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Association between miR-21/146a/155 level changes and acute genitourinary radiotoxicity in prostate cancer patients: A pilot study

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ABSTRACT

Introduction: Nearly sixty percent of patients with prostate cancer (PCa) undergo radiation therapy (RT). During the course of treatment patients may experience normal tissue reactions. It is a well established fact that genetic and epigenetic mechanisms, such as microRNA (miRNA) level changes might be associated with radiotoxicity, as a response to irradiation.

Materials and methods: This is the first study that has investigated levels of radiosensory miRNAs in association with acute genitourinary radiotoxicity extracted from peripheral blood mononuclear cells (PBCs), in three points; before RT (BRT), after RT (ART) and on the first control examination (FCONT). We measured levels of miR-21/146a/155 expression by quantitative real-time PCR (qRT-PCR), comparative ΔΔCt method, in fifteen patients with localized prostate cancer, treated with three-dimensional conformal radiotherapy (3DCRT). Nine subjects have experienced acute genitourinary (GU) radiotoxicity whereas six where without GU radiotoxicity.

Results: Firstly, we detected the highest levels of miR-21 in ART group ($p = 0.043$) in the patients with acute GU radiotoxicity. Secondly, we found trend towards higher miR-21 levels and significantly higher levels of miR-146a/155 within the patients with acute GU toxicity than in patients without ($p = 0.068$, $p = 0.016$, and $p = 0.010$, respectively). Thirdly, we detected significant change in miR-146a/155 levels within the patients without acute GU radiotoxicity during RT $p = 0.042$, and $p = 0.041$, respectively).

Conclusion: miR-21/146a/155 might be useful potential factors of radiosensitivity and acute genitourinary radiotoxicity in prostate cancer patients. miRNA might have great potential as predictors of various pathological conditions extracted from PBMCs.

1. Introduction

Prostate cancer (PCa) is the second most frequently detected male cancer and the fifth leading cause of cancer mortalities worldwide [1,2]. Fifty to sixty percent of patients with PCa undergo radiation therapy (RT) [3]. During the treatment patients may experience acute or late normal tissue reactions. Acute toxicity occurs during, and shortly after the completion of RT (approximately within 120 days from the start of RT) [4]. Acute radiotoxicity is usually reversible, and frequently manifests as inflammation in highly proliferative tissues, such as skin, bladder, and intestine, resulting in dermatitis, cystitis or diarrhea, respectively [5]. Late effects, such as fibrosis, manifest after a longer period of time compared to acute normal tissue adverse reactions, usually more than 120 days after the start of treatment [4,5].

There are still no defined and validated adequate biomarkers for the prediction of radiotoxicity in patients undergoing radiation treatment, so further research in this area is necessary. For example, levels of proinflamatory factors are significantly modified after radiotherapy and/or androgen treatment [6,7]. On the other hand, genetic and epigenetic mechanisms, among them changes in microRNA (miRNA) expression levels also lay in the basis of radiotoxicity, and in the response to irradiation. miRNA belong to the group of small non-coding

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silencers, involved in translational repression. When the 'seed' region of miRNA 'finds' incompletely complementary sequence at the 3′ untranslated region (3′UTR) of a messenger RNA (mRNA), it leads to the translational repression, or mRNA degradation. As a result, translation and protein synthesis stop [8]. Changes in miRNA levels cause changes in protein levels. The imbalance in protein levels involved in immune response cause inflammation in the irradiated area. Response to RT, as well as radiotoxicity, is an individual processes, which indicates that personalized approach to RT might be of great importance for patients undergoing radiation treatment. miR-146 and miR-155 are both immunomodulatory miRNA molecules. Their expression is frequently co-induced in immune response [9]. Both miR-146a and miR-155 overexpress in response to various inflammatory stimuli, and can be induced by tumor necrosis factor (TNF), interleukin 1 (IL-1), interferons (IFNs) and Toll-like receptor (TLR) ligand in various cell types, monocytes, and T and B lymphocytes [10]. miR-21 is an oncogenic miRNA that promotes proliferation and invasion of prostate cancer cells, involved in apoptosis [11], and has been proven as radiosensitive miRNA [12,13]. miR-21 has the ability to reduce radiosensitivity of cancer cells [14]. Reduction of miR-21 levels might be promising additional therapy for tumor sensitization to radiation treatment [15], because miR-21 has already been characterized as an inducer of radioresistance in breast cancer cells [16].

Peripheral blood mononuclear cells (PBMCs) represent an important source of various miRNA molecules [17]. There is a hypothesis that miRNA profiling might increase the chances for prediction of developing radiotoxicity in a single patient [18]. Our research was based on examination of three miRNAs miR-21, miR-146a, and miR-155, whose levels, as we assume, might change during RT. Majority of studies, up to now, have examined miRNA level changes in cancerous tissue, cells, or plasma and serum in response to radiation treatments [18–21], while our study aims to investigate normal tissue reactions.

Our previous study [22] which showed the link between clinical and individual parameters on the one hand and acute genitourinary (GU) radiotoxicity on the other, has led us to start this pilot study of possible impact of genetic/epigenetic factors such as miRNA as potential biomarkers for acute GU radiotoxicity. This is the first study that has described miRNA as factors, potential future biomarkers monitored in PBMCs. To summarize, the goal of this study was to examine the potential link between miR-21/146a/155 expression changes and acute GU radiotoxicity, by comparing patients with and without acute GU, and to investigate if miRNA levels change during the radiation treatment in PMBCs in the patients with prostate cancer, who underwent three-dimensional conformal radiotherapy (3DCRT) at the Institute for Oncology and Radiology of Serbia.

2. Materials and methods

2.1. Study population

Fifteen subjects (15 patients with prostate cancer) treated with 3DCRT at the Institute of Oncology and Radiology of Serbia were included in this study. Blood samples were collected at baseline (BRT group), immediately after the last fraction of 3DCRT (ART group), and on the first control examination, a month after the end of radiotherapy (FCONT group). In this study, nine patients with acute GU radiotoxicity (grade 1–3) at the moment of sample collecting (BRT, ART, and FCONT), between April 2016 and April 2017, and six subjects without acute GU radiotoxicity (grade 0) were investigated.

Patients with the diagnosis of localized prostate cancer were treated with 3DCRT as follows; patients treated with radical 3DCRT (72 Gy in 36 fractions), and patients treated with 3DCRT after the prostatectomy (postoperative or salvage RT) with a given dose of 66 Gy in 33 fractions.

The details of the study were clearly presented and explained to the participants, who wrote informed consents. The study protocol was approved by the Ethical Research Committee of the Institute for Oncology and Radiology of Serbia (ethical board approval № 3348/1-01). Acute radiotoxicity was evaluated weekly, according to RTOG/EORTC (Acute Radiation Morbidity Scoring Criteria) modified by Peeters [4], as well in our previous study [22]. Exclusion criteria were as follows; chronic infective diseases, neoadjuvant or concomitant hormonal therapy, the presence of enlarged lymph nodes (N1 stage) detected by imaging methods, the presence of distant metastasis (M1 stage) detected by imaging techniques, Karnofsky index < 80, and previous pelvic irradiation.

Table 1
Patients', clinical characteristics and acute genitourinary radiotoxicity.

Patient and clinical characteristics		Differences between groups with and without GU radiotoxicy p value
Number of patients	15	
Without GU radiotoxicity	6	
With GU radiotoxicity	9	
Mean age ± standard deviation		
Without GU radiotoxicity	68 ± 5.40	0.450
With GU radiotoxicity	70.57 ± 6.29	
Smoking status		
Active/former		
Without GU radiotoxicity	33.3%	0.315
With GU radiotoxicity	66.7%	
Non-smokers		
Without GU radiotoxicity	66.7%	
With GU radiotoxicity	33.3%	
Diabetes mellitus		
Yes		0.400
Without GU radiotoxicity	16.7%	
With GU radiotoxicity	0.0%	
No		
Without GU radiotoxicity	83.3%	
With GU radiotoxicity	100.0%	
Chronic hypertension		
Yes		1.000
Without GU radiotoxicity	66.7%	
With GU radiotoxicity	55.6%	
No		
Without GU radiotoxicity	33.3%	
With GU radiotoxicity	44.4%	

GU-genitourinary.

2.2. Clinicopathological characteristics of examined subjects

Clinical patients'' characteristics, such as mean age, smoking status, diabetes mellitus, and chronic hypertension are shown in Table 1, as well as the differences between groups with and without acute GU radiotoxicity. Characteristics of tumor, such as Gleason's score, and risk category. Radiation treatment characteristics, such as radical, or postoperative/salvage are shown in Table 2. In this study we examined the expression levels of three miRNAs, miR-21/146a/155, measured at the three points defined above, from PBMCs in 15 patients with prostate cancer.

2.3. Peripheral blood mononuclear cell extraction

Peripheral blood mononuclear cells were extracted with Histopaque-1077, Sigma-Aldrich solution according to the manufacturer's instructions. All steps were performed in RNase-free conditions at low temperatures (4 °C), and were stored at −80 °C for further analysis.

2.4. miRNA expression measurements and analysis

Total RNA was extracted from PBMCs by using TRI Reagent

Table 2
Tumor, treatment characteristics and acute genitourinary radiotoxicity.

Tumor charactersitics		Differences between groups with and without GU radiotoxicy, p value
Number of patients	15	
Without GU radiotoxicity	6	
With GU radiotoxicity	9	
Gleason score median (minimum-maximum)		
Without GU radiotoxicity	7.50 (6-9)	0.313
With GU radiotoxicity	7 (6-8)	
Risk category		
Low risk		
Without GU radiotoxicity	16.7%	1.000
With GU radiotoxicity	11.1%	
Intermediate risk		
Without GU radiotoxicity	16.7%	
With GU radiotoxicity	22.2%	
High risk		
Without GU radiotoxicity	66.7%	
With GU radiotoxicity	66.7%	
Radiation treatment characteristics		
Radical		
Without GU radiotoxicity	33.3%	1.000
With GU radiotoxicity	44.4%	
Salvage and postoperative		
Without GU radiotoxicity	66.7	
With GU radiotoxicity	55.6	

GU-genitourinary.
PSA-prostate specific antigen.

(Ambion, Foster City, CA, USA), followed by chloroform and precipitated with isopropanol then washed with 75% diethylpyrocarbonate (DEPC)-ethanol, and dissolved in DEPC-water.

For the reaction of reverse transcription and quantitative real-time PCR (qRT-PCR) of miR-21/146a/155, and small nuclear RNA, RNU6B, we used 10 ηg of total RNA. Reverse transcription reaction and qPCR were performed with TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, California, USA), Taqman Universal master mix, No UNG amperase, and has-miR-21 (ID: 000397), hsa-miR-146a (ID:000468); has-miR-155 (ID: 002623) and RNU6B (ID:001093), assays following standard thermal conditions (30 min 16 °C, 30 min 42 °C, and 5 min at 85 °C). The amplification reactions were run on Real Time PCR 7500 (Applied Biosystems, Foster City, California, USA) system under the following thermal conditions. Relative quantity (RQ) values, i.e. expression levels of miRNAs were presented in relative units, and were normalized to endogenous control (RNU6B). Samples with the lowest relative quantity levels were used as calibrator reference, and then were analyzed with by 7500 System SDS Software (Applied Biosystems, Foster City, California, USA), with comparative ΔΔCt method, by the following equation; RQ sample = $2^{-(\Delta Ct\ sample - \Delta Ct\ calibrator)}$. $\Delta Ct = Ct_{miR\text{-}21/146a/155} - Ct_{RNU6B}$.

2.5. Statistical analysis

The differences between the two groups were analyzed with either parametric Student's t-test, or non-parametric Mann–Whitney U test. For the frequency distribution analysis, we used Fisher's exact test. The differences between the two matched pairs of samples were investigated by Wilcoxon's signed rank test or Friedman's test for three group comparison). For the correlation analysis we used Pearson's parametric, or Spearman's non-parametric tests. P values ≤ 0.050 were described as statistically significant, while P values between 0.1 and 0.05 were pointed out as a statistical trend. Statistical data analysis was performed using IBM SPSS Statistics 22 (IBM Corporation, Armonk, NY, USA), and GraphPad Prism 5 (GraphPad Software, Inc. CA).

3. Results

In this study, we investigated expression levels of three miRNAs, miR-21/146a/155 in 15 patients with prostate cancer. miRNA expression levels were measured at three described points of time before and after RT. Fifteen patients were divided into two groups: the group with acute radiotoxicity (9 subjects), and the group without acute radiotoxicity (6 subjects) at the observed point.

3.1. Patients', clinical characteristics and acute GU radiotoxicity

We have not found any significant association in the groups of patients' divided according to acute GU radiotoxicity with clinical characteristics such as age at diagnosis, smoking status, diabetes mellitus, and chronic hypertension (Table 1). Furthermore, we examined potential association of acute GU radiotoxicity with Gleason's score, Risk category, and radiation treatment, and found no significant association (Table 2).

3.2. miR-21/146a/155 relative expression levels measured before radiotherapy, after radiotherapy, and after the first control examination

In the group of patients without acute GU radiotoxicity, we detected significantly higher levels of miR-21 in ART group compared with FCONT group (P = 0.043, Wilcoxon's signed rank test, Fig. 1A). In FCONT group, we noticed statistical trend towards higher miR-21 levels within the patients with acute GU radiotoxicity compared with group without GU radiotoxicity (p = 0.068, Mann Whitney U test, Table 3, Fig. 2A). Within the group of patients who experienced GU RT, we detected significantly higher levels of miR-21 expression in ART compared with FCONT group (p = 0.046, Wilcoxon's signed rank test, Fig. 1B).

In the group without GU radiotoxicity the lowest levels of miR-146a were found in FCONT group compared with BRT and with ART groups, respectively (p = 0.028, p = 0.046, Friedman's test, Fig. 1C). Patients experienced acute GU radiotoxicity had significantly higher levels of miR-146a compared with patients without radiotoxicity in FCONT group (p = 0.016, Table 3, Mann Whitney U test, Table 3, Fig. 2B).

miR-155 levels were significantly lower in FCONT group compared with BRT in the group of patients without radiotoxicity (p = 0.041, Friedman's test, Table 3, Fig. 1D), and as well as in the case of miR-146a, significantly higher in the group with acute radiotoxicity compared with the patients without GU toxicity in FCONT group (p = 0.010, Mann Whitney U test, Table 3, Fig. 2C).

3.3. The correlation between miR-21, miR-146a, and miR-155

Because the expression of miR-146a and miR-155 is frequently co-activated in immune response related to ionizing radiation, we examined a potential correlation between these two miRNAs, and compared with levels of miR-21, because of potential synergistic effect.

As we had expected, there was a strong positive correlation between miR-146a and miR-155 in all of the three examined groups of patients, BRT, ART, and FCONT (p < 0.00001, rho = 0.912, 0.875, and 0.951, Spearman's correlation test, respectively, Table 4). Significant positive correlations were found between miR-21 and miR-146a in all three groups, respectively (p = 0.001, rho = 0.819, p = 0.011, rho = 0.636, p = 0.001, rho = 0.867 Spearman's correlation test, Table 4), and between miR-21 and miR-155, as well, (p = 0.026, rho = 0.636, p = 0.025, r = 0.575, p < 0.001, rho = 0.891, Spearman's correlation test, respectively, Table 4).

4. Discussion

This is, to our knowledge, the first study investigated expression levels of miRNAs extracted from PBMCs at three points before during

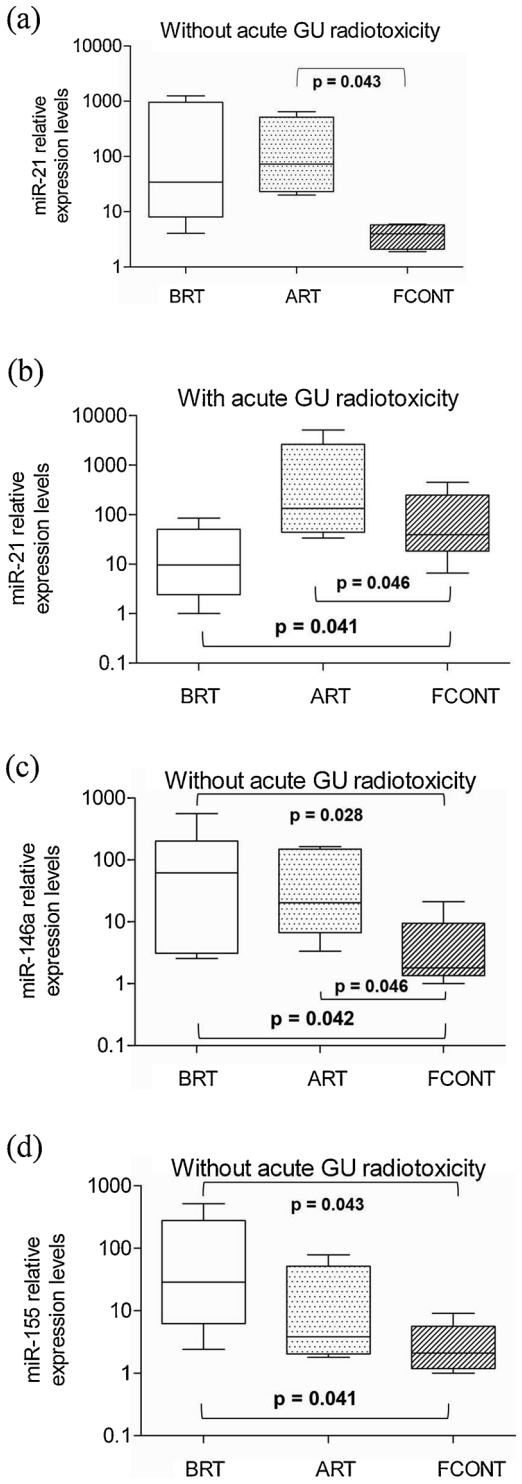

Fig. 1. Differences in miR-21/146a/155 expression levels in relative quantity units (RQ) before radiotherapy-BRT, after the last fraction of radiotherapy-ART, and at the first control (FCONT) group. A-miR-21 expression level changes within the patients without acute genitourinary (GU) radiotoxicity. B-miR-21 expression level changes within the patients with GU radiotoxicity. C-miR-146a expression level changes within the patients without GU radiotoxicity. D-miR-155 expression level changes within the patients without GU radiotoxicity.

and after radiation treatment, as potential factors of radiotoxicity in the patients with localized prostate cancer.

We have investigated and quantified miR-21 expression levels, because it has been reported that miR-21 is sensitive to radiation [12–14].

Table 3
Differences in miR-21/146a/155 relative expression levels between the patients without and with acute genitourinary radiotoxicity, and BRT, ART, and FCONT groups.

[a]Relative expression levels of miR-21

	Without acute GU radiotoxicity	With acute GU radiotoxicity	p values
BRT	38.84 (4.05-1258.00)	26.59 (1.00-1871.00)	0.661
ART	128.01 (20.03-647.34)	179.83 (33.72-5141.00)	0.289
FCONT	5.23 (1.89-49.45)	34.57 (6.61-454.90)	**0.068**
p values	0.105	**0.041**	

[a]Relative expression levels of miR-146a

	Without acute GU radiotoxicity	With acute GU radiotoxicity	p values
BRT	61.76 (2.56-560.28)	5.78 (0.001-3986.76)	0.724
ART	20.33 (3.35-163.71)	53.97 (6.58-391.54)	0.556
FCONT	1.79 (1.00-21.21)	18.29 (6.71-187.27)	**0.016**
p values	**0.042**	0.311	

[a]Relative expression levels of miR-155

	Without acute GU radiotoxicity	With acute GU radiotoxicity	p values
BRT	28.70 (2.41-520.3)	3.96 (1.64-2757.22)	0.549
ART	3.07 (1.63-78.36)	11.71 (2.43-153.17)	0.157
FCONT	1.73 (1.00-9.06)	21.16 (3.52-155.552)	**0.010**
p values	**0.041**	0.867	

GU- genitourinary.
BRT-before radiotherapy.
ART-after radiotherapy.
FCONT-first control.
[a] Median values of relative miR-21/146a/155 expression with minimum and maximum in parentheses. p values equal or less than 0.05 were considered significant according to the result of Wilcoxon's signed rank test (between 2 groups) and Friedman's test for 3 groups comparisons (in bold style). p values between 0.1 and 0.05 were considered as statistical trend, presented in bold style.

miR-21 has the ability to silence genes whose protein products regulate cell cycle propagation [16], apoptosis [23], and DNA repair [24]. miR-21 is not a sufficient factor by itself for prediction of PCa chemo-radiosensitivity [20], so we investigated proinflamatory radiosensory miR-146a and miR-155 [19,25]. miR-146a and miR-155 have been proven to participate in the intercellular regulation of immune response [26]. Furthermore, miR-155 regulates the expression of genes involved in DNA damage repair [27], and is associated with response to chemo-RT or RT solely [28,29].

PBMCs contain and produce large amounts of miRNA molecules [17]. PBMCs are very active in terms of miRNA expression [17]. miRNA regulate lymphocyte maturation, function and differentiation [30], and response to radiation exposure, as well [31,32].

Our results suggest that miR-21/146a/155 levels in PBMCs change during radiotherapy of patients diagnosed with prostate cancer and among patients with and without radiotoxicity. miR-21 levels rose during the radiotherapy, in the ART group, then and were significantly lowered after the first control, a month after the RT, indicating that RT potentially influences on miR-21 expression in PBMCs. Statistical trend towards higher levels of miR-21 in the group with acute GU radiotoxicity, and significantly higher levels of miR-146a and miR-155 show that examined miRNA molecules have great potential to be investigated as future biomarkers of acute GU radiotoxicity. miR-21 followed the same trend of expression changes in both groups (with and without radiotoxicity). miR-21 levels firstly increased from BRT towards ART groups, and then significantly decreased at first control examination. miR-146a/155 act slightly differently than miR-21 in the group of

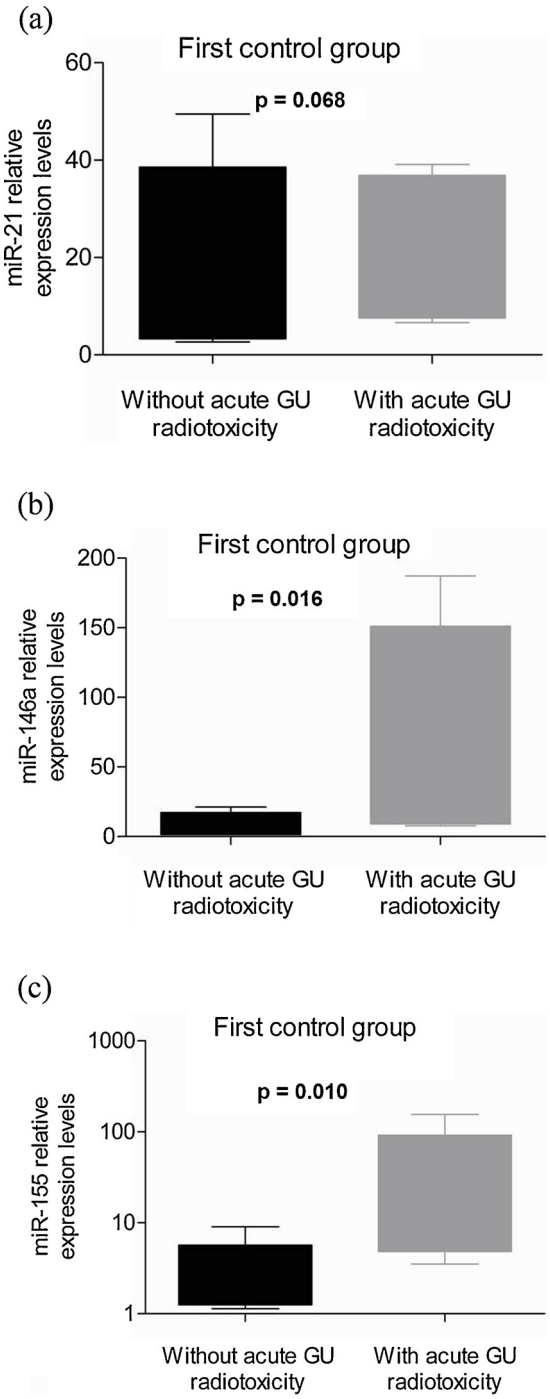

Fig. 2. Differences in miR-21 (A), miR-146a (B), and miR-155 (C) expression levels in relative quantity units (RQ) between groups of patients without and with acute genitourinary (GU) radiotoxicity.

patients with toxic effect. Furthermore, miR-146a/155 time profile (through the radiotherapy) is different between the groups divided according to toxicity. Within the group of patients without acute radiotoxicity levels of miR-146a and miR-155 significantly decreased from BRT towards FCONT, while in the group that experienced radiotoxicity, their levels did not significantly change. Higher levels of miR-146a and miR-155 in the patients from the group who experienced radiotoxicity might be associated with higher rates of inflammatory response, while higher levels of miR-21 might indicate higher apoptosis rates and higher radiosensitivity levels [16].

Lymphocytes as the predominant fraction of PBMCs are activated during immune response due to radiation, and produce higher levels of miR-155 then in physiologicaly normal conditions. It has been shown that miR-155 can increase or decrease, depending on the type of radiation exposure, dose and rates of [25,33]. Furthermore, Chaudhry et al. [25] have shown that miR-155 levels increased after 10 cGy of chronic exposure, decreased after 10 cGy of acute ɣ-rays 3 h doses, and decreased after 400 cGy of 3 h and 8f acute doses, indicating that rates, doses and type of exposure (chronic or acute) represent important factors in the cellular response to irradiation, which reflects on the different patterns of particular miRNA expression. High positive correlation among miR-21, miR-146a and miR-155 might also indicate that the three examined miRNAs might have synergistic effect on its target genes, amplifying the effect on response to radiation treatment, and should be recognized as future biomarkers of acute GU radiotoxicity, from tissue, serum [18], and PBMCs, as well. Furthermore, it has been experimentally proven that miR-21 participates in radiation-induced bystander effect [34], which means that miR-21 level changes during and after the irradiation even in surrounding non-irradiated cells through the intercellular signaling and communication between irradiated and non-irradiated cells, through the body, emphasizing the importance of measuring those miRNAs at different time points during the radiation treatment.

We have not detected any significant associations between miRNA expression levels and standard clinicopathological parameters of patients, and the type of RT, indicating that miR-21/146a/155 levels in our experiment are independent factors of acute GU radiotoxicity.

In general, limitations of the miRNA-based studies are related to the fact that whole miRNA amount is a result of different biological mechanisms, such as production and excretion of miRNA from tumor cells, circulation as free miRNAs and exosome or vesicle coated, and their trafficking [35], and in this case, probably the result of immune, proinflammatory response due to radiation treatment, which is why our research focuses on the pattern of level expression changes instead of quantifying the absolute levels of miRNAs.

Table 4
Significant correaltions between miRNA-21/146a/155 levels.

Variable 1	Variable 2	[a]N	Measured point	[b]Correlational coefficient	p value
miRNA-21/146a/155 correlations					
miR-21	miR-146a	13	BRT	rho = 0.819	**p = 0.001**
miR-21	miR-155	12	BRT	rho = 0.636	**p = 0.026**
miR-21	miR-146a	15	ART	rho = 0.636	**p = 0.011**
miR-21	miR-155	15	ART	rho = 0.575	**p = 0.025**
miR-21	miR-146a	10	FCONT	rho = 0.867	**p = 0.001**
miR-21	miR-155	11	FCONT	rho = 0.891	**p < 0.001**
miR-146a	miR-155	14	BRT	rho = 0.912	**p < 0.001**
miR-146a	miR-155	15	ART	rho = 0.875	**p < 0.001**
miR-146a	miR-155	12	FCONT	rho = 0.951	**p < 0.001**

BRT-before radiotherapy.
ART-after radiotherapy.
FCONT-first control.
[a] N-number of patients.
[b] Correlation coefficient. p values equal or less than 0.05 were considered significant.

5. Conclusions

According to our results, miR-21/146a/155 levels change in PBMCs during the RT, which suggests that examined miRNA expression might be sensitive to RT. miR-21/146a/155 might serve as future independent predictive biomarkers of acute genitourinary radiotoxicity in the patients with prostate cancer. Furthermore, miR-21/146a/155 extracted form PBMCs might be used as candidates for miRNA panel for the prediction of radiotoxicity in near future. Our results add a new level in miRNA-based biomarker researches indicating that apart from

circulating and tissue-derived miRNAs, miRNAs isolated form PBMCs might serve as additional biomarkers of radiotoxicity.

Conflict of interest

All authors declare that they have no conflict of interest.

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