ l of RT product, using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The reaction mixture contained the FastStart Universal SYBR-Green Master (Roche Diagnostics) and 200 nM of each primer (UTRLC1 and UTRLC2). The PCR profile consisted of 50°C for 2 min, 95°C for 10 min, followed by 40 cycles: 95°C for 15 sec and 60°C for 1 min.

For each PBMC sample, Ct values for both HCV RNA strands were determined. The relative levels of the genomic

and antigenomic strands were calculated from Ct values after their normalization ( $Ct_n$ ), to the GAPDH Ct value (see real-time PCR analysis of BIC RNA expression) using the formula  $2^{-Ct_n}$ , according to the supplier's guidelines (Applied Biosystems).

**Measurement of HCV RNA in serum.** Serum HCV RNA titer was measured by the Amplicor HCV test, version 2.0 (Roche Diagnostics).

**Real-time PCR analysis of miRNA expression.** Reverse transcription was performed with 10 ng of RNA (<200 nt), using the TaqMan miRNA Assay (Applied Biosystems) specific for miRNA-155 and miRNA-196b, according to the manufacturer's instructions. RT product (3.3  $\mu$ l), miRNA-specific primers and probe mix and the TaqMan Universal PCR Master mix (Applied Biosystems) were used for the real-time PCR. The reaction, performed on the ABI PRISM 7000 Sequence Detection system (Applied Biosystems), was as follows: 95°C for 10 min, then 45 cycles: 95°C for 15 sec and 60°C for 1 min. The average miRNA-155 and miRNA-196b Ct values were calculated from triplicate reactions and normalized to average Ct values, obtained for constitutively expressed small nuclear RNA U6 (snRNA U6) (endogenous control) to yield  $\Delta Ct$  values. The expression of each miRNA relative to snRNA U6 was calculated using the formula  $2^{-\Delta\Delta Ct}$ , according to the supplier's guidelines (Applied Biosystems).

**Real-time PCR analysis of BIC RNA expression.** Random cDNAs were synthesized according to the manufacturer's instruction by using 100 ng of RNA (>200 nt) and the RT kit ImProm-II (Promega) and then amplified with the TaqMan Gene Expression assay (Applied Biosystems) specific for BIC (Hs01374570\_m1) and the TaqMan Universal PCR Master mix (Applied Biosystems) as follows: 95°C for 10 min, then 45 cycles: 95°C for 15 sec and 60°C for 1 min. Endogenous control of gene expression (GADPH) was performed in the same cDNA samples, using the same RT-PCR profile as described above and the TaqMan Gene Expression assay (Applied Biosystems) specific for GADPH (4333764F).

**Western blot analysis of Dicer protein expression.** The protein concentration in lysates of PBMCs was determined by the Bio-Rad protein assay (Bio-Rad). Protein lysates (20  $\mu$ g of protein/lane) were separated on 8% SDS-polyacrylamide gel. The immunoblot analysis was performed using primary rabbit antibodies against Dicer (1:100) and goat antibodies against  $\beta$ -actin (1:300) (Santa Cruz Biotechnology; H-212, sc-30226 and C-11, sc-1615, respectively) followed by secondary goat anti-rabbit (1:50,000) and rabbit anti-goat (1:10,000) IgG HRP-conjugated antibodies (Sigma). The bound antibodies were visualized using the Western Blotting Luminol Reagent (Santa Cruz Biotechnology). The expression of  $\beta$ -actin and Dicer in PBMCs was revealed by the presence of 43 and 218 kDa bands, respectively.

**Statistical analysis.** Statistical analyses were performed with STATISTICA 8.0 PL software (StatSoft). Data are presented as the median (and range). Groups were compared using the Mann-Whitney U test. The  $\chi^2$ -square test was used to analyze differences in the frequencies between the examined groups

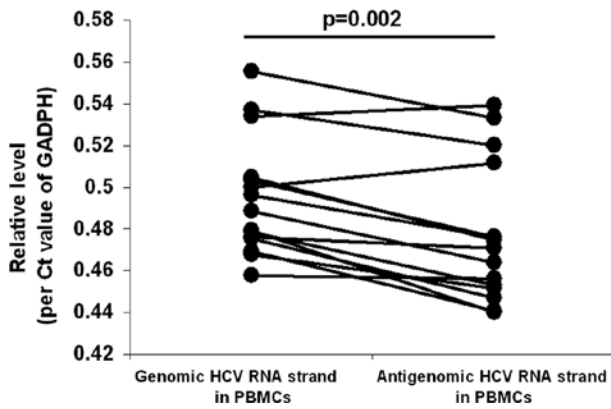


Figure 1. Differences in the relative level of the genomic and antigenomic HCV RNA strands in PBMCs from CHC patients (Wilcoxon signed-rank test).

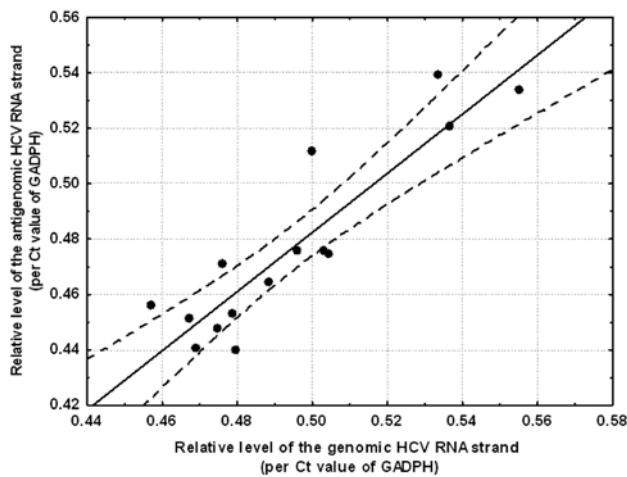


Figure 2. The correlation between the relative levels of the genomic and antigenomic HCV RNA strands in PBMCs (Spearman's Rank Correlation test).

of patients. Spearman's correlation analysis was applied to measure the strength of relationships between the parameters. Differences were considered statistically significant at  $p < 0.05$ .

## Results

**The genomic and antigenomic HCV RNA strands levels in PBMCs.** The presence of the genomic HCV RNA strand in PBMC samples, collected from 47 CHC patients, was demonstrated in 85% of the cases. In the half of this group (20 patients) the antigenomic strand was also confirmed.

When both HCV RNA strands were detected in PBMCs, the relative level of the genomic strand was significantly higher than that of the antigenomic strand (Fig. 1). High positive correlation ( $r = 0.82$ ,  $p = 0.0002$ ) between the relative level of the genomic and antigenomic HCV RNA strand in PBMCs was also revealed (Fig. 2).

**HCV RNA level in serum.** HCV RNA presence in sera was detected in all of the CHC patients included in this study. The comparison between viral load in serum and the HCV RNA replication status in PBMCs demonstrated that the viral load in

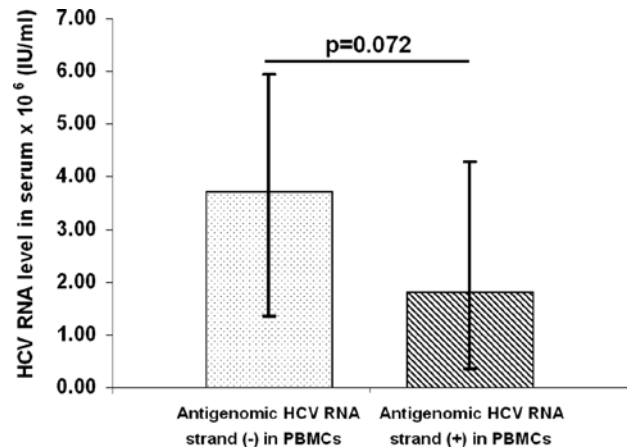


Figure 3. HCV RNA levels in sera from CHC patients: negative and positive for the antigenomic HCV RNA strand in PBMCs (Mann Whitney U test). The results are presented as median values and quartiles.

serum tended to be elevated ( $3.72 \times 10^6$ ,  $1.37 \times 10^6$ - $5.95 \times 10^6$  IU/ml) in patients without the antigenomic strand in PBMCs and diminished, when both HCV RNA strands were present in PBMCs ( $1.82 \times 10^6$ ,  $3.54 \times 10^5$ - $4.28 \times 10^6$  IU/ml), (Fig. 3).

**miRNA-155 and miRNA-196b expression level in PBMCs.** The expression of miRNA-155 and miRNA-196b was detected in all PBMC samples included in this study. Due to the highly consistent level (Ct range, 24.72-26.03) across the studied PBMCs, expression of snRNA U6 was used to normalize the Ct values of miRNA-155 and miRNA-196b in each sample. The analysis revealed that CHC patients who were positive for the antigenomic strand in PBMCs, presented significantly higher expression levels of miRNA-155 than those without the antigenomic strand ( $p = 0.024$ ) (Fig. 4A). The expression level of miRNA-196b in PBMCs showed a similar increase when the viral replicative RNA strand was detected in PBMCs ( $p = 0.024$ ) (Fig. 4B). As was demonstrated using the Spearman's test the significant correlation ( $r = 0.70$ ,  $p < 0.0001$ ) was found between the expression levels of miRNA-155 and miRNA-196b in PBMCs (Fig. 5).

**Frequency of BIC RNA detection in PBMCs.** The expression of BIC RNA was not detected in all studied PBMC samples and the observed frequency of BIC RNA detection showed an upward tendency when the antigenomic HCV RNA strand was detected in PBMCs ( $\chi^2 = 3.22$ ,  $p = 0.073$ ). In a group of patients that were positive for both HCV RNA strands, pri-miR-155 was expressed in 82% of PBMC samples, whereas only in 47% of PBMC samples without the antigenomic HCV RNA strand (Fig. 6). For comparison, the expression level of GAPDH remained unchanged (Ct range, 28.29-31.12), independently of the presence of antigenomic HCV RNA strand or BIC RNA expression.

**Frequency of Dicer protein detection in PBMCs.** Western blot analysis revealed that Dicer protein expression was not detected in PBMCs from all CHC patients, but the number of Dicer-positive samples was significantly different, depending on the antigenomic HCV RNA presence in PBMCs ( $\chi^2 = 4.11$ ,

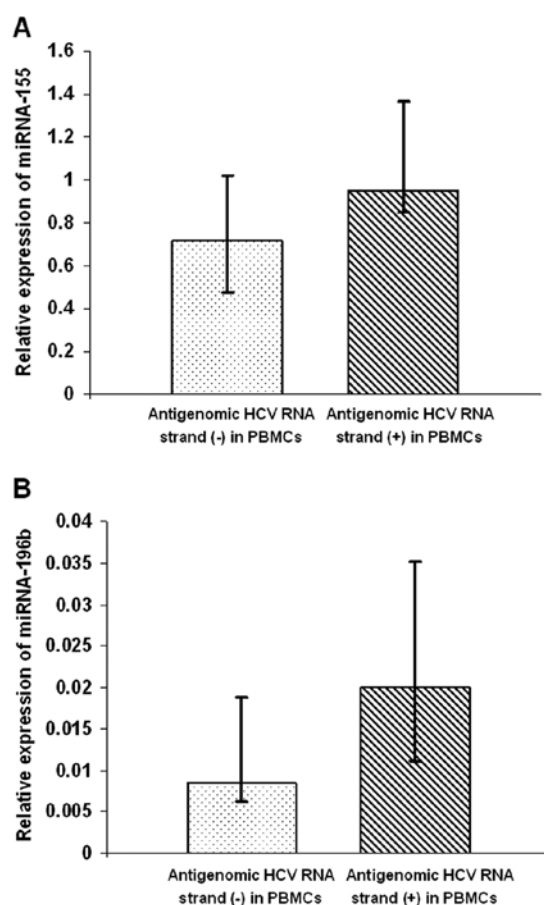


Figure 4. Expression levels of (A) miRNA-155 and (B) miRNA-196b in PBMCs from CHC patients: negative and positive for the antigenomic HCV RNA strand in PBMCs (Mann-Whitney U test). The results are presented as median values and quartiles.

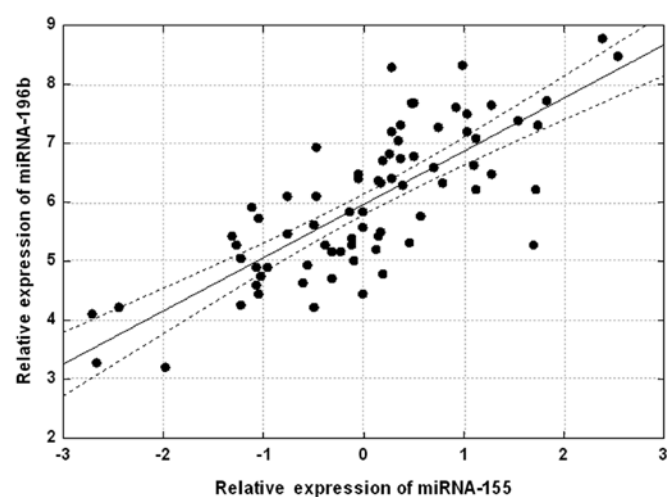


Figure 5. Correlation between the relative expression of miRNA-155 and miRNA-196b in PBMCs (Spearman's Rank Correlation test).

$p=0.042$ ). The higher frequency of the Dicer protein detection was observed when the antigenomic HCV RNA strand was present in PBMCs (77%). Lack of the antigenomic HCV RNA strand in PBMCs was associated with a lower frequency of Dicer (39%). For comparison, the expression of  $\beta$ -actin protein

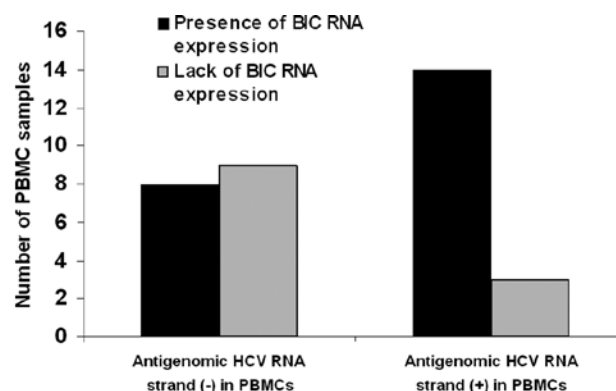


Figure 6. Differences in the frequency of BIC RNA detection between PBMC samples negative and positive for the antigenomic HCV RNA strand ( $\chi^2$  test).

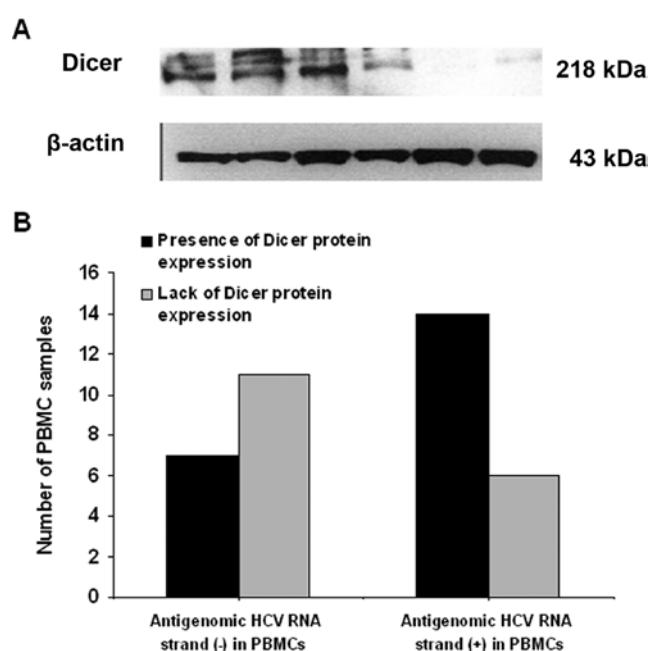


Figure 7. (A) Representative examples of Western blot analysis of Dicer and  $\beta$ -actin protein expression in PBMCs. (B) Differences in the frequency of Dicer protein detection between PBMC samples negative and positive for the antigenomic HCV RNA strand ( $\chi^2$  test).

in PBMCs remained consistent, regardless of antigenomic HCV RNA strand status and Dicer protein expression (Fig. 7).

## Discussion

Evidence is accumulating that the precise regulation of gene expression patterns by miRNA is indispensable for a well-balanced function of cells. Thus, an altered expression or function of miRNA might be expected as a result of viral infection, on the one hand enabling amplification of the virus in host cells and on the other hand it can illustrate the cells' endeavor to defend an invader. miRNA profiles identified in PBMCs are increasingly recognized as the 'footprints' for many viral infections (13). Our study is the first report evaluating if/how the expression of miRNA-155 and miRNA-196b is related to the HCV RNA replication in PBMCs from chronic hepatitis C

patients. Previous studies have confirmed that HCV RNA can replicate in PMBCs from chronically infected patients (3,4). As the replication of the genomic HCV RNA strand involves the synthesis of the antigenomic strand (the replication intermediate) we used the quantitative strand-specific RT-PCR to distinguish PMBCs, depending on the presence of the antigenomic strand. In the group where both, genomic and antigenomic HCV RNA strands were detected in PMBCs, the level of the genomic strand was significantly higher than that of the antigenomic one. Moreover, in the case of ongoing viral replication, the expression of genomic RNA was strongly positively correlated with the antigenomic strand. Our results remain in line with the findings of others, who earlier observed the predominance of a genomic strand when both strands were present in cells (14,15). The evaluated differences between the presence of genomic and antigenomic strand in PMBCs represent an additional proof of the specificity of the strand-specific method used in our study. Interestingly, in our study serum viral load tended to be lower when both genomic and antigenomic strands were present in PMBCs. This phenomenon could be partially explained by earlier studies (16-18) that showed a different regulation of HCV RNA level in serum and PMBCs. The strong relationship was found between serum and liver HCV RNA levels, but not between serum or liver HCV RNA levels and those of PMBCs. It has been suggested that serum HCV RNA levels reflect the hepatic viral burden, but not the HCV RNA level in PMBCs.

It has been shown that viral replication in target cells and the host miRNA system may interact in various ways. Previous studies have demonstrated that in Huh-7, HCV replication can be affected by cellular miRNAs (8-10). These studies, demonstrate that *in vitro* miRNA-196 serves as a negative regulator of HCV RNA accumulation (9,10). Nonetheless, the *in vivo* role of miRNA-196 in HCV infection, especially in PMBCs, has remained unknown. Similarly, despite miRNA-155 abundance in PMBCs and its contribution to the immunological response (12), no studies have yet been performed to find out how modulation of miRNA-155 expression may change the function of PMBCs and influence HCV replication. In the current study we managed to analyze miRNA-155 and miRNA-196b expression in PMBCs from naive chronically HCV-infected patients. Moreover, according to our purpose, we distinguished two groups of patients, depending on the presence of antigenomic HCV RNA strand in their PMBCs.

The PMBC-specific miRNA, miRNA-155, has been shown to be involved in lymphocyte activation (11) upon virus infection (12,19). Kaposi's-sarcoma-associated herpesvirus (KSHV), as the dsDNA virus, has been shown to encode the viral equivalent of host miRNA-155, which largely regulates the expression of viral gene products (20). Additionally, the increased level of miRNA-155 was demonstrated in Epstein-Barr virus (EBV)-infected lymphocytes displaying type III latency. In this study, an elevation of miR-155 was found to be responsible for the alteration of EBV gene expression profile (21). Our study is the first report showing that the higher expression level of miRNA-155 is associated with the HCV RNA replication in PMBCs. Similarly, the expression level of the primary precursor of miRNA-155 (BIC RNA), in the present study, demonstrated an upward tendency when the antigenomic HCV RNA strand was present in PMBCs. Our

previous report showed that HCV RNA persistence in PMBCs observed after interferon treatment, was also accompanied by increased expression of pri-miR-155 (22). These findings suggest that miRNA-155 might be involved in regulation of HCV RNA synthesis in PMBCs, and as a potential factor of immunological response, may influence a course of the viral infection.

According to earlier suggestions (23) miRNA-196 may not only recognize viral infection but can also be an effector in the humans' immune system. Recently, IFN $\beta$  was reported to mediate an inhibition of hepatitis C replication *in vitro* (9) through an elevation of miRNA-196 expression. A perfect match between miRNA-196 and the nonstructural 5A coding region of HCV genome suggests that miRNA-196 may target HCV RNA directly (9). Our *ex vivo* analysis of miRNA-196 in PMBCs collected from CHC patients exhibited a relationship between miRNA expression and HCV replication similar to miRNA-155. We can suggest here that a strong positive correlation between miRNA-155 and miRNA-196 expression may be connected with the same causality and a mode of action of these miRNAs *in vivo*. According to our findings, these PMBCs, where the presence of replicating HCV RNA strand was confirmed, represent a significantly higher level of miRNA-196 expression than PMBCs, in which HCV replication did not occur. These seemingly discrepancies between our results and earlier studies can be explained dually. First of all, it is important to realize that the study of Pedersen *et al* (9) and Hou *et al* (10) were performed *in vitro*. In comparison to the intracellular relation in the selected Huh-7, the *in vivo* impact of miRNAs on HCV replication can be more complex. As was demonstrated, by evaluation of miRNA-122 expression in HCV infection, the results obtained from *in vitro* experiments cannot be simply extrapolated to those *in vivo* (8,24,25). Secondly, in previous study HCV expression in HCV replicon cell lines and in J6/JFH1-based HCV cell culture system was inhibited after transfection with miRNA-196 (10). We cannot exclude that the increase of miRNA-196 expression observed in our study constitutes part of a natural response to the HCV infection. Thus, on the basis of our results, we can hypothesize that increased levels of miRNA-196, which were observed in the case of HCV RNA replication in PMBCs may reflect the 'attempts' of infected cells to limit HCV RNA replication and protect themselves. When this 'intervention' is unnecessary, in PMBCs inside which HCV RNA does not replicate, the level of miRNA-196b can be lower.

Additionally, our analyses of the miRNA-155 and miRNA-196b expression were completed with the assessment of the enzyme Dicer protein level that belongs to the RNase III family and is responsible for the pre-miRNAs cleavage and maturation. Recently obtained data showed that the level of cellular miRNAs may reflect differences in Dicer protein expression (26). It was also pointed out that an impairment of the miRNAs maturation may, as a consequence, influence the level of HCV RNA replication (27). Indeed, in our study, the lower frequency of Dicer protein detection was found in these PMBCs where the antigenomic HCV RNA was undetected and was consistent with the reduced expression level of both analyzed miRNAs.

In summary, we have demonstrated for the first time that miRNA-155 and miRNA-196b expressions are positively

correlated in PBMCs from CHC patients and their expression remains strictly associated with HCV replication in these cells. Future studies are required for a better understanding of the mechanism of miRNA-155 and miRNA-196b induction in the course of CHC. Nevertheless, the observation that antigenomic HCV RNA strand presence in PBMCs is accompanied with the significant increase of both miRNAs shows promise for the development of new therapeutics targeting the expression of miRNA-155 and miRNA-196 in HCV-infected humans.

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