Original paper

In silico analysis of CpG islands and miRNAs potentially regulating the JAK-STAT signalling pathway

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Abstract

Introduction: Searching for new therapeutic possibilities constitutes a challenge for modern medicine and an answer to better understanding of molecular mechanisms of pro-inflammatory diseases. The JAK-STAT pathway plays an important role in the inflammatory processes, which is supported by the fact that its inhibitors are used to treat, for instance, psoriasis and rheumatoid arthritis.

Aim: To determine whether the epigenetic mechanisms – methylation of gene promotion regions and miRNAs may serve as a new therapeutic strategy for JAK-STAT pathway inhibition.

Material and methods: Basing on MethPrimer (plus CpG Island Prediction) program and microrna.org database of the said mechanism in the regulation of the JAK-STAT signalling pathway, the gene expression was performed, indicating or excluding the possibility of their use as new potential therapeutic strategies.

Results: A different number of CpG islands (CGI) for each gene (JAK1-4 CGI; JAK2-2 CGI; JAK3-5 CGI, TYK2-6 CGI; STAT1-2 CGI; STAT2-1 CGI; STAT3-3 CGI; STAT5A-4 CGI; STAT5B-3 CGI) might be a new therapeutic goal. What is more, our results show that genes associated with JAK-STAT signalling pathways can be regulated by miRNAs (JAK1-42 miRNAs; JAK2-47 miRNAs; JAK3-15 miRNAs, TYK2-4 miRNAs; STAT1-17 miRNAs; STAT2-30 miRNAs, STAT3-36 miRNAs, STAT4-15 miRNAs; STAT5A-10 miRNAs; STAT5B-23 miRNAs).

Conclusions: The epigenetic mechanisms of the regulation of the JAK-STAT signalling pathway gene expression constitute a promising new therapeutic strategy for treatment of those diseases, during which disorders are observed in gene expression models of the analysed signalling pathway.

Key words: DNA methylation, miRNAs, JAK-STAT cascade, epigenetic, in silico analysis, modern treatment strategy.

Introduction

Development of the molecular biology technologies enabled familiarisation with and better understanding of the role of molecular signalling pathways in inducing the development of inflammatory processes and also ensured an opportunity to determine new potential molecular markers [1]. In our previous works, we showed that the *TGF*β *1-3* expression profile may serve as a marker of the efficacy of cyclosporine A therapy [2]. In turn, expression of genes connected with the histaminergic system and micro RNA particles (miRNAs) regulating their expression may constitute new markers of response to adalimumab therapy [3]. What is more, we observed that

adalimumab has influenced the expression pattern of genes associated with the JAK-STAT signalling pathway and miRNAs regulating their expression in Normal Human Dermal Fibroblasts (NHDFs) *in vitro* model [4].

Induction and development of an inflammatory process, during which cytokine concentration profile changes are observed [5] are connected with the activation of specific signalling cascades. JAK-STAT, which may be activated by interleukins (ILs): IL-12 and IL-23, constitutes an important signalling pathway, which has already been partially described by us. However, it must be noted that the constituents of the said signalling cascade activated by an interaction between IL-12 and IL-23 with specific

receptors are the following: Janus 1-3 (JAK 1-3) kinases, tyrosine kinase 2 (Tyk2), proteins of STAT – STAT1-5 family [6]. It plays a significant role, for instance, in inflammatory bowel diseases, psoriasis vulgaris and psoriatic arthritis, which is supported by the fact that IL-12/23 inhibitors [7] and JAK kinase inhibitors [8] are used to treat these diseases.

The first group of the inhibitors is ustekinumab, a monoclonal antibody directed against the p40 subunit, common for IL-12 and IL-23. It leads to a loss of bond-formation possibility between these interleukins and receptors and activation of the JAK-STAT signalling pathway. The medicine was registered for psoriasis vulgaris treatment in adults, enabling to achieve remission of disease symptoms [9–11].

Well-tested inhibitors of JAK kinases are the following: tofacitinib (inhibitor JAK1 and JAK3) and ruxolitinib (JAK1 and JAK2 inhibitor) [12]. The former contributes to reduce expression of the interleukins: IL-17A, IL-17F, IL-22, IL-22. The latter was initially registered to treat myeloproliferative tumours, however, over time, it was also used to treat a group of psoriatic patients and those with arthrosis [13].

Nevertheless, all the time one should look for new therapeutic strategies which will enable inhibition of specific signalling pathways, thus preventing induction and development of the inflammatory process. It seems that new methods to suppress the JAK-STAT pathway could involve the use of epigenetic mechanisms of the expression of genes connected with the said pathway. The epigenetic strategies were described as a new possibility to treat, for instance, cancers [14, 15], mental diseases [16] and psoriasis [17, 18].

An important stage involves an *in silico* determination which of the signalling cascade constituents may serve as therapeutic objectives and which mechanisms may be used as the basis to modify expression of the specific genes. The *in silico* analyses are an extremely important first step to properly plan next work stages with the use of experimental study models [19, 20].

Aim

The aim of this paper was to determine, *in silico*, whether methylation and sequentially-specific suppression expression of the JAK-STAT signalling pathway by miRNAs may become new therapeutic strategies, inhibiting the said signalling cascade.

Material and methods

The first stage involved, basing on the bioinformatics databases (https://www.ncbi.nlm.nih.gov/, http://www.urogene.org/cgi bin/methprimer/methprimer.cgi) an *in silico* analysis of the effect of methylation on the expression of the analysed genes. The assessment was based on an accession number to the reference gene sequence in the NCBI database (NCBI Reference Sequence). The first step of analysis using MethPrimer (plus CpG Island Prediction) program was associated with pasting sequences of interesting genes in the FASTA format into the empty space which is there for pasting nucleotide sequences. The second step was to tick the option "Use CpG island prediction for primer selection?" and "Pick MSP primers". The third stage was connected with choosing the values of "island size" > 100 nucleotide, "observed/expected CpG ratio"= 0.60 and "percentage of G plus C" = 50.0. These values are standard [21]. The last step was to read the quantity, size and location of CpG islands in every single gene sequence that was to be analysed. The second stage concerned searching for miRNAs potentially regulating expression of genes: *JAK1, JAK2, JAK3, Tyk2, STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b*, basing on the bioinformatics database (www.microrna.org). The miRanda-mirSVR algorithm, which makes it possible to look for an adequate miRNA (miRanda) and determine a potential strength of interaction between mRNA-miRNA (mirSVR), was used to find the relevant miRNA molecules.

Results

The first stage of the analysis involved determination of the possible role of methylation in the regulation of genes of the JAK-STAT signalling pathway, with the use of the bioinformatics database [21]. On the basis of the presented data, it was possible to observe that CpG islands, in the quantity between 1 and 6 (Table 1), were present in the sequence of each gene, except for *STAT4*. This study shows that in the nucleotide sequences of analysed genes one can observe the appropriate number of CpG island (CGI) JAK1-4 CGI; JAK2-2 CGI; JAK3-5 CGI, TYK2-6 CGI; STAT1-2 CGI; STAT2-1 CGI, STAT3-3 CGI, STAT5A-4 CGI; STAT5B-3 CGI.

The second stage of the analysis concerned the search for miRNAs, which are potentially capable of regulating expression of genes of the JAK-STAT signalling pathway (Table 2). The value of mirSVR stated in the Table 2, for which the name of a specific miRNA regulating expression of a given gene was stated in the same Table, amounted to ≤ –0.7, as in our previous work [22].

It may be observed that for genes: *JAK3, TY2, STAT2, STAT5a*, assuming the said cut-off point for the mirSVR parameter, no miRNAs were found. The ratio between the number of miRNAs complying with the mirSVR prerequisite ≤ –0.7 and the number of all molecules potentially regulating expression of the specific gene is as follows for the individual genes: *JAK1* (19/42), *JAK2* (22/47), *STAT1* (7/17), *STAT3* (8/36), *STAT4* (11/15), *STAT5b* (7/23). The highest impact probability was determined between *JAK1* and both miR-520d-3p and miR-520c-3p (mirSVR = –1.135), *JAK2* and miR-9 (mirSVR = –1.305), *STAT1* miR-

Table 1. Location and situation of CpG islands for the analysed genes of the JAK-STAT signalling pathway

mRNA	Accession number	Number of CpG islands	Size of CpG [bp] island	Location of a CpG island in a sequence
JAK1	NM_002227	4	206	47–252
			132	547–678
			174	1551–1724
			102	2236–2237
JAK2	NM_004972	2	164	48–211
			165	270–434
JAK3	NM_000215	5	242	672–913
			268	921–1188
			115	2770–2884
			254	2524–3139
			126	3265–3392
TYK2	NM_003331	6	178	47–224
			230	985–1214
			130	1570–1699
			109	2354–2462
			358	2967–3324
			25	3371–3595
STAT1	NM_007315	2	800	48–327
			113	1681–1793
STAT2	NM_005419	1	146	48–193
STAT3	NM_003150	2	177	48–224
			184	843–1026
STAT4	NM_001243835	0	–	–
STAT5A	NM_003152	4	607	59–665
			107	1375–1481
			150	2445–2594
			162	2893–3054
STAT5B	NM_012448	3	141	47–187
			107	902–1008
			157	2413–2569

590-3p (mirSVR = –1.081), *STAT3* miR-590p (mirSVR = –0.773), *STAT4* miR-200a (mirSVR = –1.234), *STAT5b* miR-134 (mirSVR = –1.070).

The last stage involved searching among the miRNAs, for which mirSVR ≤ –0.7, which are potentially capable of regulating expression of more than one specific gene from all analysed mRNAs (Table 3). Common miRNAs for *JAK1* and *STAT3*: miR-17, miR-106b, miR-20b; for *JAK2* and *STAT1*: miR-590-3p, miR-144, *JAK2* and *STAT4*: miR-320d, miR-320c, miR-320a, miR-9, miR-320b, for *STAT4* and *STAT5b*: miR-141, miR-200a.

Discussion

The epigenetic regulation of the expression of genes has recently become an issue of increasing importance [23–25]. Among the most important mechanisms of the epigenetic control of transcription we may list the following: methylation, the RNA interference, in which miRNAs play a significant role and modifications of histone pro-

teins. The first process usually occurs within DNA areas rich in CG dinucleotide (CpG islands) that are present in the pro-motoric areas of genes. It leads to reduced expression of a specific gene and lower amount or a lack of the protein encoded by it [15, 26]. In turn, the second mechanism engages 19–23 nucleotide particles capable of bonding to a target transcript. The mechanism of gene suppression depends on the degree of complementarity between miRNA base pairs and target mRNA [27, 28]. Post-translation modifications of histone proteins have connections with their, for example, acetylation, methylation, phosphorylation, ubiquitination, sumoylation, which – depending on the type of modification – leads to an activation or repression of transcription [23].

All of our previous research was focused on searching and describing therapeutic strategies and new supplementary molecular markers of sensibility of cells for the treatment of psoriasis vulgaris and psoriasis arthritis [3, 7, 8]. This way, the results presented in this article can be related to psoriasis, but also to other diseases in which

Table 2. MiRNAs potentially regulating expression of genes JAK1-3, TYK2, STAT1-5 (microrna.org)

mRNA	miRNA potentially regulating expression (mirSVR score ≤ –0.70)	mirSVR score	The number of all miRNAs regulating mRNA expression	mRNA	miRNA potentially regulating expression (mirSVR score ≤ –0.70)	mirSVR score	The number of all miRNAs regulating mRNA expression
JAK1	miR-520d-3p	–1.135	42	JAK3	None		15
	miR-520c-3p	–1.135		TYK2	None		4
	miR-520e	–1.131		STAT1	miR-590-3p	–1.081	17
	miR-302e	–1.131			miR-203	–1.068	
	miR-520b	–1.131			miR-144	–1.055	
	miR-520a-3p	–1.131			miR-223	–1.009	
	miR-372	–1.129			miR-495	–0.914	
	miR-302d	–1.129			miR-599	–0.913	
	miR-302c	–1.129			miR-494	–0.797	
	miR-302b	–1.129		STAT2	None		30
	miR-302a	–1.129		STAT3	miR-590p	–0.773	36
	miR-373	–1.129			miR-21	–0.766	
	miR-17	–1.097			miR-106b	–0.74	
	miR-106b	–1.097			miR-20b	–0.74	
	miR-20b	–1.095			miR-519	–0.736	
	miR-20e	–1.095			miR-93	–0.736	
	miR-30e	–1.036			miR-17	–0.711	
	miR-125a-3p	–0.877			miR-106a	–0.711	
	miR-455-5p	–0.82		STAT4	miR-200a	–1.234	15
JAK2	miR-9	–1.305	47		miR-141	–1.233	
	miR-216a	–1.274			miR-384	–1.19	
	miR-101	–1.271			miR-132	–1.166	
	miR-197	–1.253			miR-320d	–1.166	
	miR-204	–1.243			miR-320c	–1.166	
	miR-135b	–1.207			miR-320b	–1.166	
	miR-135a	–1.207			miR-320a	–1.166	
	miR-144	–1.155			miR-490-3p	–1.058	
	miR-211	–1.125			miR-212	–1.05	
	miR-374a	–1.095			miR-9	–0.879	
	miR-374b	–1.095		STAT5a	None		10
	miR-590-3p	–1.001		STAT5b	miR-134	–1.07	23
	miR-320d	–0.825			miR-496	–1.021	
	miR-320c	–0.825			miR-200a	–0.991	
	miR-320b	–0.825			miR-141	–0.991	
	miR-320a	–0.825			miR-23b	–0.969	
	miR-181c	–0.797			miR-23a	–0.969	
	miR-181d	–0.793			miR-758	–0.931	
	miR-181b	–0.793					
	miR-181a	–0.793					
	miR-370	–0.787					
	miR-216b	–0.749					

the JAK-STAT pathway plays a key role [7, 8]. According to recommendations of the Polish Dermatological Society, the choice of therapy depends on the severity of the changes in the sickness. In the treatment of psoriasis we use: phototherapy, conventional treatments (cyclosporine A, methotrexate), biological treatments (inhibitors of TNF: adalimumab, infliximab, etanercept; anti-IL12/23 – ustekinumab, anti-IL17 – ixekizumab, secukinumab) [10, 11].

The newer, interesting medicines which might be dedicated to psoriatic patients are: B-cell inhibitors (rituximab), T-cell inhibitors (alefacept and efalizumab), IL23p19 inhibitors (guselkumab and tildrakizumab), IL-23 inhibitors (tildrakizumab), anti-IL-17 agents (secukinum-

ab, ixekizumab, and brodalumab), phosphodiesterase 4 (PDE4) inhibitors, and Janus kinase (JAK) inhibitors (ruxolitinib).

The introduction of newer treatment methods, by the means of IL-23 and JAK inhibitors, emphasizes the validity of the JAK-STAT cascade in the establishment and development of the layer state on the basis of many diseases [7, 8]. Nonetheless, the dynamic progress in designing new medicines and new strategies of treatment suggest that searching for other therapeutic goals, not only by using new tools or mechanisms to inhibit known signalling paths but also better understanding of changes on the molecular level are absolutely necessary.

Using JAK inhibitors as a new therapeutic strategy can be a response to emerging drug resistance, for example in psoriasis [2–4].

In our study, we focused on the possibilities to use methylation and miRNAs as new therapeutic strategies to suppress the JAK-STAT signalling pathway. To this end, we used bioinformatics tools, which enabled us to ascertain the possibility to use the said mechanisms to interrupt the signalling cascade. The *in silico* analyses constitute an important element of therapeutic strategy planning which enables to determine potential directions of action at the first stage. These analyses are extremely important to starting research projects because they indicate which mechanisms are potentially involved in the regulation of signalling cascades and they help to express which genes should be upregulated or down-regulated [19, 20]. mRNA and miRNAs regulating their expression have to be expressed at the same time and in the same cell. This statement is supported by our previous studies. We examined the influence of adalimumab on changes in the expression of mRNA and miRNAs in NHDFs cell culture after 2 h, 8 h and 24 h of exposure to an anti-TNF drug. These works show that one mRNA can be regulated by more than one miRNA and one miRNA has a connection with other mRNAs. Besides, an important complement to the results of the microarray profiles was obtained from the *in silico* analysis which allowed to determine the potential strength of interaction between mRNA-miRNA [3, 4]. These observations indicated that *in silico* analyses are the first step to a deeper study with the use of modern and sophisticated methods. During the first stage, we determined the occurrence of CpG islands within the nucleotide sequence of genes belonging to the JAK-STAT signalling pathway. It may be observed that, apart from *STAT4*, all analysed sequences of genes show areas rich in GC pairs, within which an incorrect degree of methylation may be noted. The number and size of CpG islands is different between the analysed genes and fluctuates between 1 and 6.

The JAK-STAT signalling pathway is activated, which involves a change in the expression profile of genes engaged in the said signalling cascade in pro-inflammatory [6–8, 12, 13] and neoplastic processes [29]. Taking into account the above statement and the observed possibility of methylation effect on the expression of these genes, the reduced methylation of pro-motoric areas of the JAK-STAT signalling cascade may be assumed. Consequently, it seems that one of the possible new therapeutic strategies would involve restoration of the correct methylation degree.

The methods formerly used to restore the correct methylation model focused on the use of substances demethylating DNA (DNA methyltransferase inhibitors), with two distinguished mechanisms of operation. The first one involves the fact that a medicine whose structure imitates a cytosine is embedded during DNA replication, thus it inhibits methyltransferase. In turn, the

Table 3. MiRNAs (mirSVR ≤ –0.7) which may potentially regulate expression of more than one of the analysed genes of the JAK-STAT cascade

mRNAs	miRNA
JAK1 and *STAT3*	miR-17 miR-106b miR-20b
JAK2 and *STAT1*	miR-590-3p miR-144
JAK2 and *STAT4*	miR-320d miR-320c miR-320a miR-9 miR-320b
STAT4 and *STAT5b*	miR-141 miR-200a

second strategy concerns the use of non-nucleoside inhibitors, which do not need to be embedded into the DNA helix structure in order to block the action of DNA methyltransferases. One must remember to determine an adequate dose of the medicine that does not cause toxic action towards regular cells [14, 30, 31]. Thus, it is possible that the strategy which enables to reconstruct the correct methylation formula should focus on the genes encoding SOCS and PIAS proteins. They are inhibitors of the JAK-STAT signalling pathway [6]. It may be presumed that deregulation of the described signalling cascade may be connected with an excessive methylation of genes encoding inhibitors of the JAK-STAT pathway.

Methods based on the change in the degree of methylation seem to be promising and relatively safe due to the process reversibility [14, 30, 31]. It is also possible that the potential therapeutic objective could involve restoration of the correct enzyme activity of methyltransferases.

The second stage of the analyses presented in this work was devoted to the potential role of miRNAs in post-transcription regulation of the expression of the JAK-STAT signalling pathway. The bioinformatics database microrna.org was used for that purpose, and, basing on the mirSVR parameter, molecules that were selected were the most likely present in the analysed gene inhibition.

The cut-off criterion for mirSVR is ≤ –0.7, similarly as in our previous work [22], although it seems that the threshold ≤ –0.1 would be enough [32, 33]. The use of such restrictive criteria enables us to focus only on those molecules which are the most capable of inhibiting the analysed transcripts. We also observed that some selected miRNAs may potentially regulate expression of more than one gene of the JAK-STAT signalling pathway. Paying particular attention to those molecules makes it possible to influence, basing on potential use of one miRNA, several molecular objectives, which seem to constitute a new paradigm in designing medicines [34].

MiR-17, miR-106b, miR-20b are potentially engaged in the regulation of *JAK1* and *STAT3* expressions. It is emphasised that miRNAs considerably affect the activity of pro- and anti-apoptotic genes, contributing to the regulation of, among others, a cell cycle [35, 36].

Moreover, the miRNAs belonging to the miR-17-92 family constitute a promising objective to counteract lost response to treatment [37].

MiR-590-3p miR-144 influence the *JAK2* and *STAT1* expressions to the greatest extent. The said miRNAs also play an important role in the regulation of a cell cycle. Moreover, depending on the level of miR-144 expression, it facilitates the promotion of the proliferation process or apoptosis of cells. Whereas, miR-590-3p may be used as a prognostic marker in patients with cancers. It is also noted that miRNAs could be used as a promising therapeutic strategy [38, 39].

In turn, the activity of *JAK2* and *STAT4* is subject to the greatest post-transcription control on the part of miR-320a-c and miR-9 molecules, the latter being assigned to have a significant role in differentiation of T lymphocytes to Th17 phenotype. The correlation between miR-9 expression and miR-106a-5p expression is also emphasised [40], which seems to confirm the complex nature of the interaction between miRNAs and gene expression. The last group of genes regulated by a given miRNA is composed by *STAT4* and *STAT5b* transcripts, with the greatest degree of complementarity shown with miR-141 miR-200a, conditioning cell response to cell stress [41].

The confirmation that *in silico* analyses are the key stage to more detailed research is our observation.

Kurdyukov and Bullock in their study showed the place of the MethPrimer database in research. They indicated this program as a useful tool to design primers to Methylation-Specific Polymerase Chain Reaction (MS-PCR) and search for CpG islands (CGI) [42].

Comparing the findings of our previous work associated with analysing the microarray profiles of mRNA and miRNA related to the JAK-STAT signalling pathway with the current work one can observe that *in silico* analysis is valuable. It provides complementation of *in silico* analysis with *in vitro* and undoubtedly *in vivo* tests [4].

In this study our results show that miR-106a is connected with *STAT3*, on the contrary miR-132 is associated with *STAT4*, in the same observation we had described while we analysed the influence of adalimumab on the JAK-STAT signalling pathway in NHDFs. We highlighted that these mRNAs and miRNAs can be new supplementary molecular marker psoriasis treatments [4].

For example, Pivarcsi *et al.* observed changes in the expression profile of miR-106b, miR-26b, miR-142-3p, miR-223 and miR-126 during etanercept therapy. Furthermore, there was no difference in the expression profile of these miRNAs between psoriatic patients and healthy volunteers. Therefore, it can be said that the treatment changes the expression of the miRNAs [43]. The results of our study show a correlation between miR-106a (a small difference in the sequence compared to miR-106b) and *STAT3*. It suggests miRNAs are to be confirmed as the candidate targets.

Singling out the miRNAs showing the greatest potential of to interact with target mRNA, basing on the mirSVR parameter, indicates that the phenomenon of RNA interference may constitute another mechanism in the regulation of the expression of genes of the JAK-STAT signalling pathway. The *in silico* analyses of the role of miRNAs constitute mere preliminary studies, nevertheless, they show which of these molecules are interesting and promising objects for further studies on the selection of molecular markers or new therapeutic strategies. The comparison between our data and the information from the literature on the role of selected miRNAs enables us to observe their role in the regulation of cell cycle, their effect on cell death processes, regulation of proliferation, i.e. processes disturbed in the majority of diseases.

Conclusions

The *in silico* analysis of epigenetic mechanisms of regulation of the JAK-STAT signalling pathway, presented in this work, emphasises their role in the regulation of the expression of genes engaged in the said signalling pathway. They underline the multidimensional character of the regulation of transcription activity of genes, complexity of biological processes and the interdependence of several different mechanisms. It may be observed that the bioinformatics tools constitute an interesting and promising screening method when elaborating on new therapeutic strategies. They make it possible to better depict which mechanisms and to what extent may become a new promising therapeutic tool. Extension of the range of new possibilities to interrupt the JAK-STAT signalling pathway will be also favourable for patients who show an incorrect expression pattern of the JAK-STAT signalling path components.

Conflict of interest

The authors declare no conflict of interest.

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