

Original Paper

Psoriasis Treatment Changes the Expression Profile of Selected Caspases and their Regulatory MicroRNAs

Dominika Wcisło-Dziadecka^a Klaudia Simka^b Agata Kaźmierczak^c
Celina Kruszniewska-Rajs^c Joanna Gola^c Beniamin Grabarek^c
Jolanta Hybiak^d Catherine Grillon^e Urszula Mazurek^c Marek J. Łos^{e,f}

^aDepartment of Skin Structural Studies, Chair of Cosmetology, School of Pharmacy with the Division of Laboratory Medicine in Sosnowiec, Medical University of Silesia, Katowice, ^bDepartment of Internal Medicine, School of Public Health in Bytom, Medical University of Silesia, Katowice, ^cDepartment of Molecular Biology, School of Pharmacy with the Division of Laboratory Medicine in Sosnowiec, Medical University of Silesia, Katowice, Poland, ^dDepartment of Pathology, Pomeranian Medical University, Szczecin, ^eCentre de Biophysique Moléculaire, Orleans, France, ^fMałopolska Centre of Biotechnology, Jagiellonian University, Krakow, Poland

Key Words

adalimumab • Psoriasis • Caspase • Micro-RNA • Inflammosome • Clinical • Humira • Exemptia

Abstract

Background/Aims: Psoriasis, an autoimmune diseases of the skin, characterized by patches of abnormal/inflamed skin, although not usually life-threatening, it causes severe discomfort, esthetic impairments, and may lead to impaired social functions and social withdrawal. Besides UV-phototherapy, various anti-inflammatory treatments are applied, depending on the severity of symptoms. In 2008, adalimumab (fully humanized human anti-TNF antibody) was launched for the treatment of psoriasis. In the quest to better understand the pathomechanism of adalimumab's therapeutic effects, and the acquired resistance to the drug, we have investigated how its administration affect the regulation of the expression of selected caspases, including those activated by inflammosome. **Methods:** The research was initially carried out on normal human dermal fibroblasts (NHDF) treated with adalimumab for 2, 8 and 24 hours *in vitro*. Then, expression profile of genes encoding caspases and their regulatory micro-RNAs was determined with the use of oligonucleotide microarray. The validation of the microarray results was carried out by qRT-PCR. The *in vitro* study was followed by *ex-vivo* investigation of adalimumab's effects on the expression of caspase-6 in blood of the psoriatic patients. The samples were collected before, and 2 hours after adalimumab's administration and the analysis was determined by qRT-PCR. **Results:** The result of the analysis indicated that introduction of adalimumab to the NHDF culture resulted in the change of the transcription activity of genes encoding caspases and genes encoding miRNAs. The analysis revealed 5

different miRNA molecules regulating the expression of: CASP2, CASP3 and CASP6. There were no statistically significant differences in the expression of gene encoding caspase-6 in the patients' blood before and 2 hours after the anti-TNF drug administration. **Conclusion:** We have found that adalimumab administration affects caspases expression, thus they may be used as molecular markers for monitoring the therapy with the use of an anti-TNF drugs, including adalimumab. It is likely that the mechanisms responsible for changed expression profiles of genes encoding caspase-2,-3, and -6, may be caused by the upregulation of the respective microRNA molecules. Increased expression of genes encoding specific caspases may induce inflammatory processes, as well as trigger apoptosis. Furthermore, the proapoptotic activity of caspases may be enhanced by miRNA molecules, which exhibit proapoptotic function. The overexpression of such miRNAs was observed in our study.

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Introduction

Caspases are intracellular proteolytic enzymes, whose normal activity is crucial for maintaining homeostasis in human body. These enzymes belong to the cysteine protease family and are commonly known for their ability to regulate apoptosis, [1, 2] (either at its initiation and execution stages), as well as to induce inflammatory process [3-6]. However, the latest studies prove that caspases also participate in other cellular processes, i.e. proliferation, differentiation, cell migration [7-10] and pyroptosis [11]. Caspases also participate in autophagy [12-14]. The studies show that induction of autophagy may directly trigger apoptosis through caspase activation [15].

So far, 18 various caspases have been found, out of which 13 are present in humans [16]. Depending on the main function, the following pro-inflammatory caspases are distinguished: -1,-4,-5,-11,-12 (in mouse and in man, listed together) and proapoptotic ones, which, in turn, can be divided into initiator caspases: -2, -8, -9, -10 and effector caspases: -3, -6, -7 [16, 17]. The role of the other caspases is still not clear.

Caspases are generated in the form of inactive precursors – procaspases and require activation in order to form a functional enzyme [18]. Procaspases are formed by N-terminal prodomain and two subunits: a large one marked as p20 (with the mass of about 20kDa) and a small one - p10 (with the mass of about 10kDa). The active form of the enzyme is heterotetramer, formed by two large subunits p20 and two small subunits p10 [18]. Pro-inflammatory caspases and initiator caspases have long regulatory domains, which are characterized by a structural motive, called death domain (DD), enabling aggregation with other proteins. These caspases are activated within a large protein complexes, for example inflammasome, while recruitment of procaspases to the complexes is possible thanks to a relevant domains: DED (*death effector domain*) or CARD (*caspase recruitment domains*) [19-21]. Effector caspases have short prodomains and they are activated by initiator caspases [18].

Irregularities in the expression of genes encoding caspases are observed in the course of numerous pathological processes, including, in tumors [22], neurodegenerative and autoimmune diseases [10]. For instance, in psoriatic lesions there occurs overexpression of caspase-5 [23] and irregular activity of caspase-1 is observed in the course of many other pro-inflammatory diseases [10].

Autoimmune diseases, among others, psoriasis, rheumatoid arthritis or inflammatory bowel diseases, pose a serious challenge for contemporary medicine. Incidence of these diseases is still growing. The autoimmune diseases are manifested by chronic inflammatory condition, the etiopathogenesis of which is still not fully known [24]. Hence, lack of good knowledge of etiopathogenesis impairs the development of an effective treatment. However, recent studies of molecular mechanisms in psoriatic patients give a new promise for effective therapy [25-27].

Therapy of autoimmune diseases mainly involves targeting of the underlying inflammatory process. To this end, biologic drugs with anti-TNF effect are used. TNF (*tumor*

necrosis factor- α) plays a crucial role in the pathogenesis of many inflammatory diseases [28]. In the course of psoriasis, it causes keratinocytes activation and their excessive proliferation. It is also responsible for an increased proliferation of T- and B-lymphocytes and differentiation of B-lymphocytes. Blocking of the TNF activity ensures high efficacy of therapy [29]. An example of the biological drug with anti-TNF activity is adalimumab (Humira, Exemptia)- a fully-humanized recombinant monoclonal antibody, generated through expression in the ovarian cells of a Chinese hamster. adalimumab neutralizes the biological effect of TNF by forming complexes with it, hence blocking its ability to engage specific receptors. The anti-TNF therapy is effective, however, it causes some side effects arising mostly from its immunosuppressive functions [30]. Moreover, patients subjected to long-term therapy with the use of anti-TNF drugs, often develop therapy resistance [31].

In order to increase the efficacy of the therapy, it is very important to pinpoint the causes for the loss of patients' response to treatment, as well as to choose a molecular marker(s), which could indicate decreased efficacy of treatment before occurrence of phenotypic symptoms. It would enable to early diagnose the patient's decreasing response to treatment and choose another form of therapy before re-occurrence of the disease symptoms.

Numerous studies have lately shown the potential of microRNAs as diagnostic- and/or prognostic markers. MiRNAs are short (about 23-nucleotide), single-stranded, non-coding fragments of RNA of endogenous origin, which are responsible for regulation of ~1/3 of all human genes [32]. miRNAs expression pattern is tissue-specific. Hence, the level of miRNA expression depends on the tissue, but it is also affected by the cell type, its metabolic state and pathophysiological changes, which occur i.e. during disease [33]. MiRNAs are responsible for negative posttranscriptional regulation of gene expression [34, 35]. This is achieved by miRNA's binding to the 3'UTR (untranslated) region of the target transcript. The complementarity of the sequences of these two molecules plays the key role in the said process. There are two possibilities to regulate gene expression through miRNA: (i) transcript degradation and (ii) translation inhibition. One miRNA type may regulate transcription of hundreds of genes, while one gene may be regulated by many types of miRNA molecules [34]. Changes in the expression of genes encoding caspases and miRNAs involved in their regulation after treatment with adalimumab have not been studied so far.

MicroRNAs may constitute highly-specificity and highly-sensitivity markers for ongoing metabolic changes. Moreover, they are stable and resistant to ribonucleases, changes of temperature and pH. They are characterized by high availability and easy acquisition, with simultaneous low invasiveness of miRNA-dependent tests. Furthermore, the miRNA molecule expression may be analyzed with the use of basic molecular biology tools, such as qRT-PCR or oligonucleotide microarray techniques [32]. Results of studies conducted by Hlavaty and colleagues show that diversified response of patients to anti-TNF therapy is connected with caspase-9 polymorphism [36]. Thus, it is possible that the activity of other caspases also correlate with patients' sensitivity to such therapy. Consequently, the study was guided by a hypothesis that a change in transcription activity of genes encoding caspases may be connected with the mechanism of patients' lost response to adalimumab therapy. With regard to the foregoing, the genes encoding caspases and miRNAs regulating their activity could become a molecular markers and/or additional targets in the anti-TNF therapy. The aim of the study was to assess whether the genes encoding caspases, or miRNAs that change their transcription activity are affected by anti-TNF therapy.

Materials and Methods

Material used for the study

Material used to study expression of genes encoding caspases involved a line of normal human dermal fibroblasts (NHDF) cell line and peripheral blood of the psoriatic patients undergoing the anti-TNF therapy.

Cell culture

The NHDF cells (Lonza, Basel, Switzerland) from the 4th passage were cultured at 6x10⁵ cells per culture plate (area of 57 cm²). The cells were grown for 48 hours in Direct Heat CO₂ Incubator (Thermo Fisher Scientific, Waltham, MA, USA) under standard conditions: temp. 37°C, at humidity of 95% and 5% CO₂ enriched atmosphere. The dedicated FBM culture medium (Fibroblast Basal Medium; Lonza) was used, with an addition of insulin and gentamicin (FGM™ SingleQuots™; Lonza). adalimumab, in its final concentration of 8 µg/ml medium was added to the culture. The cells were treated with adalimumab for 2, 8 and 24 hours (2H, 8H, 24H). Three biological replicates were performed. The control (C) consisted of normal human dermal fibroblasts, incubated in the same conditions as the tested group, without addition of adalimumab.

Peripheral blood

Peripheral blood was taken from 8 psoriatic patients, treated with the anti-TNF biological drug – adalimumab. The patients took 40 mg of adalimumab in pre-filled syringes every two weeks. Once per 3 months, the patients' had their skin examined and routine blood tests (so-called monitoring) were performed. Two blood samples were taken each time during such monitoring: the first one directly before adalimumab administration and the second one two hours later. Tested samples were taken from various monitoring tests (a and b), during which different PASI (*Psoriasis Area and Severity Index*) values were noted (Table 1).

Cytotoxicity test

The cytotoxicity of adalimumab in the culture of NHDF cells was evaluated with the use of XTT test (*In vitro* Toxicology Assay Kit, XTT based; Sigma-Aldrich, St Louis, MO, USA).

Total RNA extraction

Extraction of total RNA from NHDF was performed with the use of TRIzol® (Invitrogen Life Technologies, Carlsbad, CA, USA) reagent, according to the producer's protocol. Then RNA was purified with the use of RNeasy Mini Kit (QIAGEN, Hilden, Germany) and DNase I (Fermentas International Inc., Burlington, ON, Canada). The quantitative and qualitative evaluation of extracts was performed with the use of GeneQuant II (Pharmacia LKB Biochrom Ltd., Cambridge, UK) spectrophotometer and the agarose electrophoresis method. Extraction of total RNA from patient's blood was conducted with the application of the phenol-chloroform method, using the Fenzol (A&A Biotechnology, Gdynia, Poland) reagent. The quantitative and qualitative evaluation of extracts was performed with the use of GeneQuant II (Pharmacia LKB Biochrom Ltd., Cambridge, UK) spectrophotometer and the agarose electrophoresis (not shown).

Oligonucleotide microarray procedure

Expression profile of genes encoding caspases was determined with the use of oligonucleotide microarray HGU-133A 2.0 (Affymetrix, Santa Clara, CA), according to the producer's protocol. 250 ng of total RNA was used for the procedure. The first stage involved synthesis of cDNA, then the biotinylated RNA was synthesized, purified and fragmented (GeneChip 3'IVT Express Kit, Affymetrix, Santa Clara, CA, USA). RNA hybridization with the microarray was performed for 16h in the temperature of 45°C at 60 RPM (rotations per minute). The Fluidics Station 450 (Affymetrix) and Hybridization Wash and Stain Kit (Affymetrix) were used to stain the microarray with streptavidin-phycoerythrin. The intensity of transcriptome fluorescence was determined with the use of Affymetrix Gene Array Scanner 3000 7G and Gene Chip® Command Console® Software (Affymetrix, Santa Clara, CA).

Table 1. PASI values for patients under study observed during various monitoring tests

PASI value	Patient							
	A	B	C	D	E	F	G	H
Monitoring a	21	14	15	18	16,9	18	16	1,9
Monitoring b	5	5,7	5	5	-	6	5	1

miRNA microarray procedure

Expression profile of genes encoding microRNA molecules was determined with the use of microarray Gene Chip® miRNA 2.0 Array (Affymetrix), according to the producer's protocol (FlashTag™ Biotin HSR RNA LabelingKits). 750ng of total RNA was used for the analysis. Evaluation of the staining efficiency was made with the use of ELOSA QC Assay (FlashTagBiotin HSR RNA Labeling Kit; Affymetrix), according to the producer's protocol. RNA hybridization with the microarray was performed for 16h in temperature 48°C at 60 RPM (rotations per minute). Washing and staining of the microarray were made according to the manufacturer's protocol.

QRT-PCR procedure

Analysis of the expression of the caspase-6 encoding gene was performed by qRT-PCR. The CASP6 gene expression in the NHDF (2H,8H,24H) was identified comparing to the control group. The reaction was conducted with the use of QuantiTect SYBR Green RT-PCR kit (Qiagen, Valencia, CA, USA) and primers with complementary sequences to the tested gene: FH1_CASP6 and RH1_CASP6 (Sigma-Aldrich). The Opticon™ DNA Engine SequenceDetector (MJ Research Inc., Watertown, MA, USA) was used. Thermal profile of the reaction included reverse transcription(50 °C, 30 min), polymerase activation (94°C, 15 min), and then 40 cycles of: denaturation (94°C, 15 s), annealing (60°C, 30 s), extension (72°C, 45 s).

Statistical and bioinformatic analyses

The comparative analysis covering the results of the oligonucleotide microarray experiment was performed with the use of PL-Grid Infrastructure and GeneSpring 12.6.1 program (Agilent Technologies, Inc., Santa Clara, CA, USA). The statistical analysis of results obtained from miRNA microarray was conducted with the use of miRNAQCtool version 1.1.10 (Affymetrix) and Transcriptome Analysis Console 2.0 (Affymetrix) software. The miRNA molecules that have potential ability to regulate expression of genes encoding caspases were identified in the microRNA database (<http://www.microrna.org>). The miRanda-mirSVR algorithm, a combination of the miRanda and mirSVR algorithms, was used to find the relevant miRNA molecules. The statistical analysis of qRT-PCR results was performed with the use of Statistica 12 PL (StatSoft, Tulsa, Oklahoma, USA). $p < 0.05$ was assumed as a level of statistic significance. The statistical analysis of the results comprised the one-way ANOVA test ($p < 0.05$) and the post-hoc Tukey's test in order to identify the statistically important differences in the expression of genes encoding caspases, between the groups under study (fibroblasts exposed for 2, 8 and 24 h to adalimumab), compared to the control. In order to identify the statistically important differences in the expression of gene encoding caspase-6 in the patients' blood before the anti-TFN drug administration and 2 h after its administration, the Mann-Whitney U test was conducted.

Results

Cytotoxicity test

The XTT did not show any cytotoxic activity of adalimumab towards normal human dermal fibroblasts (NHDF), data not shown.

Analysis of expression of genes encoding caspases

The analysis included assessing the diversity of transcripts in normal human skin fibroblasts (NHDF) exposed to adalimumab by: 2, 8 and 24h (2H, 8H, 24H) compared to the control. The first stage of the differentiation degree assessment was the analysis of the expression profile of the ID mRNA of caspases encoding genes by hierarchical clustering using Euclidean distances. The result of the analysis indicated that introduction of the tested drug to the culture resulted in the change of the transcription activity of genes encoding caspases. The most prominent differences in the expression profile of this set of genes was observed after 2 h of incubation, whereas, after 24 h the profile returned almost to the level observed in the control sample (Fig. 1).

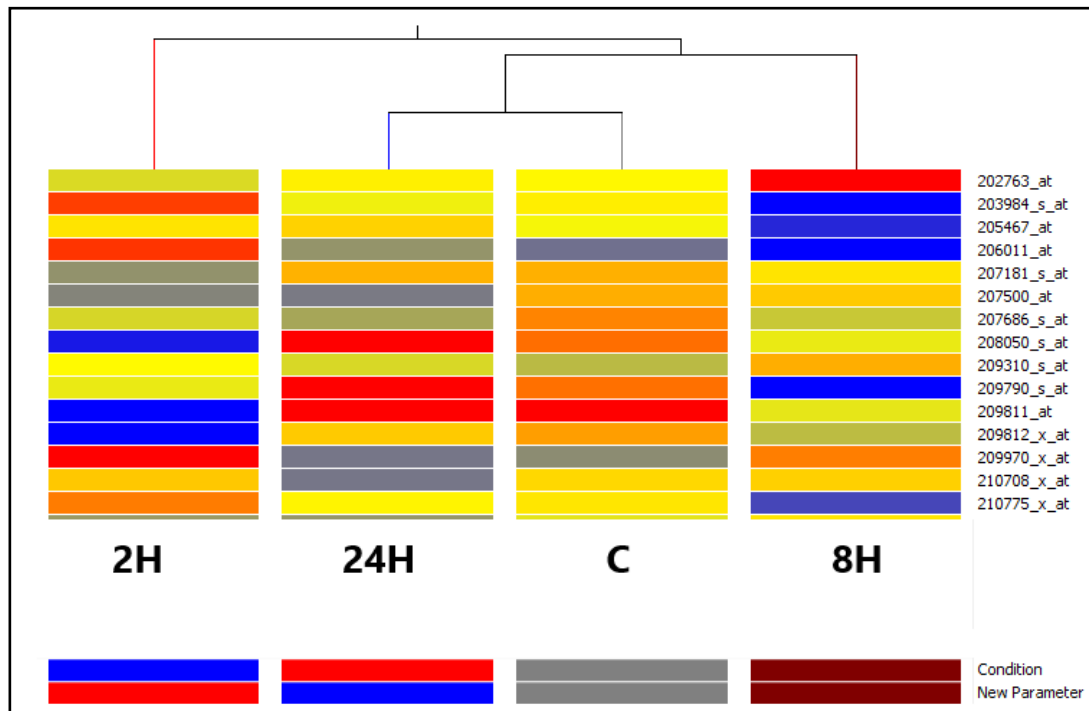


Fig. 1. Hierarchical clusterization of transcriptomes for genes related to caspases in normal human dermal fibroblasts (NHDF), depending on the time of exposure to adalimumab in vitro. C=control (NHDF not treated with adalimumab), 2H, 8H, 24H represent respectively: 2 h, 8 h, and 24 h incubation time with adalimumab.

The one-way ANOVA analysis with Benjamini-Hochberg's correction, showed the statistically important differences in the expression of 11 transcripts (Table 2). The differentiating transcripts corresponded to 6 genes encoding the following caspases: CASP1, CASP2, CASP3, CASP4, CASP6, CASP9. Post-hoc Tukey's test revealed mRNAs differentiating the individual transcriptome groups (C, 24H, 8H, 2H) (Table 3). The number of differentiating mRNA IDs depended on the time of exposure to adalimumab. When comparing the average fluorescence signals for 2H vs C - 5 differentiating mRNA IDs were identified, for 8H vs C - 7 mRNA IDs, and for 24H vs C - 1 mRNA ID.

A next stage involved evaluation of mRNA ID characteristic for studied groups of transcriptomes (2H, 8H and 24H) in comparison to control, with the use of Venn diagram (Fig. 2, Table 4). adalimumab administration immediately changed 5 mRNA IDs of caspases, including 4 specific only for 2H, and 1 common for 2H and 8H. After 8 hours of adalimumab administration - 5 specific

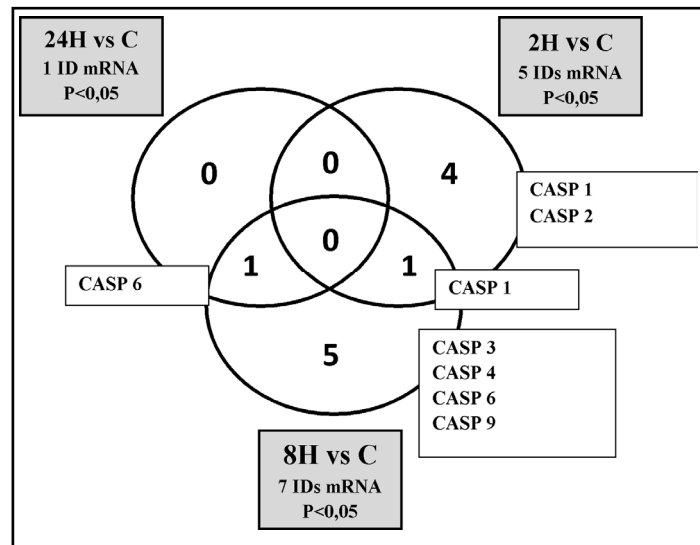
Table 2. Quantitative changes in mRNA expression upon challenging NHDF with adalimumab. A) Number of mRNA IDs differentiating transcriptomes of all tested groups, determined with the use of a one-way ANOVA test with Benjamini-Hochberg's correction

ID mRNA	P<0.05	P<0.02	P<0.01	P<0,0050	P<0.0010
25	11	10	10	10	8

Table 3. Quantitative changes in mRNA expression upon challenging NHDF with adalimumab. mRNAs differentially expressed among studied groups; significance determined with the post-hoc Tukey's test.

Transcriptome group	mRNA			
	C	2H	8H	24H
C	11	5	7	1
2H	6	11	7	6
8H	4	4	11	7
24H	10	5	4	11

Fig. 2. The Venn diagram showing mRNA IDs characteristic for transcriptomes of NHDF exposed to adalimumab in vitro in comparison to control. C=control, 2H, 8H, 24H represent respectively: 2 h, 8 h, and 24 h incubation time with adalimumab. The statistical evaluation was performed with the use of the post-hoc - Tukey's test (Table 4 legend).



mRNA IDs were observed. After 24-hour incubation with adalimumab, only 1 differentiating mRNA ID for caspase-6 was observed, compared to the control sample, common for 8H and 24H. No mRNA ID that would be common for all tested groups of fibroblasts was observed.

Analysis of miRNA expression profile

Statistical analysis of the results of miRNA microarray showed statistically significant ($p < 0.05$) change in the expression of 24 various miRNA molecules in NHDF, treated with adalimumab for: 2, 8 and 24h when compared with the control (Fig. 3).

Bioinformatic analysis

In order to identify which out of 24 differentiating miRNA molecules may regulate the expression of genes encoding caspases (CASP1, CASP2, CASP3, CASP4, CASP6 and CASP9), miRanda-mirSVR algorithm was used, based on microRNA.org database (<http://www.microRNA.org>).

The analysis revealed 5 different miRNA molecules regulating the expression of: CASP2 (three miRNAs), CASP3 (two miRNAs) and CASP6 (one miRNA) (Table 5).

Table 4. Quantitative changes in mRNA expression, with the indication of caspase family member and transcript name, upon challenging NHDF with adalimumab. Post-hoc Tukey's test results showing mRNA IDs differentiating studied groups - legend for the Venn diagram (Fig. 2). FC - fold change

Compared groups	Gene symbol	ID mRNA	p-value correction	p-value	FC (log ₂)
2H vs C - 5mRNA					
2H vs C	CASP2	209812_x_at	4,60E-02	2,95E-02	- 1.2782
	CASP1	211366_x_at	6,60E-09	1,06E-09	1.4062
	CASP1	211367_s_at	8,03E-06	2,25E-06	1.5849
	CASP1	211368_s_at	1,96E-10	7,82E-12	1.4671
2H vs C and 8H vs C	CASP1	209970_x_at	3,80E-10	4,56E-11	1.3233 1.1636
8H vs C - 7mRNA					
8H vs C	CASP3	202763_at	5,02E-08	1,00E-08	1.2272
	CASP9	203984_s_at	0,003068	1,47E-03	- 1.2692
	CASP9	210775_x_at	0,00365	1,90E-03	- 1.1922
	CASP6	211464_x_at	1,15E-05	3,69E-06	- 1.2842
2H vs C and 8H vs C	CASP4	213596_at	8,24E-05	2,96E-05	1.4506
	CASP1	209970_x_at	3,80E-10	4,56E-11	1.3233 1.1636
8H vs C and 24H vs C	CASP6	209790_s_at	7,01E-08	1,68E-08	- 1.3559 1.1529
24H vs C - 1 mRNA					
8H vs C and 24H vs C	CASP6	209790_s_at	7,01E-08	1,68E-08	- 1.3559 1.1529

Fig. 3. The Venn diagram showing miRNAs characteristic for transcriptomes of NHDF exposed to adalimumab in vitro in comparison to control. C=control, 2H, 8H, 24H represent respectively: 2 h, 8 h, and 24 h incubation time with adalimumab. The statistical evaluation was performed with the use of the post-hoc – Tukey’s test.

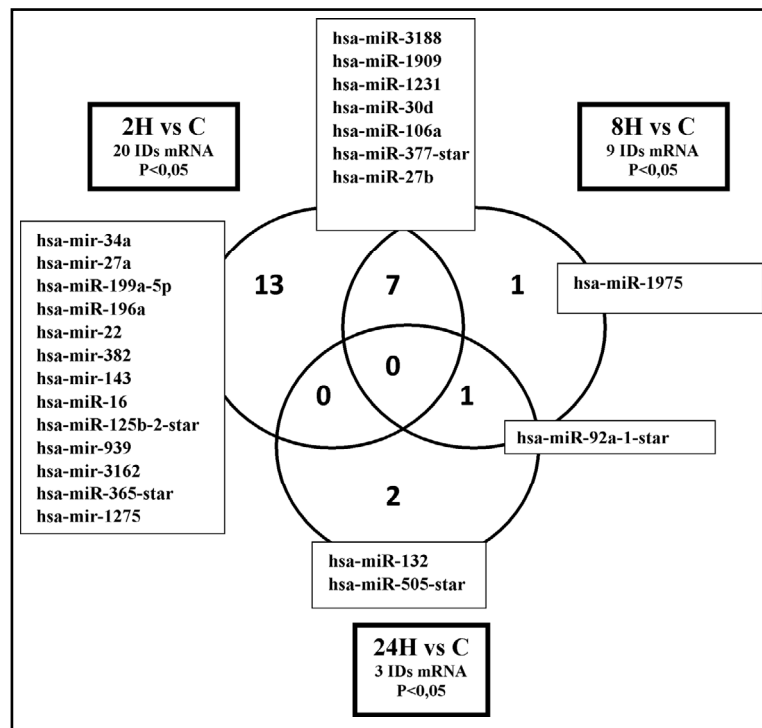


Table 5. Changes in the expression of CASP2, CASP3, CASP6 genes and miRNAs capable of regulating expression of these genes in fibroblasts exposed to adalimumab for 2, 8 and 24h, when compared to the control sample

Gene symbol	CASP 2 209812_x_at				hsa-mir-34a				hsa-mir-377				hsa-mir-106a				CASP 3 202763_at				hsa-mir-30d				hsa-mir-382				CASP 6 209790_s_at				CASP 6 211464_x_at				hsa-mir-106a			
	p-value	FC (log2)	p-value	FC (log2)	p-value	FC (log2)	p-value	FC (log2)	p-value	FC (log2)	p-value	FC (log2)	p-value	FC (log2)	p-value	FC (log2)	p-value	FC (log2)	p-value	FC (log2)	p-value	FC (log2)	p-value	FC (log2)	p-value	FC (log2)	p-value	FC (log2)	p-value	FC (log2)										
2H	0.029	↓1.278	0.043	↑16.52	0.023	↑12.7	0.001	↑12.09					0.015	↑12.19	0.009	↑12.82																0.001	↑12.09							
8H					0.013	↑12.32	0.007	↑12.45	1,00E-08	↓11.227	0.045	↓12.55					1,68E-08	↓1.356	3,69E-06	↓1.284	0.007	↑12.45																		
24H																	1,68E-08	↑1.152																						
mirSVR			↓0.2232		↓0.3218		↓0.4468						↓0.3918		↓1.289																	-0.1209								

2 hours after adalimumab administration, 3 miRNAs showed overexpression: hsa-mir-34a, hsa-mir-377, hsa-mir-106a potentially capable of regulating expression of gene encoding caspase-2. At the same time, lower expression of the tested gene was observed. After 8hours, expression of the gene encoding caspase-2 reached the level observed in the control.

The hsa-mir-106a molecule also shows potential capability to regulate caspase-6. Increased expression of hsa-mir-106a occurred with the lower expression of CASP6 after 8h of incubation with adalimumab. The return of miRNA expression to the level observed in the control after 24h after administration of the drug corresponded to the increase in the expression of the caspase-6 encoding gene.

One miRNA molecule was determined for caspase-3 - hsa-mir-30d. Expression of both CASP3 gene and hsa-mir-30d was down regulated in cells treated with adalimumab for 8h comparing to control cells.

Table 6. Result of the post hoc Tukey's test for CASP6 in the tested groups (2H, 8H, 24H), compared to the control sample

Time	2H	8H	24H
p-Value	NS	0,002748	0,047468

Fig. 4. The expression profile of CASP6 gene in normal human dermal fibroblasts (NHDF), depending on the time of exposure to adalimumab in vitro. C=control (NHDF not treated with adalimumab), 2H, 8H, 24H represent respectively: 2 h, 8 h, and 24 h incubation time with adalimumab. The expression level of caspase-6 mRNA was determined by qRT-PCR.

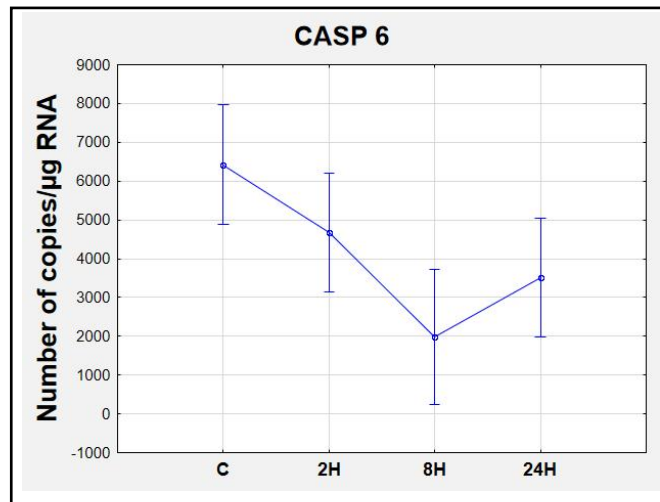
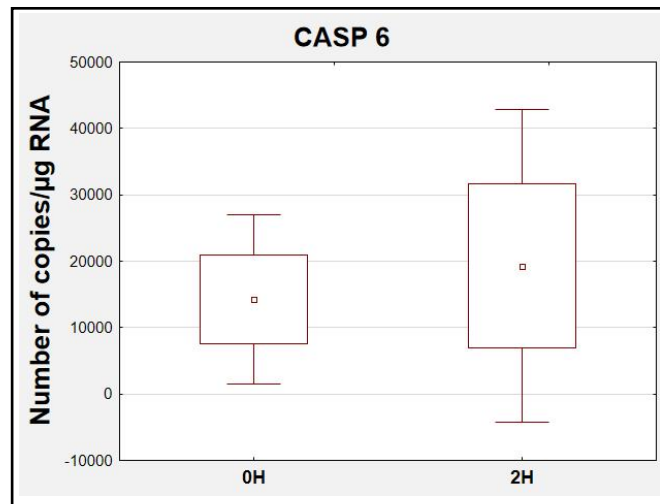


Fig. 5. Expression of gene encoding caspase-6 in the psoriatic patients' blood. The blood was collected before (0H) and 2 hours (2H) after adalimumab administration. The expression level of caspase-6 mRNA was determined by qRT-PCR.



Validation of the oligonucleotide microarray results

The last stage of the analysis was the validation of the microarray results with the use of qRT-PCR method. Only CASP6 gene was analyzed, because during the microarray experiment statistically significant changes in the expression profile of this gene were observed in the groups under study (2H,8H,24H) when compared to the control. Moreover, it was shown that CASP6 expression might be regulated by hsa-mir-106a molecule. The results showed a statistically significant difference in the expression of the tested gene in the cells treated for 8 and 24 hours with adalimumab, when compared to the control sample (Table 6, Fig. 4).

Analysis of the expression of gene encoding caspase-6 in patients' blood

The analysis did not show any statistically significant differences in the expression of gene encoding caspase-6 in the patients' blood before and 2 hours after the anti-TNF drug administration (Fig. 5). The expression level of caspase-6 mRNA was determined by qRT-PCR as described in the method section.

Discussion

The anti-TNF therapy constitutes one of the most effective therapies of the pro-inflammatory diseases. It is mainly used in the treatment of serious forms of psoriasis and other pro-inflammatory diseases, as well as in those cases where standard anti-inflammatory therapies are ineffective. adalimumab, an anti-TNF drug, is a fully-humanized monoclonal antibody. The adalimumab and other anti-TNF drugs are highly effective, however, they cause many side effects [30]. Moreover, some patients treated with anti-TNF drugs do not respond to the therapy. Many of them show the primary non-response (PNR), whereas some others show a loss of response to treatment (LOR) after some time of effective therapy [31]. Reduced response to anti-TNF treatment may be caused by polymorphism in genes encoding proteins associated with apoptosis, such as caspase-9 or NLRP3 gene encoding one of the proteins that constitutes an element of inflammasome [36]. Hence, molecular markers, which could indicate decreased efficiency of treatment before occurrence of symptoms, are being sought.

This work focused on the determination of expression profile of genes encoding caspases and miRNA in dermal fibroblasts exposed for adalimumab for 2-, 8- and 24h. The oligonucleotide microarray technology facilitates simultaneous evaluation of expression of many genes, including those encoding caspases and miRNA molecules. Evaluation of the gene expression at various intervals since drug administration enabled full analysis and more accurate identification of the mechanism responsible for changes.

The results obtained in the course of the study showed that adalimumab affected the transcription activity of genes encoding caspases in dermal fibroblasts, which may suggest that genes encoding caspases participate in cell response to the administered drug. Changes in the expression profile of genes encoding caspases observed in fibroblasts depended on time of exposure to tested drug. adalimumab showed its activity as early as 2h after its administration, however, the greatest variations in the expression profile of the tested genes was observed after 8 hours of incubation, when compared to the control sample. After 24h, the expression profile of genes encoding caspases returns almost to the pretreatment level, or level observed in the control cells. This may be indicative of adaptive mechanisms triggering in cells, in response to the drug treatment, or simply represent the end of the response. However, it is also possible that the effect may be connected with the reduced concentration of the drug in the medium due to the fact that drug molecules adsorb to the plate surface.

Expression of genes is strictly regulated on various levels, including numerous epigenetic mechanisms. Initial studies of Carmen Castro-Villegas and colleagues [37] proved that circulating miRNAs could be used as diagnostic markers in monitoring anti TNF drug therapies in patients with rheumatoid arthritis. The bioinformatic analysis showed several possible interactions between differentiating mRNAs and miRNAs described in this paper. Hsa-mir-34a, hsa-mir-377, hsa-mir-106a may be responsible for changes in the expression of gene encoding caspase-2, whereas hsa-mir-30d and hsa-mir-106a molecules may regulate activity of genes encoding: caspase-3 and caspase-6, respectively, however, this mechanism must be further examined. None of the differentiating miRNAs found in this study may interact with mRNA of genes encoding caspases: -1, -4 and -9.

Validation of the results of the oligonucleotide microarray with the use of qRT-PCR confirmed the effects of adalimumab on the expression of gene encoding caspase-6 in NHDF

cell line. Caspase-6 is one of the effector caspases in apoptosis. It is responsible, among others, for inactivation of nuclear structural proteins - lamins, which leads to the condensation of chromatin and fragmentation of cell nucleus [38]. Two isoforms of caspase-6 have been identified, which occur as a result of an alternative splicing of the gene [39]. Caspase-6 expression and activity increases early after excitotoxic event in neurons. With regard to the foregoing, it is assumed that caspase-6 may also play an initiating role in an apoptotic cascade [40].

Expression of gene encoding caspase-6 in dermal fibroblasts changes with the time of fibroblasts incubation with adalimumab. The mechanism underlying these changes may be connected with miRNA molecules. For the analysis 19 miRNA molecules were chosen from the microRNA.org (<http://www.microRNA.org>) database. According to miRanda-mirSVR algorithm - may be capable of regulating the gene encoding caspase-6, among them being Hsa-mir-106a molecule. Hsa-mir-106a plays either the role of neoplastic process suppressor, or an oncogene, depending on tissue type [41-43]. The studies showed that the molecule is capable of promoting apoptosis [44].

The mirSVR software interaction analysis for hsa-mir-106a and caspase-6 indicates poor consensus for interaction between target mRNA and miRNA molecule. However, the obtained results show certain correlation between the direction of expression of both molecules. The correlation between the direction of expression of the gene encoding caspase-6 and hsa-mir-106 gene may indicate the regulating role of this miRNA, however this thesis requires further studies. The studies revealed that adalimumab did not affect the profile of caspase-6 expression in psoriatic patients' blood 2h after drug administration.

In this study for the first time expression profile of genes encoding caspases and miRNAs that may regulate their activity under anti-TNF treatment were assessed. Summing up: the obtained results initially confirm that caspases may participate in cell response to the administered drug, thus, in the future they may be used as molecular markers supplementing monitoring the therapy with the use of an anti-TNF drugs, including adalimumab. It is likely that the mechanism responsible for the change of the expression profile of genes encoding caspases-2,-3 and -6 may be connected with microRNA molecules. Increased expression of genes encoding specific caspases may induce inflammatory processes, as well as trigger apoptosis. Furthermore, the proapoptotic activity of caspases may have been enhanced by miRNA molecules, which exhibit proapoptotic function. The overexpression of such miRNAs was observed in this study.

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Disclosure Statement

The authors declare that no conflicts of interest exist.

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