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.
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 arthritis [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 suppression of genes connected with the said pathway. The epigenetic strategies 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, STAT4-4 CGI; STAT5-3 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-
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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.

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

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 proteins. 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
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 (secukinumab, 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.

| mRNA | miRNA potentially regulating expression (mirSVR score ≤ –0.70) | The number of all miRNAs regulating mRNA expression |
|------|---------------------------------------------------------------|---------------------------------------------------|
| JAK1 | miR-520d-3p, miR-520c-3p, miR-520e, miR-520a-3p, miR-372, miR-302d, miR-302c, miR-302b, miR-302a, miR-373, miR-17, miR-106b, miR-20b, miR-20e, miR-30e, miR-125a-3p, miR-455-5p | 42 |
| JAK2 | miR-9, miR-216a, miR-101, miR-197, miR-204, miR-135b, miR-135a, miR-144, miR-211, miR-374a, miR-374b, miR-590-3p, miR-590-5p | 47 |
| JAK3 | None | 15 |
| TYK2 | None | 4 |
| STAT1 | miR-590-3p, miR-203, miR-144, miR-223, miR-495, miR-599, miR-494 | 17 |
| STAT2 | None | 30 |
| STAT3 | miR-590p, miR-21, miR-106b, miR-20b, miR-519, miR-93, miR-17, miR-106a | 36 |
| STAT4 | miR-200a, miR-141, miR-384, miR-132, miR-320d, miR-320c, miR-320b, miR-320a, miR-400-3p, miR-212, miR-9 | 15 |
| STAT5a | None | 10 |
| STAT5b | miR-134, miR-496, miR-200a, miR-141, miR-23b, miR-23a, miR-758 | 23 |

Table 2. MiRNAs potentially regulating expression of genes JAK1-3, TYK2, STAT1-5 (miruv.org)
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 downregulated [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 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-transcriptional regulation of the expression of the JAK-STAT signalling pathway. The bioinformatics database miRmac.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 $\leq -0.7$, similarly as in our previous work [22], although it seems that the threshold $\leq -0.1$ 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].

**Table 3.** MiRNAs (mirSVR $\leq -0.7$) 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 |
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 expression 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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