Genome-Wide DNA Methylation Profiling Identifies Differential Methylation in Uninvolved Psoriatic Epidermis

Deepti Verma¹,², Anna-Karin Ekman¹,², Cecilia Bivik Eding¹ and Charlotta Enerbäck¹

Psoriasis is a chronic inflammatory skin disease with both local and systemic components. Genome-wide approaches have identified more than 60 psoriasis-susceptibility loci, but genes are estimated to explain only one-third of the heritability in psoriasis, suggesting additional, yet unidentified, sources of heritability. Epigenetic modifications have been linked to psoriasis and altered DNA methylation patterns in psoriatic versus healthy skin have been reported in whole-skin biopsies. In this study, focusing on epigenetic modifications in the psoriatic uninvolved skin, we compared the lesional and non-lesional epidermis from psoriasis patients with epidermis from healthy controls. We performed an exhaustive genome-wide DNA methylation profiling using reduced representation bisulphite sequencing, which interrogates the methylation status of approximately 3–4 million CpG sites. More than 2,000 strongly differentially methylated sites were identified and a striking overrepresentation of the Wnt and cadherin pathways among the differentially methylated sites was found. In particular, we observe a strong differential methylation in several psoriasis candidate genes. A substantial number of differentially methylated sites present in the uninvolved versus healthy epidermis suggests the presence of a pre-psoriatic state in the clinically healthy skin type. Our exploratory study represents a starting point for identifying biomarkers for psoriasis-prone skin before disease onset.

INTRODUCTION

The genetic component in psoriasis pathogenesis is well recognized, and genome-wide association studies have identified more than 60 psoriasis-susceptibility loci (Ellinghaus et al., 2010; Nair et al., 2009; Tang et al., 2014; Tsoi et al., 2012). However, it is estimated that the identified genes explain only 28% of the heritability of psoriasis, suggesting additional, yet unidentified, sources of heritability. Epigenetic approaches have identified more than 60 psoriasis-susceptibility loci, but genes are estimated to explain only one-third of the heritability in psoriasis, suggesting additional, yet unidentified, sources of heritability (Tsoi et al., 2015).

The incomplete concordance in disease status between monozygotic twins suggests a role for environmental factors in psoriasis pathogenesis (Das et al., 2009). Environmental triggers, such as infections, stress, injuries, smoking, alcohol, and lithium medication, can induce an auto-inflammatory response mediated by DNA methylation in genetically predisposed individuals (Baker et al., 1997; Tsankov et al., 2000). Because parental exposure to environmental triggers has been shown to affect the transgenerational transmission of altered DNA methylation (Tirerotola et al., 2015), epigenetics may contribute to the missing heritability in psoriasis.

RESULTS

Global DNA Methylation Profiling of PP, PN, and NN Epidermis

Using reduced representation bisulphite sequencing to map the global CpG methylation state, we obtained approximately 21–52 million total reads in the samples, with a mean value of 35,899,496 per sample. The mapping efficiency ranged from 48% to 59%, with an average of 19,060,737 sites successfully aligning back to the human genome (UCSC hg19) and the read depth ranged from six to nine-fold. The technical validation of reduced representation bisulphite sequencing data using pyrosequencing...
showed highly consistent results for all the tested sites (data not shown). Using flow cytometry, the CD45+ leukocyte population was found to contribute <2.4% of the total cells (data not shown).

The number of covered CpG sites and DMS are shown in Table 1. The genomic distribution of these sites are represented in Supplementary Figures S1a–S1d (online).

The top 2,000 DMS showing >33% differential methylation in the pairwise comparisons, demonstrated several over-represented pathways relevant for psoriasis (Supplementary Table S1 online). Using a significant threshold of a Bonferroni-adjusted P-value of 0.05, we found common enrichments of pathways like cadherin (PN/NN \( P_{\text{Bonferroni}} = 2.8 \times 10^{-9} \), PP/PN \( P_{\text{Bonferroni}} = 8.5 \times 10^{-21} \), PP/NN \( P_{\text{Bonferroni}} = 6.2 \times 10^{-9} \)) and Wnt (PN/NN \( P_{\text{Bonferroni}} = 2.8 \times 10^{-9} \), PP/PN \( P_{\text{Bonferroni}} = 2 \times 10^{-20} \), PP/NN \( P_{\text{Bonferroni}} = 3.5 \times 10^{-10} \)). The pathways exclusively detected in the lesional skin when compared with the non-lesional or healthy skin were heterotrimeric G-protein signaling (PP/PN \( P_{\text{Bonferroni}} = 1.19 \times 10^{-3} \), PP/NN \( P_{\text{Bonferroni}} = 1.13 \times 10^{-2} \)) and inflammation mediated through cytokines and chemokines (PP/PN \( P_{\text{Bonferroni}} = 3.6 \times 10^{-2} \), PP/NN \( P_{\text{Bonferroni}} = 2.9 \times 10^{-2} \)), which might reflect accelerated cell growth and sustained inflammation.

### Table 1. Distribution of differentially methylated sites in the pairwise comparisons

<table>
<thead>
<tr>
<th>Variable</th>
<th>PP vs. NN</th>
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</tr>
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<tbody>
<tr>
<td>Total sites</td>
<td>2,072,217</td>
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<td>DMS</td>
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Abbreviations: DMS, differentially methylated sites; NN, healthy; PN, uninvolved; PP, involved.

The CpG Methylation Pattern in the Psoriasis-Susceptible and the Normal Epidermis Compared with the Normal Epidermis

The PN/NN comparison revealed a substantial number of DMS (Figure 1a). The DMS overlapping between the PN/NN and PP/NN comparisons likely represent a methylation pattern that is intrinsic to the psoriatic skin, rather than resulting from the inflammatory process (Figure 1b, Supplementary Appendix S1 online). The overlapping genes show an overrepresentation of the Wnt and cadherin pathways (\( P_{\text{Bonferroni}} = 3.1 \times 10^{-6} \) and \( 4 \times 10^{-8} \), respectively). Using the recent Reactome resource in PANTHER (Mi et al., 2017), we found that these genes were enriched in non-canonical Wnt signaling (\( P_{\text{Bonferroni}} = 2 \times 10^{-2} \)). The Wnt-pathway regulating genes including Wnt7B, Nfatc1, Celsr2, and Fzd7 demonstrated >33% differential methylation at multiple sites. Fzd7, the Frizzled receptor for Wnt proteins, is differentially expressed in psoriasis (Gudjonsson et al., 2010). Il23r, which has a strong pathogenic association with psoriasis (Harden et al., 2015), was hypermethylated in both the PN/NN and PP/NN.

### Methylation Levels Distinguish Involved, Uninvolved, and Normal Epidermis

To determine whether the PN and NN can be distinguished based on the differences in methylation pattern, we selected the 100 most DMS between the PP and NN comparisons. We performed an unsupervised hierarchical clustering, including the PN samples, which revealed a distinct clustering between the PP, NN, and PP (Figure 2a). The PN samples, albeit in a distinct cluster, displayed a methylation pattern that more closely resembled the NN than the PP epidermis.

### Altered Methylation at the Psoriasis Risk-Associated Loci

Two recent meta-analysis identified 59 replicated psoriasis risk-associated genes (Tsoi et al., 2015, 2017). The genes are listed in the Supplementary Appendix S4 online. We investigated the level of DNA methylation for all the CpG sites in

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these psoriasis risk-associated genes, including their promoters. Using a permutation test, we found 38 sites with >33% DMS among the psoriasis candidate genes, which was greater than expected by chance ($P < 0.01$). These sites mainly annotated to the candidate genes TRAF3IP2, ZMIZ1, and CARD14. The number of such DMS in the top 15 cis-acting eQTLs in psoriatic lesional skin (Ding et al., 2010) was not found to be greater than expected by chance ($P = 0.92$).

Transcriptomic Profiling of Epidermis

We found a distinct differential gene expression in the PP/PN (496 differentially expressed genes) and PP/NN comparisons (587 differentially expressed genes). The hallmark genes (IL36G, KNYU, RHCG, ATP12A, HPSE, CCL20, SERPINB13, SOX7, and C20orf24), universally shown to be upregulated in psoriatic full-thickness skin (Swindell et al., 2014), displayed an increased expression in our epidermal samples from the PP/NN and PP/PN comparisons.

An unsupervised hierarchical clustering of the top differentially expressed sites resulted in the absence of clustering between the PN and the NN samples, indicating that the expression differences between them are subtle (Figure 2b).

Association of mRNA Expression with Methylation Levels in the Epidermis

Using a differential methylation cutoff of 33%, we found 44 shared genes in the PP/NN and 41 shared genes in the PP/PN. The genes DUSP1 and GATA3, both previously shown to be downregulated in psoriasis (Kjellerup et al., 2013; Racz et al., 2011), demonstrated several >33% DMS, along with a corresponding transcriptional downregulation in the PP/NN comparison. Additional genes with key skin-regulatory functions, where hypermethylation correlated with a downregulated expression, included EXPH5, AHNAK, PTPN14, NR1D1, and PTPN21 (Gerber et al., 2013). Strongly hypomethylated genes with an upregulated transcription in the PP/NN comparison included FOXE1, GJB2, RAB31, WWOX, ATP1B1, SLC7A5, and SOX7, all of which have been implicated in psoriasis (Suarez-Farinas et al., 2012).

DISCUSSION

We report a comprehensive genome-wide analysis using epidermal samples to study global DNA methylation and RNA expression patterns in psoriasis. We utilized the next-generation sequencing–based technique RRSB to investigate DNA methylation in the epidermis derived from lesional and non-lesional psoriasis and from normal skin. The epidermis was removed using ammonium thiocyanate, a
method that does not affect gene expression (Clemmensen et al., 2009). In order to minimize the confounding effects of cell heterogeneity, we specifically analyzed the epidermis rather than the full-thickness skin.

A few studies using the Illumina’s 27–450K resolution in full-thickness skin, have reported a difference in methylation between the PP skin and the PN or NN skin (Roberson et al., 2012, Zhou et al., 2016). Using almost 10 times the number of probes, we now provide additional, comprehensive data, and validate the previous findings. Using the Illumina 27K, Roberson et al. (2012) identified 1,108 differentially methylated genes, of which 443 were found in our pairwise PP/NN comparison. We also identified six (MANIC1, SPIRE2, AHDC1, DLGAP4, ECE1, and CYP2S1) of the nine differentially methylated loci recently described by Zhou et al. (2016). Several DMS mapped to the genome-wide association studies psoriasis-risk genes and altered methylation at multiple sites were observed for TRAF3IP2, ZMIZ1, and CARD14.

Substantial differential methylation was evident in the comparison of PN and NN. Intriguingly, these DMS included several sites (328 genes) identified by Roberson et al. (2012), as well as five of the nine sites described by Zhou et al. (2016) in the PP/NN comparisons, showing that the methylation differences not only delineate the lesional and healthy skin, but also the clinically normal, uninvolved skin from the healthy skin. When comparing DMS present in both PP and PN compared with NN, a strong over-representation of differentially methylated Wnt and cadherin pathway genes was found, which were significantly enriched for the non-canonical Wnt/Ca^{2+} signaling. Wnt signaling plays critical roles in embryogenesis and tissue homeostasis. NFATc1, a WNT5A downstream gene implicated in non-canonical Wnt signaling, demonstrated DMS at multiple sites. Interestingly,
NFATc1 was recently shown to be important for imiquimod-induced psoriasiform dermatitis by suppressing IL-10 in B cells (Alrefai et al., 2016).

When comparing the PP skin with the PN (or NN) skin, we attempted to filter out the changes in methylation that are a consequence of the inflammatory reaction in lesional skin to distinguish them from the changes that underlie disease susceptibility. An over-representation of DMS associated with Wnt signaling was found and included SFRP4, a negative regulator of Wnt signaling, which was recently shown to be downregulated in psoriasis through an epigenetic mechanism (Bai et al., 2015). SFRP4 directly inhibited keratinocyte proliferation triggered by proinflammatory cytokines in vitro (Bai et al., 2015). Moreover, we found differential methylations of Wnt5A, as well as its receptor, FZD2. Wnt5A is an important pro-inflammatory factor that has been implicated in several inflammatory diseases, such as rheumatoid arthritis, atherosclerosis and psoriasis (Pashirzad et al., 2017). The expression of Wnt5A is increased in psoriatic epidermis, together with its receptors, FZD2 and FZD5 (Gudjonsson et al., 2010; Reischl et al., 2007; Romanowska et al., 2009).

Interestingly, Wnt5A is one of the very few genes that retain increased expression in resolving psoriatic lesions (Suarez-Farinas et al., 2011).

As reported previously (Roberson et al., 2012; Zhang et al., 2013; Zhou et al., 2016), we found that relatively few, albeit functionally relevant, subsets of genes overlapped between methylation and expression. Unlike the clear distinction in the methylation pattern between the PN and NN samples, the differences in gene expression between these groups were subtle. This suggests that DNA methylation does not directly translate into altered expression but may poise the associated genes for expression in response to future triggers.

The differences between PN and NN might be influenced by the underlying systemic/local inflammation or genetic factors. The increased inflammatory activity in PN skin was suggested by Johnson-Huang et al. (2012), who demonstrated infiltrating CD3+ T cells, and inflammatory DCs in PN skin in patients with psoriasis (Johnson-Huang et al., 2012). In favor of an intrinsic abnormality in PN skin is our recent finding of an apoptosis-resistant phenotype in cultured uninvolved psoriatic skin. Interestingly, this effect persisted after several passages of culture (Bivik Eding and Enerback, 2017). Moreover, uninvolved skin demonstrated abnormal barrier function and differential in vitro keratinocyte spreading (Chen et al., 2001, Ye et al., 2014).

In conclusion, we demonstrate substantial methylation differences between uninvolved psoriatic skin and healthy skin, suggesting that the uninvolved skin might represent a pre-psoriatic state. Further studies are required to identify the biological mechanisms underlying the altered methylation patterns in PN versus NN skin.

MATERIAL AND METHODS

The detailed protocols and statistical analysis are described in the Supplementary Materials and Methods (online).

Study Population and Samples

Written informed consent was obtained from the patients and control subjects, and the ethical principles of the Declaration of Helsinki were followed. The study was approved by the Regional ethics committee in Linköping. Skin punch biopsies (4 mm) were obtained from psoriasis patients and controls. The psoriasis diagnosis had been confirmed by a dermatologist. The participating patients were not receiving any systemic treatment, and the lesions from which the skin biopsies were obtained were untreated. The PP biopsies were taken from the lower back in an active psoriasis lesion and PN skin was obtained from a non-lesional area on the lower back of the same patient at least 10 cm from the active, lesional skin. A total of 12 biopsies, 6 from psoriasis patients (including 3 pairwise PP and PN) and 6 NN biopsies, were obtained. All participants were males, to avoid confounding gender-specific methylation patterns (Liu et al., 2010). The epidermis was detached by soaking the biopsy in 3.8% sterile ammonium thiocyanate in phosphate buffered saline for 30 minutes, as described previously (Clemmensen et al., 2009). DNA was immediately isolated using the Blood and Tissue DNA extraction kit (Qiagen, Hilden, Germany).

Methylation Analysis

The DNA samples were processed for genome-wide DNA methylation sequencing analysis by Zymo Research (Irvine, CA) (Supplementary Materials and Methods). The CpG sites having a minimum read-depth of five and a methylation difference cutoff ±10%, were annotated into promoter, exon, intron, or CpG island. The gene boundary was defined as ±1 kb from the transcription start site of the gene. The methylation level of each sampled cytosine was estimated as the number of methylated cytosines divided by the total number of cytosines. Pairwise comparisons were done for PP/NN, PP/PN, and PN/NN samples. The methylation difference for each site was calculated by subtracting the median methylation value of the sample of interest from the reference sample. For increased stringency, we based our analyses on the top 2,000 DMS with a differential methylation cut off of 33%.

Gene Ontology Annotation

The PANTHER (http://www.PANTHERdb.org/) database was used for the gene ontology and pathway over-representation analysis. Official gene symbols were used as input to calculate the statistical over-representation of biological process gene ontology terms.

Gene Expression Profiling

Gene expression was determined using Affymetrix Beadchip 2.0 (Affymetrix, Santa Clara, CA), following the manufacturer’s instructions. The differential expression of genes was determined using the GeneSpring GX software (Agilent Technologies, Santa Clara, CA), with a cutoff of ±1.5 fold change.

Statistics

The statistical significance of the methylation difference was determined by the Student t-test or Fisher’s exact test. Statistical over-representation of PANTHER pathways was determined using a Bonferroni correction. The differential methylation of CpG sites in psoriasis candidate genes and eQTL loci were investigated using a permutation test.

Data Access

The DNA methylation data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database under accession number GSE 103038.

CONFLICT OF INTEREST

The authors state no conflict of interest.
SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org. See the references.

REFERENCES


