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MINIREVIEWS

What have we learned about non-involved psoriatic skin from large-scale gene expression studies?

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Abstract

Psoriasis is a chronic inflammatory skin disorder; its genetic background has been widely studied in recent decades. Recognition of novel factors contributing to the pathogenesis of this disorder was facilitated by potent molecular biology tools developed during the 1990s. Large-scale gene expression studies, including differential display and microarray, have been used in experimental dermatology to a great extent; moreover, skin was one of the first organs analyzed using these methods. We performed our first comprehensive gene expression analysis in 2000. With the help of differential display and microarray, we have discovered several novel factors contributing to the inherited susceptibility for psoriasis, including the EDA+ fibronectin splice variant and PRINS. The long non-coding PRINS RNA is expressed at higher levels in non-involved skin compared to healthy and involved psoriatic epidermis and might be a factor contributing cellular stress responses and, specifically, to the development of psoriatic symptoms. This review summarizes the most important results of our large-scale gene expression studies.

Key words: Non-involved psoriatic skin; Differential display; cDNA microarray; EDA+ fibronectin isoform; PRINS long non-coding RNA; mRNA maturation

Core tip: Large-scale gene expression studies, including differential display and microarray, have provided valuable data on the molecular background of psoriasis pathogenesis. This review summarizes the most important results of the available literature and our large-scale gene expression studies obtained from the clinically non-involved psoriatic skin: we identified the EDA+ fibronectin splice variant as an autocrine proliferation signal for psoriatic hyperproliferative keratinocytes and PRINS, a long non-coding regulatory RNA. We believe that the characterization of new candidate genes and proteins might establish new therapeutic approaches, which may allow treatment of already existing psoriatic lesions as well as non-involved psoriatic skin by affecting molecular aberrancies, and may lead to the development of prophylactic interventions.

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INTRODUCTION

Psoriasis is a hyperproliferative inflammatory skin disorder affecting approximately 2%-3% of the European population[1]. However, in some other parts of the world, this disease is almost unknown: *e.g.*, in Africa the occurrence of psoriatic cases is remarkably rare[2]. The exact trigger of the disease is still obscured and the subject of several investigations. Inherited and environmental factors (*e.g.*, mechanical trauma, UV exposure, stress) are responsible for the development of psoriatic symptoms[2,3].

In the most typical cases, hyperproliferative psoriatic plaques are formed on the skin of the knees, elbows and the scalp. In addition, the disorder can affect skin annexes and joints. In the case of some patients with severe psoriasis, the entire body is covered with lesions. Severe psoriasis is often associated with metabolic syndrome; hence, psoriasis patients also have elevated cardiovascular and stroke risks[2]. Unfortunately, psoriasis has a negative effect on the patient's quality of life due to serious psychosocial and emotional stress[4]. A number of emerging arguments support the idea that psoriasis is a systemic disorder rather than simply a skin disease. Psoriasis has many common features with chronic autoimmune inflammatory disorders, such as progressive arthritis. Moreover, psoriasis and autoimmune syndromes often share common genetic loci[5-7]. Similarities are especially evident when psoriasis is compared to chronic inflammatory bowel disorders, such as Crohn's disease, where internal barriers are involved[8,9].

Similarly to autoimmune disorders, immune-activation plays an important role in psoriasis: the development of the characteristic erythematosus, demarcated and scaly lesions is related to the abnormal functioning of the cellular immune system[10-14]. Cytokines produced by aberrantly functioning T-lymphocytes are able to stimulate keratinocytes, which show an elevated sensitivity to these proliferative signals[14,15]. However, it is still unknown whether the primary triggers of the disease phenotype are the professional immune cells or the keratinocytes. Inherited susceptibility of keratinocytes has been partially established. Identification and characterization of these factors may greatly facilitate the understanding of the molecular background of psoriasis. Large-scale gene expression profiling methods developed and used in the 1990s might be useful tools to answer these exciting questions.

DAWN OF THE LARGE-SCALE GENE EXPRESSION STUDIES: DIFFERENTIAL DISPLAY AND SAGE

In recent years, we and others have tried to characterize molecular factors responsible for the hyper responsiveness of keratinocytes to various stimuli[8,12,14,16]. To reveal these processes, researchers need suitable and powerful methods that can detect more than one possible target. Previously, altered expression of only a few candidate genes or proteins was possible. The development of large-scale gene expression analysis methods marked a significant breakthrough in this field. With the help of microarrays and their predecessors, differential display (DD) and the serial analysis of gene expression (SAGE), gene-expression patterns of serial samples can be compared for large data sets.

For DD, gene expression profiles are analyzed for pairs of corresponding sample sets. The most important steps of the method are the isolation of total RNA from the samples and its reverse transcription into cDNA. Subsequently, cDNA is amplified, subjected to gel electrophoresis and, after the expression pattern has been compared, bands representing differentially expressed genes are cut out from the gel and the DNA content is cloned into a plasmid vector. It should be mentioned that the DD method has some limitations due to the relatively frequent incidence of false positive results. Hence, the results must be validated using an independent technique. Validation is usually carried out by reverse Southern blot analysis, followed by sequencing the differentially expressed transcripts[17-19]. The great advantage of DD is that it is an "open ended" analysis system, allowing unannotated differentially expressed transcripts to be identified.

Another sequence-based approach, SAGE, was developed at the Oncology Center of the Johns Hopkins University by Velculescu and his co-workers. Changes in gene expression patterns are detected by sequencing reverse transcribed cDNAs. Application of short oligonucleotide sequence tags allows quantitative changes to be monitored, in addition to the qualitative analysis[20,21].

MICROARRAYS

Microarrays provide more extended and comprehensive methods for analyzing gene expression profiles than DD and SAGE. The biggest advantage of this approach is that it allows thousands of genes to be measured simultaneously. Moreover, complex regulatory networks can be assessed[22,23]. In contrast to DD and SAGE, microarrays are a "closed" analysis system, allowing only known sequences to be screened. The introduction and widespread use of microarrays has facilitated advances in several branches of science, including experimental dermatology. In fact, the skin was one of the first human organs to be analyzed with this technique[24-27].

Microarray technologies rely on complementarity for sequence-specific recognition of the DNA segments[28]. Most commonly used probes are cDNAs derived from bacterial libraries and BACs or oligonucleotides. Long oligonucleotide probes (50-120 nt) might support a higher degree of specificity and sensitivity than short (15-25 nt) probes[22]. The probes are fixed to a solid support, such as glass or plastic that are referred to as "chips" in common laboratory jargon[23].

For microarray experiments, total RNA is isolated

from samples and reverse-transcribed with fluorescent dyes such as Cy3 and Cy5 or with radioactive isotope to label the synthesized cDNA. After hybridizing the labeled probes to the chips for approximately 16-24 h, the chips are washed and the fluorescence is scanned with a confocal microscopy. Data are then analyzed using specially developed software. Like DD, this method can identify false positives and, therefore, must be validated by RT-PCR, northern blot analysis or RNAse protection assay[22,28].

The outstanding advantage of microarray techniques is the simultaneous investigation of thousands of genes and, thus, the possibility to explore novel molecular pathways. This technique can be a powerful tool in tumor and biomarker research and may serve as the basis of personalized therapies[27].

LARGE-SCALE GENE EXPRESSION STUDIES OF PSORIASIS: IDENTIFICATION OF MOLECULAR FACTORS CONTRIBUTING TO PATHOGENESIS

The use of large-scale gene-expression analysis methods has been fruitful for experimental dermatology. Microarrays have been used to study several disorders, such as melanoma, atopic dermatitis and autoimmune skin diseases. In the past decade, DD and microarray techniques have been widely employed alone or in combination with other methods in psoriasis research[29].

Gene-expression profiling of peripheral blood cells and epidermis samples from healthy, psoriatic involved and psoriatic non-involved skin proved to be a powerful tool for the characterization of aberrant molecular patterns in the disease[30]. The results of cDNA microarrays supported previous findings and were useful to describe novel pathways implicated in psoriasis pathogenesis. Psoriasis research was dominated by the so-called "immune theory" for many years, and microarray studies further proved the involvement of genes related to inflammation and immune responses. One of the earliest microarrays identified several inflammation- and immune-related genes (*IL4R*, *CD2*, *CD24* and *INF-γ* induced genes) that were not previously reported to contribute to the pathogenesis of this disorder[30,31]. Moreover, Oestreicher *et al*[31] performed a longitudinal analysis in which they characterized changes in gene expression in response to recombinant human IL-1 or cyclosporine in therapy responder and non-responder populations. A study from Zhou, which compared samples from healthy, involved psoriatic and non-involved psoriatic skin biopsies further supported the involvement of the activated T-cell product INF-γ and transcription factors induced by this pro-inflammatory lymphokine[30,32]. The role of IL-17 signaling was also demonstrated in large-scale gene-expression studies[13]. In addition, Gudjonsson *et al*[3] emphasized the role of altered innate immune functions in psoriasis. Differential expression of genes encoding chemokines and their receptors were also described by several research groups[32-34].

Other important cellular pathways related to psoriasis regulate epidermal keratinocyte proliferation and apoptosis. The implication of *PPAR-δ*, *mTOR*, *NFκB*, *BCL-2* and *BAX* expression was verified for these mechanisms[35-37]. In a study of Wnt pathways responsible for stem cell proliferation and differentiation, Reischl *et al*[38] found that only *Wnt5a* expression was higher in psoriatic involved skin than in non-involved samples. In addition, actin cytoskeleton organization can be affected: the *CCNA2* gene is responsible for the G_2/M transition in the cell cycle and affects intracellular cytoskeleton organization and cell migration[39,40].

The clinical association of psoriasis and metabolic syndrome is a well-known phenomenon. Gudjonsson and co-workers were able to show that lipid metabolism pathways were altered in psoriatic non-involved epidermis compared to healthy samples[3]. In this comparison, it was proven that lipid metabolism genes were down-regulated in non-involved skin samples as compared to healthy skin and further down-regulation was identified in psoriatic involved skin[3]. Romanowska *et al*[35] studied the role of PPARδ, a transcription factor participating in metabolic and inflammatory processes, in psoriasis. PPARδ exerts proangiogenic and antiapoptotic affects and is suspected to be involved in the enhancement of keratinocyte proliferation[35].

Most recently, bioinformatic meta-analyses were performed using publicly available databases of psoriasis-related microarray data. In one of the first microarray meta-analysis, Tian *et al*[41] analyzed the result of five previous cDNA microarrays experiments. In a subsequent meta-analysis, Manczinger *et al*[40] compared differentially expressed genes of psoriatic involved and non-involved epidermis. The findings of these two meta-analyses agreed and showed that the most important components of the molecular networks related to psoriasis are factors implicated in cell proliferation and immunomodulation. Importantly, these meta-analyses confirmed that several differentially expressed transcripts were also involved in metabolic disturbances, such as impaired glucose tolerance, insulin tolerance and atherosclerosis[40,41].

It is important to note that most of the large-scale gene expression studies for the identification of molecular patterns in psoriasis pathogenesis have compared the gene expression profiles of psoriatic involved and non-involved skin or psoriatic involved and healthy skin. This research provided extremely valuable data for the molecular events of psoriasis[40]. Much less information is available, however, on differentially expressed genes in normal epidermis compared to psoriatic non-involved epidermis. We and others believe that identifying aberrantly expressed genes and molecular patterns in non-involved psoriatic epidermis is important for understanding this disease.

DIFFERENTIAL DISPLAY AND MICROARRAY EXPERIMENTS OF OUR RESEARCH GROUP, FOR THE IDENTIFICATION OF NOVEL MOLECULAR FACTORS OF PSORIASIS

Our research group performed the first comprehensive gene-expression analysis for psoriasis in 2000 to compare psoriatic non-involved epidermal samples with control healthy epidermis. This approach allowed early and inherited molecular factors to be studied in detail and allowed novel susceptibility factors to be revealed. This study identified two known transcripts that were differentially expressed: *RAB10*, an oncogene that belongs to the small GTPase superfamily, and fibronectin, a well-known extracellular matrix component[42]. Our subsequent studies focused on the role of fibronectin in the pathogenesis of psoriasis.

Fibronectin is a complex glycoprotein composed of repetitive modules[43]. At least 24 differentially spliced variants of this gene have been described, and the presence of certain variants depends on age, developmental state and cell type[44]. Alternative processing involves three preferred sites: extra domain A (EDA), extra domain B and extra type homology B[43,45]. The splice variants containing the EDA domain play a crucial role in embryonic development and wound healing. However they are detectable only in modest amounts in adult normal tissues[44,46,47]. Because it is also abundantly expressed in different types of tumors, it is referred to as the oncofetal fibronectin splice variant[48]. Interestingly, in the brain, an organ in which fibronectin is poorly expressed, the inclusion of the EDA domain is abundant in young adults (88% as compared to fetal level) and decreases with age to 33%[46].

The presence of the EDA+ fibronectin variant is associated with several pathological conditions and is suspected to participate in the development of psoriasis as well. The oncofetal fibronectin form was found to be present in a higher ratio at the dermal-epidermal junction of psoriatic non-involved skin compared to healthy normal skin[14,49]. Unlike the conventional variant, the oncofetal EDA+ fibronectin form interacts with the α5 integrin subtype, instead of α2 and α3, and, as a result, its effect on cellular signaling processes is more robust. α5β1 integrin receptors were shown to be upregulated in both non-involved and involved psoriatic skin[14,50].

In addition, other authors reported that the EDA+ fibronectin variant is co-localized with CD11c+ macrophages. It was suggested that these cells might contribute to the production of the oncofetal variant; however, because of their relatively low number, they are likely not to be the most important source[49]. Based on our results we supposed that keratinocytes themselves might produce EDA+ fibronectin and, as an autocrine molecular factor, may contribute to the induction and maintenance of keratinocyte hyperproliferation in psoriasis[16].

We have also performed *in vitro* experiments to understand the role of EDA+ fibronectin in the regulation of keratinocyte proliferation. Subsequently, RT-PCR was carried out using immortalized HaCaT cells. Our results indicated that, after serum starvation and contact inhibition, the highest level of EDA+ fibronectin expression could be detected in the highly proliferative HaCaT cells, and the ratio of EDA+/EDA- fibronectin produced by the keratinocytes might well be a potent mitogen signal in cell cycle regulation. In contrast to fibroblasts and normal human keratinocytes, the ratio was altered in this cell line. Flow cytometry supported the RT-PCR results. The results of the HaCaT cell line experiments indicated that keratinocytes themselves might produce the oncofetal fibronectin variant[16].

In addition to proteins with known functions, the DD experiment identified a novel transcript: the corresponding gene was subsequently named psoriasis-susceptibility-related RNA gene induced by stress (PRINS, accession number AK022043). During the structural investigation of PRINS, we found that the gene consists of two exons containing several stop codons, which prevent the formation of a longer open reading frame. *In silico* sequence comparison supported the hypothesis that PRINS functions as a non-coding RNA molecule, rather than serving as a template for protein translation. In addition, PRINS contains two repetitive *Alu* sequences and has 70% sequence similarity with the *Tetrahymena thermophyla* G8 small nucleolar non-coding RNA[42].

In a quantitative RT-PCR analysis, we demonstrated that PRINS is expressed at higher levels in non-involved skin compared to healthy and involved psoriatic epidermis. Our *in vitro* experiments performed on synchronized HaCaT cells showed that PRINS expression dropped significantly when the cells were released from cell quiescence and the cells started to proliferate actively[42]. These data suggested that PRINS might be a factor disposing keratinocytes to hyperproliferation and contributing to the development of psoriatic symptoms. The exact role of PRINS is still unknown, but it is very possible that it plays an important role in cellular stress responses. Silencing PRINS did not affect the survival of the cells; however under certain stress conditions (such as serum starvation) the cells died at a much higher rate when the expression of PRINS was down-regulated[42,51]. Consequently, the PRINS-silenced cells became more vulnerable, supporting the cellular-stress response hypothesis. Moreover, our research group later showed that the G1P3 antiapoptotic protein might be regulated by the PRINS non-coding RNA[52].

Since then, we have identified nucleophosmin as one of the possible cellular interacting partners of PRINS. Nucleophosmin is a phosphoprotein which is a member of the p53 pathway, and its movement in fibroblasts, cancer cells and keratinocytes is triggered by ultraviolet (UV) exposure[53]. We also demonstrated that silencing PRINS prevents nucleolar-cytoplasmic shuttling of nucleophosmin.

This result indicates that PRINS might physically interact with the nucleophosmin protein and that the abnormal functioning of the PRINS-nucleophosmin ribonucleoprotein complex may contribute to psoriasis pathogenesis[54].

Taken together, we consider the identification of novel factors implicated in the early molecular defects in psoriasis pathogenesis-the EDA+ fibronectin splice-variant and the PRINS non-coding RNA-the most significant outcomes of our DD experiments. Due to the success of the DD, we attempted to identify novel psoriasis susceptibility factors using newly available cDNA microarray technology for large-scale gene-expression analysis. In particular, we aimed to identify molecular patterns that are responsible for the differential reactivity of normal healthy epidermis and psoriatic non-involved epidermis.

Organotypic tissue cultures were created from four healthy and four psoriatic non-involved skin samples. Half of the samples were treated with a mixture of T-cell lymphokines, containing IL-3, IFNγ and GM-CSF, cytokines previously described to be implicated in the T-cell response and the formation of psoriatic plaques[10]. After three days of treatment, the dermis and epidermis were separated. Total RNA was isolated from the epidermis, reverse transcribed and used to perform the cDNA microarray experiment. Based on the results, we selected genes that showed an altered gene expression in response to the lymphokine treatment[12].

We identified 61 transcripts that exhibited altered gene expression. Of these, eleven had been demonstrated earlier to contribute to psoriasis. Using bioinformatics tools, such as Gene Ontology and Ingenuity pathway analysis, we demonstrated that most of these molecules are implicated in two important intracellular pathways: "apoptosis" and "metabolism of small molecules and lipids." Real-time RT-PCR validation experiments revealed that many of these genes are already upregulated in non-involved psoriatic epidermis, and the lymphokine treatment did not further increase expression. In contrast, expression of these genes was inducible in healthy samples. These data indicate that keratinocytes in psoriatic non-involved epidermis are in a presensitised status, which explains their altered response to different triggering stimuli[12].

Among the differentially expressed genes, we also identified members of the serine-arginine rich (SR) proteins SR splicing factor 18 (SFRS18), peptidylprolyl isomerase G (PPIG) and luc-7 like 3 (LUC7L3), which regulate mRNA splicing. It was previously described that these proteins interact with pinin and SR-related nuclear protein[55-60]. Splicing is a post-transcriptional regulatory process and one of the most important sources of mRNA diversity, permitting the production of different mRNAs from the same DNA template. Splicing dysfunction has been shown to be involved in several disorders, and some novel therapeutic modalities have been designed to repair them[61-63].

Our research group has previously demonstrated that the fibronectin splice variants containing the EDA domain is implicated in the pathogenesis of psoriasis. This suggests the interesting question whether the identified splicing genes, *LUC7L3*, *PPIG* and *SFRS18*, contribute to the production of the EDA+ fibronectin variant. We are currently investigating the role of *LUC7L3*, *PPIG* and *SFRS18* splicing regulatory genes in the production of EDA+ fibronectin, and we aim to identify further differentially spliced mRNA variants contributing to psoriasis pathogenesis.

CONCLUSION

Taken together, recent comparisons between psoriatic non-involved and involved epidermis dominated large-scale gene expression studies related to psoriasis. Relatively few studies have focused on the comparison of gene expression differences between healthy and psoriatic non-involved epidermis samples. Nonetheless, we believe that these experiments are valuable for identifying factors that increase the risk for developing psoriatic plaques. In our microarray studies, we identified several novel candidate genes and molecular patterns that might contribute the formation of typical lesions. The altered expression of EDA+ fibronectin and that of LUC7L3, PPIG and SFRS-18 suggests that some kind of splicing anomalies have an important role in the development of psoriatic symptoms. The exploration of cellular networks related to RNA-maturation processes gave us a deeper insight into the molecular pathogenesis of psoriasis and investigation of the splicing machinery might be a very new approach in this field. Results of wide-scale gene expression studies have provided pioneering advances in psoriasis research as well as in the recognition of different types of non-coding RNAs, including *PRINS*. This RNA is a long non-coding RNA (lncRNA), and most lncRNAs have been identified in their involvements in the central nervous system and certain tumors[64-69].

The last decade has seen a rapid evolution in large-scale gene expression profiling methods. Techniques, such as RNA-Seq and digital gene expression profiling, provide an even greater resolution and wider dynamic range compared to either DD or cDNA microarray. Advancement of methods based on next-generation sequencing has accelerated the accumulation of data, and processing the results requires huge efforts. Thus, validation and interpretation of these newly discovered factors is a very important challenge. Identification of new candidates might establish new therapeutic approaches, which may allow treatment of already existing psoriatic lesions as well as non-involved psoriatic skin by affecting molecular aberrancies, and may lead to the development of prophylactic interventions.

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