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MicroRNAs and potential target interactions in psoriasis

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ABSTRACT

Background: Psoriasis is a chronic inflammatory skin disease often seen in patients with a genetic susceptibility. MicroRNAs (miRNA) are endogenous, short RNA molecules that can bind to parts of mRNA target genes, thus inhibiting their translation and causing accelerated turnover or transcript degradation. MicroRNAs are important in the pathogenesis of human diseases such as immunological disorders, as they regulate a broad range of biological processes.
Objective: We investigated miRNA–mRNA interactions in involved (PP) and non-involved (PN) psoriatic skin compared with healthy skin (NN).
Methods: Biopsies were obtained from PP, PN and NN, the miRNA and mRNA expression was analyzed by microarray techniques and a subset of miRNAs and mRNAs were validated by q-RT-PCR. Novel target interactions in psoriasis were found using PubMed, miRBase and RNAhybrid. In addition, TIMP3 protein expression was studied in PP, PN and NN. Finally, the miR-221/2–*TIMP3* target interaction was studied in primary human keratinocytes by endogenous overexpression of the miRNAs.
Results: We identified 42 upregulated miRNAs and 5 downregulated miRNAs in PP compared with NN, and only few deregulated miRNAs in PN compared with NN. Based on the miRNA and mRNA profiles miR-21, -205, -221 and -222 were found to have the following potential mRNA targets in psoriatic skin: *PDCD4*, *TPM1*, *P57*, *C-KIT*, *RTN4*, *SHIP2*, *TIMP3*, *RECK* and *NFIB*. The identified target mRNAs were likely to be involved in cellular growth, proliferation, apoptosis and degradation of the extracellular matrix. Finally we found that TIMP3 is downregulated in psoriatic skin. *In vitro* overexpression of miR-221 and miR-222 lead to degradation of *TIMP3* resulting in decreased TIMP3 protein level.
Conclusion: Our data indicate several novel important associations for miRNAs in psoriasis and in particular the miR-221/2–*TIMP3* target interaction could among others play a role in the psoriasis pathogenesis.

1. Introduction

Psoriasis is a chronic inflammatory skin disease characterized by intense proliferation and abnormal differentiation of keratinocytes. Several observations suggest T cells, dendritic cells and inflammatory cytokines as key players in the pathogenesis [1]. Subjects predisposed to psoriasis have an inherited disorder associated with several different psoriasis susceptibility loci and major histocompatibility complex alleles [1]. However, psoriasis is a complex disorder requiring not only a genetic susceptibility but also an environmental trigger [2].

MicroRNAs (miRNAs) usually bind to the 3′ untranslated regions (UTR) of mRNAs, inhibiting translation, causing accelerated turnover or degradation of the mRNA transcript [3]. The miRNA-mediated regulation is often detectable on the protein level, in some cases even without detectable changes at the mRNA level [4]. Several publications implicate miRNAs as important players in the pathogenesis of human diseases such as immunological disorders, cancers and metabolic disorders [5,6]. Although several hundred miRNAs have been identified in humans, new insight on their function is lacking [7]. To date, one study has been published on miRNA in psoriasis comparing psoriatic skin with healthy skin where miR-203 was suggested to act as a suppressor of cytokine signaling 3 (*SOCS-3*), modulating cytokine signaling, keratinocyte hyperproliferation and differentiation in psoriatic skin [8]. Little is known about other miRNA–mRNA interactions in psoriatic skin,

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and no work has been done describing the deregulated miRNAs in non-involved psoriatic skin.

In this paper we describe the statistically significant expression miRNA and mRNA in involved (PP) and non-involved (PN) psoriatic skin compared with healthy skin (NN). To find the biological significance of the miRNA expression we aimed to associate previously experimentally validated miRNA–mRNA interactions with our miRNA and mRNA expression data. Finally, we aimed to predict new potential miRNA targets among the most significant downregulated mRNAs in psoriasis and identified miR-221/2, which are likely to target tissue inhibitor of metalloprotease-3 (*TIMP3*) in keratinocytes.

2. Materials and methods

2.1. Patients

Thirteen healthy subjects of Caucasian origin (average age 38.9 years, range 23–62 years, 5 women and 8 men) and 13 patients of Caucasian origin diagnosed with psoriasis vulgaris (average age 55.5 years, range 32–73 years, 7 women and 6 men), were recruited for the study. None of the participants had used any systemically immunosuppressive medications for four weeks and none local treatment at the site of biopsies for two weeks before study participation. The study was approved by the Danish National Committee on Biomedical Research Ethics (KA-20060119) and in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Two 4 mm punch biopsies were taken from each patient (PP and PN) and one 4 mm punch biopsy from healthy subjects (NN). The skin area was either flash frozen using liquid nitrogen and immediately placed in liquid nitrogen and stored at −80 °C or fixed in formaldehyde (4%) and paraffin embedded.

2.2. Total RNA purification and analysis

Skin biopsies were ground in liquid nitrogen, immediately transferred to Lysis/Binding Buffer (Applied Biosystems, Warrington, UK) and homogenized with a rotor stator (IKA, Staufen, Germany). Human primary keratinocytes were dissolved in Trizol (Invitrogen, Taastrup, Denmark) and RNA was isolated using standard RNA extraction methods with chloroform, isopropanol and EtOH. Total RNA from the biopsies was isolated using the one-step *mir*Vana™ miRNA Isolation Kit (Applied Biosystems) following the manufacturer's instructions. The RNA quality was assessed by Agilent RNA 6000 Nano Assay (Agilent Technologies, Naerum, Denmark) accepting an RNA integrity number higher than 7.5 and the RNA concentration was determined using a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Copenhagen, Denmark).

2.3. MicroRNA microarray

Total RNA from PP ($N = 6$) as compared with NN ($N = 3$), and PN ($N = 6$) as compared with NN ($N = 3$) were analyzed using the miRNA analysis platform miRCURY™ LNA Array (v.8.1) (containing 561 of 701 miRBase 12.0 annotated human miRNAs) (Exiqon, Vedbaek, Denmark). One microgram total RNA from each sample was labeled and hybridized to the array (Tecan HS4800, Grödig, Austria), scanned (ScanArray 4000 XL, PerkinElmer, Waltham, MA) and analyzed (ImaGene 6.1.0, BioDiscovery, El Segundo, CA). The median signal of three out of four probes from each miRNA exceeding the local background by a factor of 2 was quantile normalized in dChip [9]. For multiple hypothesis testing correction and to find statistically significant deregulated miRNA data were uploaded to Significance Analysis of Microarray (SAM) [10], accepting a q-value less than 0.05, based on measures of the proportion of miRNAs being statistically significant by a t-test ($p < 0.05$) and with a false discovery rate less than 5%, calculated by 100 random permutations. All other analysis were performed using dChip.

2.4. MessengerRNA microarray

Total RNA from 4 NN and 4 matched PP and PN biopsies were analyzed using the Affymetrix GeneChip® Human Genome U133A 2.0 Array containing 22,241 probe sets representing 14,500 well-characterized human mRNAs (Affymetrix, Santa Clara, CA). 100 ng total RNA was amplified, labeled, hybridized, stained and scanned according to the manufacturer's instructions for the Affymetrix Two-Cycle Eukaryotic Target Labeling kit and Affymetrix GeneArray® (Affymetrix). The generated DAT and CEL files were imported and analyzed in dChip. Data were quantile normalized with PN7 as baseline (with a median probe intensity of 278). The PM/MM difference model was used and data were filtered accepting probe sets with a variation across samples between $0.5 < \mathrm{SD}/\mathrm{mean} < 1000$, and a difference between samples of 100 or more to account for the background level (~70). Data were log2 transformed and imported to SAM [10] to do multiple hypothesis testing correction and to identify statistically significant mRNAs ($q < 0.05$). Gene ontology analyses were performed applying gene ontology algorithms to the data (NetAffx™ Analysis Center, Affymetrix). Microarray data (miRNA and mRNA) were deposited at EMBL-EBI in accordance with MIAME guidelines (E-MEXP-2232).

2.5. Quantitative real-time PCR

Total RNA from skin biopsies from NN ($N = 4$), PN ($N = 4$) and PP ($N = 4$) were quantified for miR-21, -203, -205, -221, -222, RNU6B, PDCD4, TPM1, P57, C-KIT, RTN4, SHIP2, TIMP3, RECK, NFIB and actin by TaqMan® Real-Time PCR (Applied Biosystems). Five nanograms of total RNA was reverse transcribed (RT) using either for the miRNA analysis the TaqMan® MicroRNA Reverse Transcription Kit with miRNA-specific stem-loop primers or for the mRNA analysis the TaqMan® High-Capacity cDNA Reverse Transcription Kit with random primers (Applied Biosystems). 1.33 μl RT-product was introduced to a 20 μl PCR reaction and incubated in a 7900HT sequence detection system (Applied Biosystems) following the manufacturer's instructions. The miRNA expression was normalized to the RNU6B expression and the mRNA expression was normalized to actin. The significance was calculated by unpaired t-tests except for PP compared with PN, where a paired t-test was used (SPSS, Chicago, IL).

2.6. Experimentally validated miRNA–mRNA interactions

Experimentally validated *in vitro* or *in vivo* miRNA–mRNA interactions from the literature were assessed (www.ncbi.nlm.nih.gov/pubmed). The found miRNA targets were then compared with the downregulated mRNA expression data in PP compared with NN or PN (Table 2).

2.7. Prediction of miRNA–mRNA interactions

The 10 most downregulated mRNAs in PP were uploaded to the miRNA target prediction programs: TargetScan 5.0 [11], Pictar [12], MiRanda [13] and microRNA.org [14]. Predicted targets were compared with the deregulated miRNAs in PP and evaluated in RNAhybrid [15] accepting: hybridization conditions with a helix constraint of nucleotide position 2–8 in the 5′ seed region of each miRNA binding to the 3′UTR of the target mRNA [16], and a minimum free energy of −21 kcal/mol.