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Statements:

What's already known about this topic?; MiR-125b has previously been shown to be strongly associated with psoriasis

What does this study add? This study adds a likely molecular mechanism for the association between MiR-125b and psoriasis. Additionally it provides a possible novel pathway for therapeutical treatment-

Abstract

Background: The extensive involvement of miRNA in the pathophysiology of psoriasis is well-documented. However, in order for this information to be useful towards therapeutic manipulation of miRNA-levels, it is essential that detailed functional mechanisms are elucidated. MiR-125b has previously been shown to be strongly associated with psoriasis, and presents as an obvious candidate for further investigation. **Objectives** Elucidate the specific pathway and mechanism of interest in this association. **Methods** A three-step bioinformatical hypothesis-generation pipeline was performed to identify genes of interest. This pipeline was based on miR-125b-binding, expression in psoriatic lesions and genome-wide association study-based evidence of involvement. The identified candidate gene was then carefully evaluated using luciferase binding assays, in-vitro overexpression, siRNA knock-down and downstream gene read-outs. **Results** Based on our bioinformatical pipeline, the Ubiquitin Specific Peptidase 2 (USP2) was selected as a likely candidate for a mechanistic explanation for psoriasis association. After establishing a definite connection to miR-125b, we proceeded to show that modulation of NFkB-mediated inflammation is the likely mechanism through which this miRNA-gene pair functioned. **Conclusions** Shedding further light on the multi-factorial causes of psoriasis is essential, if the goal is to progress towards finer control of therapeutic tools in disease management. Findings, such as the ones presented herein, are therefore necessary in order to achieve the future of personalized medicine.

Introduction

Psoriasis is a common chronic inflammatory skin disease that affects approximately 2 to 3% of the population. The cause of psoriasis is still unclear and a complete cure is lacking. Nonetheless, significant progress has been made in understanding the cellular immunology and biology of psoriasis. This includes the importance of interleukin-12 and interleukin-23 and the Th1 and Th17 inflammatory pathways, as is already well-established through the success of Ustekinumab in treatment of moderate to severe psoriasis. Likewise it is unsurprising that more unspecific modulation of the immune system is beneficial in psoriasis (corticosteroids, methotrexate, and TNF-inhibitors). However, a multifactorial disease like psoriasis is likely to originate in a complex interplay among many different effector molecules and further studies into the specific pathophysiological pathways are warranted.

One particularly interesting aspect of psoriasis-specific pathophysiological pathways is the extensive studies of micro-RNAs (miRNAs), which have been extensively discussed as important regulators in this disease^{1,2}. The direct pharmacological targeting of miRNA have even been suggested³. The

broad regulatory activity of miRNA undoubtedly makes miRNA very interesting from a biological point of view, but it should be noted that it also creates the one weakness of miRNA targeting, which is that it can be problematic to pharmacologically modulate molecules with such broad consequences. It is therefore of interest to further elucidate the related molecular pathways for the purpose of gaining increased pathophysiological understanding.

In this study we set out to characterize the targets of psoriasis-associated miRNA, miR-125b, which have previously been shown to be downregulated in psoriasis lesion, playing an important role in modulating keratinocyte function^{4,5}. As with all miRNAs, a number of different targets have been proposed and characterized for each miRNA, which is not surprising given the functionality of miRNA. The first steps of our study therefore involved the search for a useful gene for further characterization as target molecule. We required such gene to be 1) a direct target of a known psoriasis-associated miRNA, 2) over-expressed in psoriatic lesions, and 3) having evidence of causal involvement in disease formation.

The first and second requirements give a direct link to psoriasis and previous discoveries. The third, tries to overcome the problem that a gene up-regulated in diseased tissue may be the cause of the disease or simply a consequence of the disease. Short of large-scale clinical drug trials and knock-out animals, it is difficult to overcome that fact. One manageable method is the use of human genetics to investigate if natural variation in the expression level of a gene coincides with variation in risk-of disease⁶. Taken together, these methods constitute the foundation for our selection of Ubiquitin specific protease 2 (USP2) as a potential target of interest.

Having established this, we set out to characterize the interplay between USP2 and miR-125b and its effects on the three hallmarks of psoriasis: differentiation, proliferation and inflammation. The overall aim of these experiments was to provide new knowledge about this aspect of psoriatic pathogenesis signaling.

Materials and Methods

Selection of target gene USP2

For miRNA target gene list Targetscan release 6.2 was used, with the default settings using hsa-mir-125b as input in the mammalian database, retrieving all genes with a probability of conserved targeting score (P_{ct}) above 0.9⁷.

Gene expression data from psoriatic skin lesions and healthy controls was downloaded from the Gene Expression Omnibus entry with accession number GSE13355, and re-normalized using RMA and log₂ transformation^{8,9}. The validation measurements in figure 1B were performed on an independent cohort from Karolinska Institute, using real-time PCR analyzed according to the 2^{-ddCt} methodology. Comparison between groups were calculated using a heteroscedastic Student's T-test. The psoriasis patients in the cohort had not received systemic treatments for at least 1 month, and topical therapy for at least 2 weeks before skin biopsy. The study was approved by the Stockholm Regional Ethics Committee, and conducted according to the Declaration of Helsinki Principles.

Accepted Article

For the investigation of genetic disease association, genome-wide association data was downloaded from the dbGap project accession number *phs000019.v1.p1* and P-values and all odds-ratios were used as reported. Genetics of gene expression data was downloaded from array express accession number E-MTAB-2232, and gene expression data was used as provided. The USP2 gene was measured using the ILMN_1755502 microarray probe in the 24 hour LPS stimulated data set. Association with genotype was calculated using an additive linear model, in which the TT was coded as 0, the CT as 1, and the CC genotype as 2.

Plasmids mutagenesis and 3'UTR luciferase-binding assays

Firefly luciferase reporter plasmids containing the 3'-UTR of the USP2 gene and empty luciferase vector were obtained from Promega (Madison, WI, USA). The nucleotides of USP2 3'-UTR containing the miR-125b binding site were cloned into psiCHECK-2 (Promega) using the restriction sites XhoI/NotI. The 3' UTR of the human USP2 gene was PCR-amplified using the primers:

USP2-FW 5'- ggctcgttgatgcattgcagt-3'

USP2-REV 5'- ggcaggactggagacc-3'

To delete the seed sequence of miR-125b on the USP2 3'UTR, we used the Quick-Change Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA) and the primers:

MUT-USP2-FW 5'- ttggaccgagtttctgccaggccacc-3'

MUT-USP2-RV 5'- ggtggcctgggcagaaactcgggtccaa-3'

For the reporter luciferase assay HEK-293 were cotransfected in a 12 well-plate with 1 µg psiCheck2-USP2 and/or psiCheck2-Mut and 100 nM of miR-125b or scrambled sequence miRNA control (Thermo Scientific). Dual Luciferase Assay (Promega) was performed 24h after transfection according to the manufacturer's instructions.

Immunostaining

USP2 protein expression was analysed in frozen skin sections using rabbit anti-human USP2 antibody (ab66556 diluted 1:100) from Abcam (Cambridge, UK) and anti-rabbit conjugated with HRP (P 0448) (DAKO) at 1: 200 dilution. Sections were mounted using Glycergel® Mounting Medium, Aqueous (DAKO). The omission of the primary antibody in the staining procedure was used as a negative control.

Cell culture and transfection

Human adult skin epidermal keratinocytes (obtained from Cascade Biologics, Portland, OR) were cultured in EpiLife serum-free keratinocyte growth medium including Human Keratinocyte Growth Supplement at a final Ca²⁺ concentration of 0.06 mM (Cascade Biologics). Third passage keratinocytes were used at 50–70% confluence for all experiments.

Transfection for keratinocytes was carried out by using Lipofectamine® RNAiMAX (Invitrogen, Carlsbad, CA), following the manufacturer's instruction. 20nM miRCURY™ LNA Inhibitor was used for inhibition of miR-125b. 50nM small interfering RNA was used for the specific knockdown of USP2 (Santa Cruz Biotechnology, Texas, USA). Silencer select negative control #1 (Ambion Inc., Austin, TX, USA) was used as negative control. Keratinocytes were treated with TNF- α (50 ng/ml; R&D system, Minneapolis, MN) at the indicated time points. Transfection efficiency for pre-mir-125b was 51.4 \pm 13.9 fold. For anti-mir-125b experiments all measurements were below the detectable range of 40 cycle thresholds.

Proliferation assay

EdU was added at a 10 μ M final concentration to the transfected cells 2 hours before harvesting. A Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit (Invitrogen) was used according to the manufacturer's instructions and analyzed by flow cytometry (Beckman-Coulter, Fullerton, CA) as described previously (Manfe et al., 2012b; Manfe et al., 2013) to determine EdU positive cells..

Quantitative real-time PCR

For the quantification of USP2, involucrin, K10, IL-8, CXCL 1 and CXCL 5, 20ng of cDNA per reaction were amplified in the presence of TaqManR universal master mix (Applied Biosystems, Foster City, CA) and TaqMan® Gene Expression Assay: USP2 (Hs00899199-g1), involucrin (Hs00846307-s1), K10 (Hs01043114-g1), IL-8 (Hs00174103-m1), CXCL 1 (Hs00605382-gH), CXCL 5(Hs01099660-g1) and GAPDH (Hs02758991-g1)(Applied Biosystems) (stage 1, 50°C for 2 min, stage 2, 95°C for 10 min and stage 3, 95°C for 15 s, 60°C for 1 min, repeated 45 times) in Stratagene Mx3005p (Agilent Technologies, Santa Clara, CA). Target gene expression was normalized based on the values of the expression of GAPDH (Applied Biosystems).

Results

Investigation of miR-125b targets that are overexpressed in psoriasis

A list of target genes of miR-125b was obtained using the targetscan software. This targetscan algorithm searches for binding motifs for specific miRNAs, typically returning hundreds of potential and real target genes. For miR125 a total of 202 genes with an aggregate binding score above 0.9 were identified.

To further narrow down the list of potential candidate genes we investigated a publically available gene expression microarray data set of psoriatic skin lesions and healthy skin biopsies. We omitted those of the 202 genes that were not at up-regulated in psoriatic lesions by with a minimum significance of $P < 2.5e-4$ and 2.5 fold (corresponding to a Bonferroni corrected P-value of 0.05 for testing 202 genes). This left 57 genes of potential interest, quite likely all having some measure of importance for the interplay of miR-125b and psoriasis. However, for the purpose of this study we chose to continue with the gene named USP2. This choice was not based on any further filtering strategies, but rather motivated by its known function as a requirement for TNF-alpha-induced

NFκB-signaling¹⁰. Overexpression of USP2 in microarray data was shown in figure 1A. To validate the over-expression finding, we measured USP2 expression using real-time PCR in an independent cohort with psoriasis lesions (n=25), non-lesional skin from psoriasis patients (n=10) and skin from healthy volunteers (n=22) (figure 1B). In accordance with the microarray data, quantitative real-time PCR results showed significantly ($p < 0.001$) increased USP2 level in psoriasis skin when compared with psoriasis non-lesion and healthy skin (figure 1B). In addition, we confirmed the presence of USP2 protein by immunohistochemistry in the corresponding frozen samples. Stronger expression of USP2 was observed in the suprabasal layer of psoriasis lesion, but not in healthy skin (figure 1C).

Investigation of human genetics resources for involvement of USP2 in psoriasis

Using a previously published genome-wide association study of 1446 psoriatic cases and 1432 healthy controls, we investigated if there were any SNPs with association to psoriasis found close to USP2¹¹. The rs10892465 SNP was found to have a psoriasis association at $P = 0.00154$ (figure 1D). This was significant at a false discovery rate of 5%, calculated over the 142 measured SNPs found within a 500 kb range of the gene. Additionally this SNP was found to affect the expression level of USP2 in monocytes. This was done in a set of LPS and IFN-stimulated monocytes for which both gene expression and genotypes were available¹². In the set of LPS-stimulated monocytes data we observed that the C-allele of the psoriasis risk-SNP caused a slight over-increase in the USP2 expression ($P = 0.027$, figure 1C). The confidences of these two findings are not sufficiently strong to support USP2 as a candidate psoriasis-gene on their own, but taken together and viewed in the light of the miR-125b-hypothesis, they strengthen the interest in further pursuing detailed characterization of USP2 in pathogenesis of psoriasis.

In-vitro confirmation of link between miR-125b and USP2

To confirm the TargetScan observation linking miR-125b and USP2, we generated a site-specific mutation in the miR-125b binding site. Luciferase reporter assay revealed a direct up to 60% inhibition of USP2 by miR-125b. When the predicted miR-125b binding site in the 3'UTR of USP2 mRNA was mutated, luciferase activity was restored (figure 2B).

We further performed an anti-miR knock-down experiment and a pre-miR over-expression experiment, which showed the expected results of pre-miR-125b reduced USP2 expression and anti-miR-125b increased USP2 expression (figure 2C and 2D). Taken together, these experiments solidified the link between miR-125b and USP2, further suggesting the involvement of USP2 in psoriasis^{4,5}. It has been shown that overexpression of miR-125b prohibits keratinocyte proliferation and promotes keratinocytes differentiation⁴. As a target of miR-125b and a pivotal gene in TNF- α -induced NFκB-signaling, we therefore hypothesize that knockdown of USP2 has effects in keratinocyte proliferation/differentiation and NFκB signaling (figure 2E).

Investigation of USP2 and miR-125 effects on keratinocyte differentiation and proliferation

A hallmark of psoriasis is the increased proliferation and altered differentiation of keratinocytes. Accordingly we investigated the influence of USP2 in these two aspects. First the differentiation markers keratin10 (K10) (figure 3A) and involucrin (figure 3B) were measured after transfection with siUSP2 at mRNA level. Knockdown of USP2 significantly increased mRNA expression of both differentiation markers. The induction of early differentiation marker, K10, was shown also in the protein level. Only a slight increase was detected in the protein expression of the late differentiation marker, involucrin (data not shown).

Further, the effect of USP2 knockdown on keratinocyte proliferation was measured. We analyzed the cell proliferation rate by 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay using flow cytometry and gating on live keratinocytes (figure 4). This showed that siUSP2 strongly reduced keratinocyte proliferation, particularly at the 48 hour time point. Since reduced differentiation and increased proliferation is the important cellular state in psoriasis, these two findings are consistent with a hypothesis that USP2-modulation could have a beneficial effect in vivo.

Exploration of USP2 and miR-125b effects on NFkB-signaling pathway

Previously published observations that USP2 works through the NFkB-signaling pathway¹⁰, prompted us to investigate the effect of miR-125b and USP2 modulation on characteristic NFkB-signaling consequences, namely regulation of IL8, CXCL1, and CXCL5. The purpose of this investigation was to gain a better understanding of the direct mechanism through which USP2 exerted any psoriasis-related function. As expected miR-125b knock down-increases the expression of these genes, whereas USP2 knock-down decreases the expression. The miR-125 knock-down is shown in figure 5A, where IL8-expression is increased 4.8-fold compared to anti-miR-con at 24 hours post transfection. CXCL5 and CXCL1 were up-regulated 3.0 fold and 1.4 fold respectively. Correspondingly, the down-regulation on siUSP2 treatment was 3.6, 1.5 and 3.3 fold for IL8, CXCL5 and CXCL1 (figure 5B). Addition of 50x fold miR-125b to keratinocytes also affected levels of IL8 and CXCL8, although in an upward direction (supplementary figure S1). The USP2 knock-down experiment was also conducted after TNF stimulation to obtain a sufficient active level of NFkB-signaling. The same suppressing effects in NFkB-signaling was shown.

Additionally we performed a combined miR-125b and USP2 double knock down experiment to confirm the interaction between the USP2 and miR-125b effects on NFkB-signalling. siUSP2 alone diminished the expression of two NFkB downstream genes to the lowest level. By combining with miR-125b knockdown, IL-8 and CXCL 1 expression recovered 21% and 43%, respectively (figure 5c). The increase in NFkB-signaling activity by anti-miR125b was only observed without TNF stimulation.

Discussion

Starting with the aim of elucidating miRNA-related pathways leading to psoriasis, we investigated potential targets of the known psoriasis-associated miR-125b. From a list of 57 miR-125b-target genes we selected USP2, fully acknowledging that this was not the only possible candidate. However, according to the notation that solid functional characterization of molecular pathways is nonetheless required, we proceeded to further elucidate the connection between miR-125b and

USP2 in psoriasis. First we firmly substantiated the relationship between miRNA and gene, using both luciferase assay and knockdown/overexpression assays. Then we asked if modulation of the gene had any direct effect on the cellular events of psoriasis, namely reduced keratinocyte differentiation and increased proliferation. Prompted by siRNA-screen findings that USP2 is a required factor for TNF- α -induced NF κ B signaling¹⁰, we proceed to analyze the relationship between USP2, miR-125b and established NF κ B-induced genes. Finally we tested if miR-125 rescues the NF κ B signaling-decrease otherwise caused by USP2-knock-down, which it did. Our overall conclusion therefore is that one effect of miR-125b is through USP2, then NF κ B-signaling and ultimately towards a psoriasis phenotype.

A central question is how to conclude on findings on separate target molecules for miR-125b, e.g. FGFR, MMP13^{4;5}. Likewise, it is puzzling how both miR-125b knock-down and overexpression can cause an increased IL8/CXCL1 expression (figure 5A and supplementary S1). It is generally established that each miRNA have many targets¹³. Thus the miRNA-regulated output of a pathway can depend on interaction with different genes with different threshold levels, and resulting complex patterns. It is therefore important to underscore that these results only shed light on the miR-125B to USP2 interaction, and further on the downstream USP2 effects. We are of course unable to conclude on all effects of miR-125b. Ultimately however, understanding the many specific arms of their regulation will lead to increased knowledge about pathogenesis of diseases.

An additional aspect of our interest in USP2 is the genetic findings showing a slight, but significant naturally occurring variation of USP2 dependent on a single SNP rs10892465. Having the high-expression USP2 variant also gives individuals a slightly higher life-time psoriasis risk. Expression levels changes from natural genetic variations are much smaller than expression level changes seen when comparing healthy and disease tissue. However, a central advantage of these genetic studies is that unlike disease gene expression studies, a causality link can be established, following theory for genetic drug-candidate selection that is further discussed elsewhere^{6;14}.

The modest strength of the genetic findings is one property of this study that could be improved. However, although larger publically funded genome-wide association studies have been described, they are not accessible by other researchers¹⁵. Likewise it would be of interest to investigate if the SNP in question has any influence on the miR-125B effect on USB. It is possible that miR-125b mediated down regulation will be decreased in patients carrying the C allele. Other limitations worth discussing are the purely in-vitro setups for experimentation. Ideally a conditional knock-out animal model would be created for USP2. However, such undertakings were outside the scope of our resources. Ultimately, however a main interest of all these investigations is the identification of novel drug targets for psoriasis. The current setting is one of unspecific immune system modulators as the first line of treatment, followed by more specifically targeting drugs such as ustekinumab. In this setting, it is beneficial to expand our knowledge towards as many other pathways into disease as possible. This is because a broader range of highly specific drugs is likely to go well together with a future setting in which personalized medicine is much more established. We hope that increased knowledge about the USP2-dependent NF κ B-signalling pathway will contribute a piece towards that goal.

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- Accepted Article
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