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Original study article

The miR-338-3p expression level in pemphigus diagnosis

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ABSTRACT

BACKGROUND: Pemphigus is a group of potentially fatal chronic cutaneous diseases in which blisters appear on the skin and mucous membranes as a result of IgG autoantibodies binding to desmosomes in the epidermis, leading to keratinocytes acantholysis. Currently, methods to monitor disease activity and therapy efficiency using various biomarkers are being investigated. MicroRNA expression, in particular miR-338-3p, has been one of these biomarkers, as changes in miR-338-3p expression may trigger the Th1/Th2 cell imbalance and possibly be involved in the pathogenesis of the disease.

AIM: This study aimed to design a protocol to evaluate the level of miR-338-3p expression in peripheral blood mononuclear cells and verify the diagnostic value of miR-338-3p expression in pemphigus.

MATERIALS AND METHODS: Experimental prospective comparative study was conducted from February 2023 to February 2024 at the Dermatology Department of Sechenov University. The study included 10 patients with pemphigus in the active stage of the disease, 3 patients in remission, and 9 participants of the control group. The expression of miRNA-338-3p was analyzed by real-time polymerase chain reaction, cDNA was obtained using StemLoop method. The evaluation of miRNA-338-3p expression level was based on its comparison with the expression of U6 gene using 2-ΔΔCt method.

RESULTS: The expression level of miR-338-3p was analyzed in 10 patients in the active stage of the disease (5 men, 50%; 5 women, 50%; mean age 46±10.7 years), 3 patients in remission (2 women, 66.7%; 1 man, 33.3%; mean age 57±8 years), 9 control group (8 women, 88.9%; 1 man, 11.1%; mean age 36±16.8 years). The mean expression level of miR-338-3p was 8.64 (SD±5.72) in patients with active disease, 3.38 (SD±1.44) in patients in remission, and 1.48 (SD±1.12) in controls. A statistically significant increase in the expression level of miR-338-3p was found in patients in the active disease stage compared to the control group (p=0.002). A statistically significant correlation was found between the level of miR-338-3p expression and the PDAI index score (p <0.001).

CONCLUSION: Based on the data obtained in this study, it can be assumed that microRNAs are important in pemphigus, and miR-338-3p expression in particular may serve as a key element in pemphigus pathogenesis. More detailed study of microRNAs and analysis of expression variability according to clinical data may provide the basis for developing new diagnostic methods and severity scoring, allowing more accurate and less invasive diagnostic methods, as well as monitoring and predicting disease progression.

Keywords: microRNAs; bullous dermatoses; pemphigus vulgaris; pemphigus foliaceus.

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Оригинальное исследование

Оценка уровня экспрессии miR-338-3p в диагностике аутоиммунной пузырчатки

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АННОТАЦИЯ

Обоснование. Аутоиммунная пузырчатка — группа потенциально смертельных хронических дерматозов, при которых возникают пузыри на коже и слизистых оболочках в результате фиксации аутоантител класса IgG на десмосомах в эпидермисе, акантолиза кератиноцитов. В настоящее время ведётся поиск методов контроля активности и терапии заболевания с помощью различных биомаркеров. Одним из таких направлений стало изучение экспрессии микроРНК, в частности miR-338-3p, поскольку изменение её экспрессии может быть пусковым фактором в дисбалансе Th1/Th2 клеток и, следовательно, играть роль в патогенезе заболевания.

Цель исследования — разработать технологию оценки уровня экспрессии miR-338-3p в мононуклеарных клетках периферической крови и оценить диагностическую ценность экспрессии miR-338-3p при аутоиммунной пузырчатке.

Материалы и методы. Экспериментальное проспективное сравнительное исследование проводилось в период с февраля 2023 по февраль 2024 года. В исследование включены 10 пациентов с аутоиммунной пузырчаткой в активной стадии заболевания (5 мужчин, 50%; 5 женщин, 50%; средний возраст 46±10,7 года), 3 пациента в стадии ремиссии (2 женщины, 66,7%; 1 мужчина, 33,3%; средний возраст 57±8 лет) и 9 участников группы контроля (8 женщин, 88,9%; 1 мужчина, 11,1%; средний возраст 36±16,8 года). Изучение экспрессии miRNA-338-3p проводили с помощью полимеразной цепной реакции в режиме реального времени, кДНК получали на основе технологии StemLoop. Оценка уровня экспрессии miRNA-338-3p основывалась на её сравнении с экспрессией малой ядерной РНК U6.

Результаты. Средний уровень экспрессии miR-338-3p у пациентов в активной стадии заболевания составил 8,64 (SD±5,72), в ремиссии — 3,38 (SD±1,44), в группе контроля — 1,48 (SD±1,12). Выявлено статистически значимое повышение уровня экспрессии miR-338-3p у пациентов в активной стадии заболевания по сравнению с группой контроля (p=0,002). Установлена статистически значимая связь между уровнем экспрессии miR-338-3p и величиной значения индекса площади поражения при пузырчатке PDAI (p <0,001).

Заключение. По результатам проведённого исследования можно сделать вывод о значимости микроРНК при аутоиммунной пузырчатке, а экспрессия miR-338-3p, в частности, может служить ключевым элементом в патогенезе данного заболевания. Более детальное изучение микроРНК и анализ вариабельности экспрессии в зависимости от клинических данных может стать основой для создания новых диагностических методов, а также оценки степени тяжести, что позволит применять более точные и менее инвазивные методы диагностики, а также контролировать и прогнозировать течение заболевания.

Ключевые слова: микроРНК; буллёзные дерматозы; вульгарная пузырчатка; листовидная пузырчатка.

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BACKGROUND

Pemphigus represents a group of rare and potentially fatal chronic autoimmune dermatoses. In pemphigus, anti-desmoglein 3 and/or 1 (Dsg3, Dsg1) autoantibodies binding in the epidermis results in acantholysis of keratinocytes with the formation of intraepidermal blisters on the skin and mucous membranes [1].

The prevalence of pemphigus varies greatly depending on the geographic region: the disease is most common in the Indian subcontinent, the Mediterranean region, and the Middle East. The annual incidence of pemphigus in Russia is 1.9 cases per 100,000 [2]. The most common types of pemphigus are pemphigus vulgaris and pemphigus foliaceus, which account for 90–95% of all variants of the disease [3]. Pemphigus vulgaris is the most common type of pemphigus and accounts for up to 65% of all cases [4, 5]. It predominantly affects females, with a male to female ratio ranging from 1:1.7 to 1:1.1 [1–5]. Almost all cases of pemphigus vulgaris develop lesions of the oral mucosa, with more than 50–70% of cases manifesting at this localization [6, 7]. Pemphigus foliaceus is the second most common type of pemphigus, which accounts for up to 27% of all cases [8].

Due to the rarity of the disease and an extended differential diagnosis search, several weeks to months may pass from first symptoms to diagnosis, which leads to delayed pathogenesis-oriented therapy start and aggravated disease course [9–14]. The standard for the diagnosis of pemphigus is the presence of a characteristic clinical presentation, histological examination, and direct and indirect immunofluorescence (IF) assays and/or enzyme-linked immunosorbent assay (ELISA); however, false-positive and false-negative results in histology and immunohistochemistry may occur, which makes the search for new diagnostic methods an urgent problem [15].

Currently, a wide range of studies is being conducted to search for methods to control disease activity using various biomarkers that would allow monitoring the progression of pemphigus and predicting response to therapy, which will optimize the choice of personalized therapy [16]. Assessment of the severity of pemphigus by the titer of anti-Dsg1 and anti-Dsg3 autoantibodies was considered particularly promising but has been shown to be ineffective because the antibody titer can remain high even in clinical remission [17, 18]. In recent years, microRNAs have been actively studied as a method for diagnosing and predicting disease outcomes [19].

MicroRNAs are noncoding RNAs about 22 nucleotides long that control gene expression at the posttranscriptional level [20]. According to recent data, there are 2,675 mature microRNAs in the human genome, which serve as potential regulators of more than 60% of coding genes [21, 22]. MicroRNAs bind to target messenger RNAs (mRNAs), causing degradation or translational suppression of mRNAs and subsequently regulate various biological processes including apoptosis, cell differentiation, intercellular communication, and immune response reactions [23]. Dysregulation of microRNAs plays an important role in the pathogenesis of autoimmune diseases by promoting T cell autoreactivity, activating inflammatory pathways and cytokine production, and regulating B cell activation and inducing apoptosis [24–26].

The results of recent studies indicate that due to tissue-specific expression, as well as the ability to rapidly exit from cells and remain stable in biological fluids, microRNAs may surpass existing biomarkers in sensitivity and specificity [27]; however, the number of publications devoted to the study of microRNA expression in the context of pemphigus is limited (Table 1) [28–34].

In the studies published to date, the expression of more than 170 microRNAs has been analyzed (see Table 1), and miR-338-3p has been of particular importance, since it has been experimentally established to suppress interferon-gamma production and increase the levels of interleukin (IL) 4 and IL-10 [31]. Therefore, altered expression of miR-338-3p may be a trigger factor in Th1/Th2 cell imbalance, which underlies the theory of pemphigus pathogenesis [35–37].

Real-time polymerase chain reaction (rtPCR) is an effective method for quantifying relative and absolute levels of microRNAs in biological samples because the method has high accuracy and sensitivity [38]; however, there is a large variability among studies with regard to the number of microRNAs analyzed (from 1 to 124), PCR modification options, and detection of rtPCR results. The choice of substrates for the study (blood plasma, mononuclear cells, exosomes, skin biopsy samples), as well as the choice of internal control for expression analysis (see Table 1), also remains open. Thus, there is no standard protocol for assessing microRNA expression levels in pemphigus.

The study aim was to develop a technology to assess the expression level of miR-338-3p in peripheral blood mononuclear cells and to evaluate the diagnostic value of miR-338-3p expression in pemphigus.

MATERIALS AND METHODS

Study design

The study is an experimental prospective comparative study.

Eligibility criteria

Inclusion criteria: Individuals of both sexes; newly diagnosed or with previously established diagnoses of pemphigus vulgaris or foliaceus; age from 18 years; voluntary willingness of the patient to enroll in the study and a signed informed consent; consent to the processing of personal data.

Table 1. Comparison of studies on microRNA expression in pemphigus

Publication	Phenotype	Total number of microRNAs studied	Key miRNAs	Substrate	Detection method	Internal control
Valentino et al., 2023 [28]	PV	86	hsa-miR-148a-3p hsa-miR-146b-5p hsa-miR-126 hsa-miR-139	Exosomes obtained from blood plasma	rtPCR TaqMan	U6
Khabou et al., 2023 [29]	PF	6	hsa-miR-17-5p hsa-miR-21-5 hsa-miR-146a-5p hsa-miR-155-5p hsa-miR-338-3p hsa-miR-21	Peripheral blood mononuclear cells /skin biopsy samples	rtPCR*	*
He et al., 2022 [30]	PV	12	hsa-miR-125b-5p hsa-miR-146a-5p hsa-miR-148a-3p hsa-miR-150-5p hsa-miR-155-5p hsa-miR-181a-5p hsa-miR181b-5p hsa-miR-326 hsa-miR-338-3p hsa-miR-423-5p hsa-miR-424-5p hsa-miR-584-5p	Blood serum	rtPCR SYBR Green I	5S рРНК
Xu et al., 2020 [31]	PV	1	hsa-miR-338-3p	Peripheral blood mononuclear cells	rtPCR SYBR Green I	U6
Lin et al., 2018 [32]	PV	1	hsa-miR-338-3p	Peripheral blood mononuclear cells	rtPCR SYBR Green I	U6
Liu et al., 2018 [33]	PV	1	hsa-miR-338-3p	Peripheral blood mononuclear cells	rtPCR SYBR Green I	18srRNA
Wang et al., 2017 [34]	PV	124	hsa-miR-424-5p	Peripheral blood mononuclear cells	rtPCR SYBR Green I	U6

Note. * Detailed information on the method is not available. ВП — pemphigus vulgaris; ЛП — pemphigus foliaceus; ПЦР-РВ — real-time polymerase chain reaction.

Inclusion criteria for the control group: Persons of both sexes; age from 18 years; voluntary willingness of the subject to enroll in the study and a signed informed consent; consent to the processing of personal data.

Non-inclusion criteria: Failure to meet the inclusion criteria; history of severe concomitant or other autoimmune diseases; pregnancy and lactation; patient's refusal to participate in the study.

Non-inclusion criteria for the control group: Failure to meet the inclusion criteria; history of severe concomitant or other autoimmune diseases; unwillingness of to participate in the study.

Exclusion criteria: Patient's wish to withdraw from the study.

Exclusion criteria for the control group: Volunteer's wish to withdraw from the study.

Facilities

The study was conducted at the Clinic for Skin and Venereal Diseases named after V.A. Rakhmanov of the I.M. Sechenov First Moscow State Medical University of the Ministry of Health of Russia. All patients signed a voluntary informed consent before inclusion in the study and consent to the processing of personal data. The study material (blood) was transported at 4 °C to the laboratory at the Department of Molecular Pharmacology and Radiobiology named after Academician P.V. Sergeev, Faculty of Biomedical Sciences, N.I. Pirogov Russian National Research Medical University, Ministry of Health of Russia.

Study timing

The study was conducted between February 2023 and February 2024.

Description of the medical intervention

The study included 10 patients diagnosed with active pemphigus, including 6 with pemphigus vulgaris, 4 with pemphigus foliaceus, and 3 patients with pemphigus vulgaris in remission (more than 2 years) receiving maintenance dose of systemic glucocorticoids (10 mg). Disease severity was assessed using the pemphigus disease area index (PDAI). Patient characteristics are presented in Table 2. The control group included 9 healthy volunteers.

The diagnosis of pemphigus was based the clinical presentation, patient history, the presence of anti-Dsg1 and anti-Dsg3 antibodies in blood serum, the results of histological examination (intraepidermal, suprabasal acantholysis with the formation of slit-like clefts containing acantholytic cells) and direct immunofluorescence test (fixation of IgG and C3 complement component at the level of intercellular connections of cells of the stratum spinosum of the epidermis).

After the diagnosis was confirmed, the patients were treated as inpatients in the Dermatovenerology Department of the V.A. Rakhmanov Clinic for Skin and Venereal Diseases of Sechenov University. During hospitalization, peripheral venous blood was drawn from patients into tubes aerosolized with EDTA K2 anticoagulant. Within not more than 5 hours after blood sample collection, peripheral blood mononuclear cells were isolated by centrifugation in the Ficoll density gradient (density: 1.077 g/cm^3) (PanEco, Russia) in the form of an interphase ring. The obtained peripheral blood mononuclear cells were placed in cryotubes and stored at –80 ºC until further processing.

MicroRNA from mononuclear cells was isolated using a reagent for extraction of total RNA and microRNA from the Lira kit (Biolabmix, Russia) according to the manufacturer's methodology. Complementary DNA (cDNA) was prepared based on StemLoop technology using the OT-1 reverse transcription kit (Syntol, Russia). The reaction was performed separately for miRNA-338-3p and U6 (small nuclear RNA) using selected and synthesized specific RT primers (Syntol, Russia) (Table 3). Each reaction mixture contained 10 µL of 2.5x Reaction Mix, 1 µL of specific reverse transcription primer, 1 µL of MMLV, 3 µL of nuclease-free water, and 10 µL of RNA solution. The reaction was carried out according to the program: 37 °C for 30 minutes, 95 °C for 5 minutes, followed by cooling of the mixture.

The study of miRNA-338-3p expression was carried out for each tested sample by triplet rtPCR using the kit "2.5x Reaction Mix for rtPCR in the presence of SYBR Green I" (Syntol, Russia) in a final volume of 25 µL. In the course of rtPCR with patient samples it was experimentally established that 5-fold dilution of cDNA and synthesized primers is optimal for obtaining melt peaks and melt curves at the level of at least 1,000 relative fluorescence units in rtPCR for the selected sequences of forward and reverse primers (see Table 3), as well as for the cDNA under study. The final PCR mixture per 25 µL tube contained 10 µL of 2.5x Reaction Mix + SYBR Green I; 1 µL of 10 pmol/µL forward and reverse primer mix in 5-fold dilution; 0.5 µL of 25 mM $MgCl_2$; 1 µL of test cDNA in 5-fold dilution; 12.5 µL of dd H_2O. The amplification program was as follows: 10 min at 95°C; followed by 40 cycles lasting 30 s at 95°C, 20 s at 60°C, 1 min at 72°C; then 1 cycle of 5 min at 95°C. Amplification was performed using Bio-Rad iCycler iQ5 (Bio-Rad Laboratories, Inc., USA).

Evaluation of miRNA-338-3p expression level was based on its comparison with the expression of U6 small

Table 2. Clinical characteristic of patients with pemphigus in active stage of the disease

Patient number	Gender	Age, years	Diagnosis	Presence of rashes on the skin/ mucous membranes	Glucocorticoid dose, mg	PDAI	Disease severity
1	M	53	PV	Skin/mucous membranes	0	66	Severe
2	M	52	PF	Skin	0	22	Moderate
3	M	36	PV	Skin/mucous membranes	0	81	Severe
4	F	45	PV	Skin/mucous membranes	15	37	Moderate
5	F	64	PF	Skin	0	20	Moderate
6	F	56	PV	Skin/mucous membranes	0	34	Moderate
7	F	30	PF	Skin	0	25	Moderate
8	M	38	PV	Skin/mucous membranes	0	30	Moderate
9	F	52	PV	Skin/mucous membranes	0	98	Severe
10	M	38	PF	Skin	0	19	Moderate

Note. ВП — pemphigus vulgaris; ЛП — pemphigus foliaceus; PDAI — pemphigus disease area index.

Table 3. Primer nucleotide sequences

Primer	Nucleotide sequence
Reverse transcription primers	
RT-oligo-miR-338-3p	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGGTCG-3'
RT-oligo-U6	5'-GTCGTGTCTGAGGCTGACTGAGACCTATTCGCACCTGACACGACGGCCATGC-3'
Amplification primers	
Forward-miR-338-3p	5'-TACGTTGTTTTAGTGACTACGACCT-3'
Reverse-miR-338-3p	5'-GTGCAGGGTCCGAGGTATTC-3'
Forward-U6	5'-GGCCGCATACAGAGAAGATTA-3'
Reverse-U6	5'-CTGAGGCTGACTGAGACCT-3'

nuclear RNA, which was used as a control due to its stable expression level. Signal intensity was expressed in relative fluorescence units. Two fluorescence signal accumulation curves were plotted for each sample, miRNA-338-3p and U6. The rtPCR result was the cycle threshold (Ct) value, i.e., the number of cycles required for the fluorescence signal to cross the threshold (baseline level) because the Ct level is inversely proportional to the amount of target nucleic acid in the sample (the lower the Ct level, the higher the amount of target nucleic acid in the sample).

Ethical review

The study protocol was approved by the Local Ethics Committee of I.M. Sechenov First Moscow State Medical University of the Ministry of Health of Russia (protocol No. 02-23 dated January 26, 2023).

Statistical analysis

Statistical analysis of the obtained data was performed using Microsoft Excel, GraphPad Prism 10.1.1, and Jamovi 2.3.28. The relative expression level of miR-338-3p was calculated using the standard $2^{-\Delta\Delta Ct}$ methodology [39].

The Shapiro–Wilk test was used to assess the normality of distribution in the groups, and the Levene's test for homogeneity of variance was used to assess the homogeneity of the general sample. To detect differences between the groups, the Games–Howell post-hoc test was used. Statistical significance of differences in the mean values of expression levels in three groups was assessed using Student's *t*-test for independent samples. Correlation analysis was performed using the Spearman and Pearson correlation coefficient.

RESULTS

Study subjects

Ten patients with the active disease were included in the study: 6 (60%) diagnosed with pemphigus vulgaris and 4 (40%) diagnosed with pemphigus foliaceus. Seven out of 10 (70%) patients were diagnosed with moderate and 3 (30%) with severe disease according to PDAI. In 9 (90%) patients, the material for the study was sampled before initiation of pathogenesis-oriented therapy with systemic glucocorticoids; 1 (10%) patient was receiving glucocorticoid therapy at a dose of 15 mg at the sampling time. The group consisted of 5 (50%) men and 5 (50%) women, corresponding to a 1:1 ratio; the mean age was 46 ± 10.7 years. Detailed characteristics are shown in Table 2.

The group of patients in remission included 3 patients: 2 (66.7%) females and 1 (33.3%) male, with a mean age of 57 ± 8 years. In all three patients, the maintenance dose of glucocorticoids was 10 mg.

Nine conditionally healthy volunteers were included in the control group: 8 (88.9%) women and 1 (11.1%) man; the mean age was 36 ± 16.8 years.

Primary findings

The results of miR-338-3p expression in mononuclear cells of patients with the active disease, patients in remission, and healthy volunteers are presented in Table 4 to Table 6.

The mean expression level of miR-338-3p in the group of patients with the active disease was 8.64 (SD±5.72), 3.38 (SD±1.44) in the group of patients in remission, and 1.48 (SD±1.12) in the control group (Figure 1). All three groups showed normal distribution as evidenced by using the Shapiro–Wilk test: p=0.27; p=0.98 and p=0.58, respectively. Based on Levene's test for homogeneity of variance and Games–Howell test, a difference in mean expression levels was found between the group of patients with the active disease vs. the control group (p=0.008). A statistically significant increase in miR-338-3p expression level was detected in patients with the active disease vs. the group of healthy volunteers (p=0.002, effect size: 1.69) (see Figure 1).

In correlation analysis using Spearman and Pearson correlation coefficient, a statistically significant relationship between miR-338-3p expression level and PDAI index value was established (Spearman: r=0.624, p=0.030; Pearson: r=0.834; p<0.001) (Figure 2).

Table 4. Relative expression levels of miR-338-3p in peripheral blood mononuclear cells of patients with active pemphigus

Patient number	miR-338-3p Ct	U6 Ct	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
1	8.13	18.65	-10.52	-3.28	9.710
2	10.52	20.36	-9.84	-2.60	6.047
3	9.82	21.05	-11.24	-3.99	15.902
4	12.49	20.88	-8.40	-1.15	2.221
5	9.67	18.9	-9.23	-1.99	3.962
6	10.04	20.25	-10.22	-2.97	7.841
7	11.77	22.63	-10.86	-3.61	12.219
8	11.92	21.41	-9.49	-2.25	4.744
9	7.84	19.38	-11.54	-4.30	19.645
10	11.56	20.83	-9.27	-2.03	4.073
Mean					8.636

Table 5. Relative expression levels of miR-338-3p in peripheral blood mononuclear cells of patients with pemphigus in remission

Patient number	miR-338-3p Ct	U6 Ct	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
1	13.85	22.05	-8.20	-0.96	1.945
2	12.03	21.0	-9.00	-1.75	3.366
3	13.47	23.0	-9.52	-2.27	4.827
Mean					3.379

Table 6. Relative expression levels of miR-338-3p in peripheral blood mononuclear cells of control group of healthy volunteers

Patient number	miR-338-3p Ct	U6 Ct	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
1	24.02	30.6	-6.54	0.71	0.612
2	24.62	31.2	-6.63	0.62	0.651
3	25.78	29.4	-3.61	3.63	0.081
4	22.50	30.6	-8.06	-0.81	1.755
5	17.39	26.5	-9.10	-1.86	3.620
6	19.93	28.5	-8.55	-1.31	2.473
7	18.65	26.4	-7.73	-0.48	1.396
8	19.15	25.9	-6.73	0.52	0.698
9	22.84	31.1	-8.27	-1.03	2.037
Mean					1.480

There was no significant difference in miR-338-3p expression level between the group of patients in remission and the group of patients with the active disease (p=0.153, effect size: 1.01), but there was a difference between the group of patients in remission and the control group (p=0.038, effect size: 1.60). The lack of differences between the group of patients in remission and the group of patients with the active disease stage may be due to the strong heterogeneity of these two samples, as well as the small sample of patients in remission.

DISCUSSION

Pemphigus is a group of severe progressive autoimmune dermatoses. In the absence of timely pathogenesis-oriented therapy, the outcome of the disease can be fatal. Currently, the

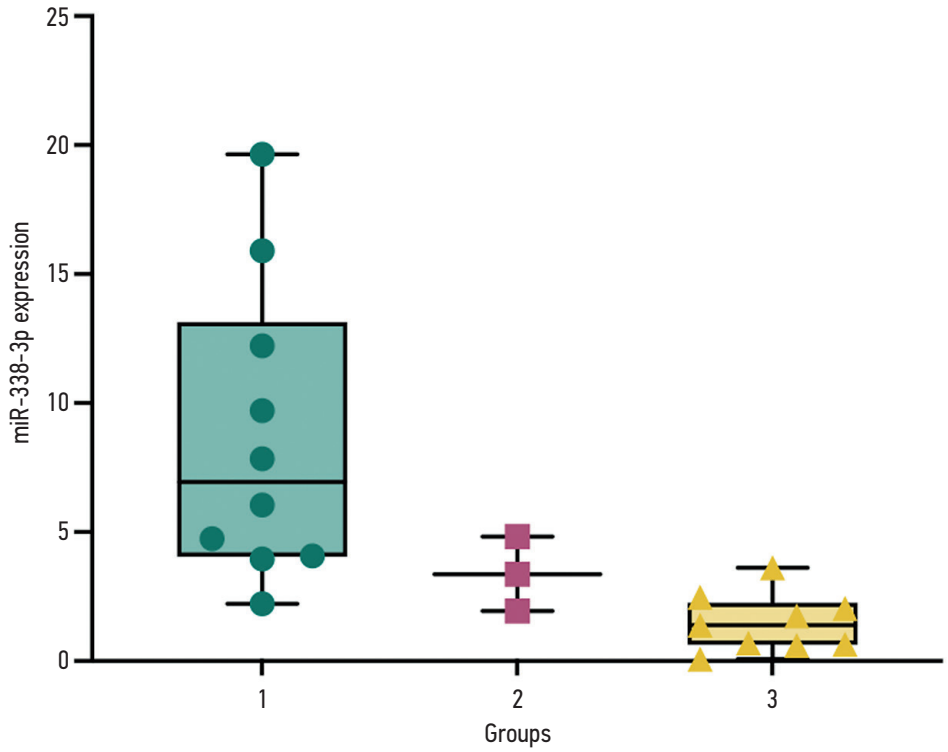

Fig. 1. Mean expression level of miR-338-3p. Groups: 1 — patients with active stage of the disease; 2 — patients in remission; 3 — control group of healthy volunteers.

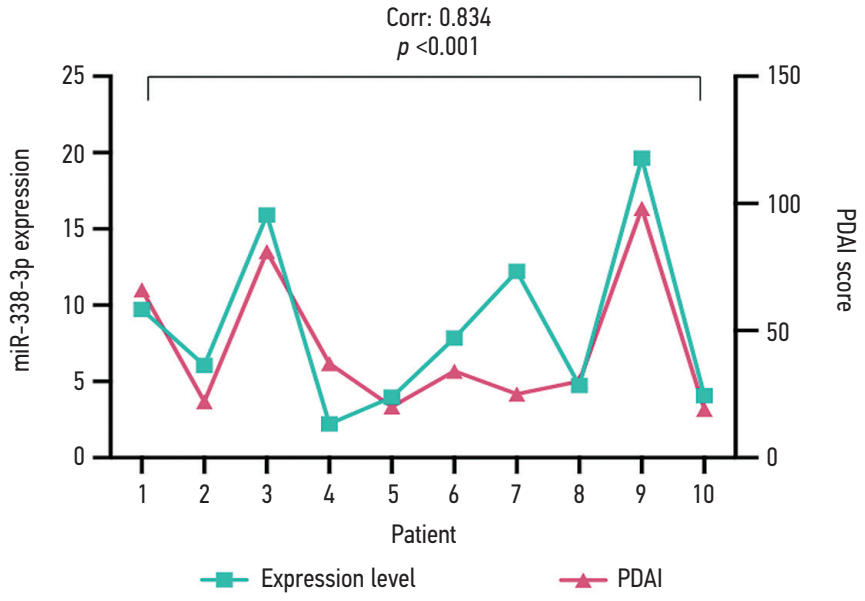

Fig. 2. Correlation between miR-338-3p expression level and PDAI index score.

diagnosis of pemphigus is based on the clinical presentation, histological examination, ELISA, direct/indirect IF. The gold standard for diagnosis of pemphigus is direct IF of the biopsy specimen with visualization of intercellular immunoglobulin G (IgG) and/or C3 complement regulator deposition in the epidermis, as well as histological examination of the skin biopsy specimen at the border between the blister and the unchanged skin [15]. However, these methods are often unavailable in clinical practice due to the lack of appropriate laboratory equipment and high cost. Thus, histopathology and direct IF are rather labor-intensive and time-consuming, but are not free of false-negative results [40].

ELISA has become a widespread and important method in the diagnosis of pemphigus due to its high sensitivity and

specificity, but false negatives [41] and false positives [42] are still possible. In addition, one study reported positive anti-Dsg-3 antibodies results in a healthy Egyptian population [43]. A recent discovery in the diagnosis of pemphigus is the indirect IF method using the BIOCHIP test system (Dermatology Mosaic 7, EuroImmun, Lubeck, Germany), which is characterized by a simplified protocol of use and lower cost compared with ELISA, as well as high sensitivity and specificity [44]. However, the BIOCHIP technology is not widely used and requires validation based on studies with a larger sample size [45].

Thus, modern diagnostic methods allow accurate diagnosis in most cases, but the frequency of misdiagnosis remains high, resulting in rapid disease progression and the development of severe complications due to delayed initiation of therapy, so the problem of finding new diagnostic methods for pemphigus remains relevant.

MicroRNAs are one of the new fields of modern studies in the search for new biomarkers [19]. MicroRNAs are proven to be stable *in vivo* because they are resistant to RNases, freeze/thaw cycles, and significant pH fluctuations [46], as well as to prolonged incubation at room temperature [47, 48]. In addition, the small size of microRNAs, compared to DNA and target mRNAs, makes them more stable and therefore less susceptible to degradation [46].

Despite the presence of different variations of PCR technology, quantitative real-time reverse transcription PCR (real-time RT-PCR) remains the most used method and the gold standard due to its high sensitivity, specificity, availability, and reproducibility [38].

If we consider the studies devoted to microRNA expression in the context of pemphigus, there is no unified approach to the choice of substrates, isolation protocols, and expression assessment, and there is no consensus on the microRNAs that are diagnostically significant for the disease (see Table 1). For example, in the study by He et al. [30] based on the study of microRNA expression in the plasma of patients with pemphigus revealed increased expression of 12 microRNAs, and the sensitivity and specificity of miR-584-5p and miR-155-5p expression for diagnosis amounted to 95.5 and 100%, respectively. The study also established a strong correlation between the expression level of miR-326 and the severity of pemphigus. Meanwhile, the study by Lin et al. [32], which focused on miR-338-3p, demonstrated that the expression level of miR-338-3p in pemphigus was not only elevated but also gradually decreased during effective therapy, although in the study by He et al. [30] miR-338-3p was eliminated from the experiment as a microRNA with low expression. In a recent study by Valentino et al. [28], it was first reported about downregulation of microRNA expression in pemphigus, particularly miR-148a-3p.

Despite ongoing research in various medical fields to find the role of microRNAs in pathological processes, they remain poorly understood in the pathogenesis of many diseases, since microRNAs are a relatively young field of science. However, the identification of disease-specific microRNAs and establishment of their role in diagnostic, prognostic, and treatment strategies offer increasing promise for modern medicine. Although many microRNAs in various substrates have been studied in bullous dermatoses in general [49] and in pemphigus in particular (see Table 1), according to experimental data, only miR-338-3p can be considered specific for this disease to date.

According to current data, the pathogenesis of pemphigus includes the formation of anti-Dsg3 and/or anti-Dsg1 autoantibodies, which leads to acantholysis of keratinocytes, but the molecular mechanism underlying the production of autoantibodies and regulation of the humoral response has not been fully determined [2, 50–52]. In their study, Lin et al. [32] hypothesized that the imbalance of Th1/Th2 cells in pemphigus may be a result of overexpression of miR-338-3p, which through transfection can downregulate the mRNA expression of *RNF114*, which in turn, as evidenced in studies, is involved in the regulation of apoptosis and activation of T cells [53, 54]. Moreover, the interaction between miR-338-3p and *RNF114* mRNA *in vitro* was confirmed by luciferase reporter assay [32]. Another study [33] hypothesized that another target gene for miR-338-3p is *TRADD*, which is also involved in apoptosis, because in both *in vitro* and *in vivo* studies, *TRADD* mRNA level was decreased with miR-338-3p overexpression.

Thus, miR-338-3p may be directly involved in the pathogenesis of the disease, which was a decisive factor in the choice of microRNAs for this study, because in other studies the hypotheses of microRNA involvement in disease pathogenesis and influence on target genes were predicted only using computer models and databases TargetScan, MiRanda MiRTarBase, etc. [28, 30]. [28, 30].

The present study demonstrated a significant difference in the expression level of miR-338-3p in the group of patients with the active disease compared to the control group. The expression level of miR-338-3p also correlated with the severity of the disease as assessed by PDAI. The data obtained are consistent with the results in the literature. For instance, in the study by Lin et al. [32] overexpression of miR-338-3p was also found, but the study included only patients with pemphigus vulgaris. Based on the literature search, we can conclude that this paper is the first study in Russian-language sources to investigate miRNA expression in pemphigus. The authors developed a technology for isolation and evaluation of miR-338-3p microRNA expression level, and the protocol (microRNA isolation, cDNA extraction, rtPCR) was based on the use of Russian-made reagents, which may make it more accessible in clinical practice.

Summary of the primary findings

The study found that the mean expression level of miR-338-3p in patients in the active stage of the disease was significantly higher than in the control group; the differences between the groups were statistically significant (p=0.002).

A correlation between miR-338-3p expression level and PDAI was found (p <0.001). The technology of isolation and evaluation of miR-338-3p expression level by rtPCR method was developed.

Study limitations

The limitation of our study was the small sample of patients, so a study with a larger sample is planned to test the preliminary results, as well as to establish reference intervals of miR-338-3p expression level for the development of a new method of diagnosis and objective assessment of the severity of pemphigus. A study of miR-338-3p expression in patients during treatment is also planned.

Another limitation of our study was the choice of one miRNA to be studied. We plan to identify correlations of miR-338-3p expression level with other microRNAs and laboratory parameters previously investigated according to the literature, as the establishment of additional correlations may become the basis for the creation of more accurate and disease-specific models, which can later be implemented in the form of a medical decision-making system.

CONCLUSION

Pemphigus is a group of rare and potentially fatal chronic autoimmune dermatoses. Currently, more and more studies are being conducted to find methods to control disease activity using various biomarkers that would allow monitoring disease progression and predicting response to therapy, which will optimize the choice of personalized therapy and avoid severe complications. Based on the results of our study and comparison of our findings with data from various literature sources, we can conclude that microRNAs play an important role in the development of pemphigus, and miR-338-3p expression may be central in the pathogenesis of the disease.

A more detailed study of microRNAs in patients with pemphigus, including a larger sample of patients and analysis of the variability of expression depending on the severity of the disease and the dose of glucocorticoids used, may become the basis for the development of new diagnostic methods, as well as methods of objective assessment of the severity of the disease, which will make it possible to apply more accurate and less invasive diagnostic methods, as well as to monitor and predict the course of the disease.

ADDITIONAL INFORMATION

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