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INVESTIGATIVE REPORT

Genes Involved in Epithelial Differentiation and Development are Differentially Expressed in Oral and Genital Lichen Planus Epithelium Compared to Normal Epithelium

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Lichen planus (LP) is a chronic mucocutaneous disease with unknown cause. Patients with LP often have both oral and genital lesions, but these conditions are often considered as separate diseases and treated accordingly. To find out which genes are differently expressed in mucosal LP compared to normal mucosa and establish whether oral and genital LP are in fact the same disease, whole genome expression analysis was performed on epithelium from 13 patients diagnosed with oral and/or genital LP and normal controls. For confirmation of keratin 4 and corneodesmosin expression, quantitative reverse-transcription PCR and immunohistochemistry were used. Many genes involved in epithelial development and differentiation are differently expressed in epithelium from LP compared to normal epithelium. Several of the differentially expressed genes are common for oral and genital LP and the same biological processes are altered which supports the fact that oral and genital LP are manifestations of the same disease. The change in gene expression indicates that differentiation is altered leading to changes in the epithelial barrier. ***Key words: oral lichen planus; genital lichen planus; epithelial differentiation; corneodesmosin; keratin 4.***

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Lichen planus (LP) is a chronic inflammatory disease affecting skin and mucosa. The origin of the disease is unknown but autoimmunity has been suggested to play a part in the pathobiology. One of the major histological hallmarks of LP is a band of inflammatory cells beneath the epithelium in which cytotoxic $CD8^+$ T cells are found (1). The antigen triggering the inflammatory response is not known even if changes on the surface of the keratinocytes have been suggested as a potential cause. Autoantibodies against factors involved in epithelial homeostasis such as members of the p53 family (2) and ELF-3 (3) have further been found in oral LP, supporting the theory of an epithelial defect. In vulvar LP an autoimmune phenotype has also been shown, characterised by an increased Th1 response and a strongly up-regulated expression of pro-inflammatory chemokines and receptors such as CXCR-3 and its ligands CXCL-10 and CXCL-11 (4).

The oral and genital mucosae are most commonly affected by mucosal LP, but oesophagus and conjunctiva can also be affected (5). The oral form of LP (OLP) affects between 1–2% of the population with a female predominance (6). A recent study showed that many of these patients also have skin and/or genital lesions. Of the men, 40% had both oral and genital lesions while the corresponding figure for women was 53% (7). The red erosive and ulcerative forms of LP can be very painful and greatly affect quality of life (8). In mucosal LP the erosive form can lead to scarring and fibrosis (9). OLP is classified by the WHO as a potentially malignant condition, a classification that is vividly discussed. No such classification is made for the other mucosal LP, even if there are reports on genital and oesophageal LP developing into squamous cell carcinoma (10, 11). Even if LP patients with both oral and genital lesions need multidisciplinary treatment including dermatologist and dentist with special interest in the oral mucosa, these conditions are in many countries and clinics considered as separate diseases and treated accordingly (7).

In order to establish whether OLP and genital LP (GLP) are in fact the same disease, we performed whole genome analysis on lesions from both locations. To specifically compare epithelial changes only, microdissection was used to isolate the epithelium. Changes found in LP lesions were further compared to status in normal oral and genital epithelium analysed in parallel.

Results showed several differentially expressed genes in common for OLP and GLP epithelium.

MATERIAL AND METHODS

Tissue samples

A total of 21 punch biopsies were collected after informed consent from 16 consecutive patients clinically and histologically diagnosed with mucosal LP (12 from oral and 9 from genital lesions). From 5 patients biopsies were collected from both oral and genital lesions. Twelve patients were women (mean age 66

years, range 45–77) and 4 were men (mean age 58 years, range 45–67) (Table SI[1]). OLP was diagnosed according to the modified WHO diagnostic criteria (12). Biopsies were embedded in Tissue TEK and snap frozen in liquid nitrogen and stored at –80°C until use. Control biopsies were collected from 8 persons with healthy oral and genital mucosa, totally 8 oral and 5 genital controls. Five of these control biopsies were taken from both oral and genital mucosa of the same individual. In the oral control group there were 6 women with a mean age of 55 years, range 45–69, and 2 men with a mean age of 59 years, range 49–69. The genital controls were all from women ($n = 5$) with a mean age of 52 years, range 45–67 (Table SI[1]). The study was approved by the Ethical Review Board at Umeå University (Dnr 09-083M).

Laser micro-dissection, RNA extraction and assessment of RNA quality

To enable analysis of epithelium only laser micro-dissection was performed using 10 µm cryosections placed on membrane coated glass slides (Carl Zeiss Micro Imaging GmbH, Bernried, Germany) and stained with HistoGene staining solution (Arcturus Bioscience, Mountain View, USA). Laser micro-dissection was performed using PALM® micro laser system (PALM GmbH, Hamburg, Germany) and epithelium was collected and placed in tubes with 850 µl TRIzol Reagent (Invitrogen). Tubes were incubated at room temperature for 30 min and then mixed for 5 min and centrifuged. Samples were stored at –80°C until RNA extraction. Total RNA was isolated using RNeasy Plus Microkit, (Qiagen, Hilden, Germany). RNA quality was assed using Agilent 2100 Bioanalyzer (Agilent, Palo Alto, USA).

Microarray and analysis

Amplification and labelling of RNA was performed using TargetAmp™-Nano labeling Kit for Illumina Expression BeadChip (Epicentre, Madison, WI, USA). Illumina BeadChip array, HumanHT-12_v4_BeadChip (Illumina, San Diego, CA, USA) was used for obtaining gene expression data. The procedure was performed as described by the manufacturer. Briefly, labelling and amplification of RNA was performed and after hybridisation and washing chips were scanned in an iScan system. Whole genome expression analysis was performed on RNA extracted from epithelium from 13 LP lesions, 7 oral and 6 genital, and from 12 controls, 7 oral and 5 genital. From 5 patients epithelium from both oral and genital lesions were used (Table SI[1]). Before normalisation and background correction, quality control of raw data was performed in GenomeStudio, Gene expression module v1.0 (Illumina). Two samples (one GLP and one genital control) with low expression of internal control genes were excluded from further analysis. Raw data were normalised and background corrected in R by the use of the Limma package (13). Significance analysis of microarrays (SAM) in Chipster (14) was used for detection of differentially expressed genes. A false discovery rate (FDR) < 2.5% was accepted. The Database for Annotation, Visualization and Integrated Discovery (DAVID) (15, 16) was used to detect enriched biological processes or pathways. The *p*-value is a modified Fischer Exact *p*-value: the smaller, the more enriched. The group of biological processes with the highest enrichment score and a *p*-value below 0.05 is presented in Table SII[1].

A heatmap was generated by the use of Chipster, with the Pearson correlation as a distance measure and average linkage for constructing the dendrogram. Array data have been submitted to and are available from the gene expression omnibus (GEO) (accession number GSE52130).

[1]http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1803

Quantitative real-time PCR

Quantitative real-time PCR was used for validation of Keratin 4 (*KRT4*) and Corneodesmosin (CDSN). Messenger RNAs from 8 OLP and 2 GLP, and 7 oral and 2 genital controls (Table SI[1]) was used. For cDNA synthesis 200 ng of RNA and the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) was used. IQ SYBR Green Supermix (Bio-Rad Laboratories Inc, Hercules, USA) and QuantiTect Primer Assay® (Qiagen, Hilden, Germany) for KRT4 and CDSN was used for PCR amplification, and all samples were run in duplicate and normalised against GAPDH (Qiagen). The relative amount of mRNA was determined by the comparative *C*t method, calculating a ratio between normalized RNA levels for LP and normal tissue, $\Delta\Delta C$t. Statistical analysis was performed using IBM SPSS Statistics 20 and the non-parametric method Mann-Whitney was used. Significance level was $p < 0.05$.

Immunohistochemistry

Immunohistochemical staining for KRT4 was performed on frozen sections from 10 OLP and 8 GLP and 8 oral and 5 genital controls using ab55392 (Abcam, Cambridge, UK) at a dilution of 1:200. Sections were fixed in methanol/ethanol (50:50) for 10 min and then air-dried for 1 h. Staining was performed in Ventana Bench Mark Ultra (Ventana Medical Systems, Inc, Tucson, AZ, USA) staining machine according to the manufacturer's recommendations.

RESULTS

Whole genome expression analysis was performed on RNA extracted from LP epithelium and normal controls. After background correction and normalisation SAM analysis was performed. A heat map was produced involving the differentially expressed genes in OLP and GLP. All LP (oral and genital) samples except 2 were clustered together. The same was seen for the normal controls (oral and genital) (Fig. 1).

Normal genital epithelium compared to normal oral epithelium

Comparison of gene expression profiles between normal oral and normal genital epithelium showed 47 differentially expressed genes when using > 2 fold change. Twenty were up-regulated and 27 down-regulated in genital compared to oral epithelium. In Table I the top 10 up- and top 10 down- regulated genes in normal gential compared to normal oral epithelium are shown. Functional annotation indicates that the most enriched biological processes in genital compared to oral epithelium are involved in development of glands, urogenital and reproductive systems (Table SII[1]).

Oral lichen planus epithelium compared to normal oral epithelium

When comparing OLP epithelium to normal oral epithelium 2,010 transcripts (FDR 1%) showed significantly different expression. A > 2-fold difference in gene expression was seen in 439 genes, of which 222

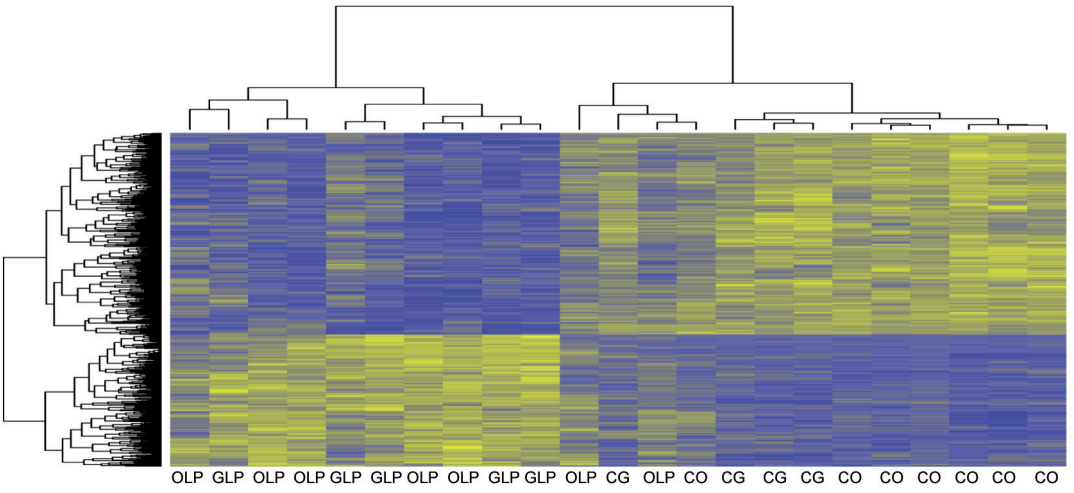

Fig. 1. A heat map of the differentially expressed genes in oral and genital lichen planus (LP). Two of the LP samples did not cluster with the other LP samples. The heatmap was generated in Chipster using Pearson correlation as a distance measure and average linkage for constructing the dendrogram. The blue colour representing up-regulated genes and the yellow down-regulated genes. The figure demonstrates that there is virtually no overlapping patterns between LP and control tissues (C).

were up-regulated and 217 down-regulated. Genes like aspartic peptidase retroviral like 1 (*ASPRV1*), found in epidermis and related to keratinocyte differentiation, several late cornified envelope genes (LCE), corneodesmosin (*CDSN*), loricrin (*LOR*), repetin (*RPTN*) and filaggrin (*FLG*) were up-regulated. The LCE genes *CDSN*, *LOR*, *RPTN* and *FLG* are all part of the epidermal differentiation complex (EDC). Among the down-regulated genes scinderin (*SCIN*), a calcium dependent actin binding protein, *KRT4*, related to differentiation, and keratin 8 (*KRT8*), a simple epithelial cell-specific keratin, were found. The top 20 up- and top 20 down-regulated genes are shown in Table SIII[1]. The biological processes most enriched in OLP using DAVID were involved in epidermal/ectodermal/epithelial development, differentiation and keratinisation (Table SII[1]).

Genital lichen planus epithelium compared to normal genital epithelium

In GLP epithelium compared to normal genital epithelium 774 genes were significantly differentially expressed (FDR 1.8%). A ≥2-fold difference was observed in 459 transcripts, 194 transcripts were up-regulated and 265 down-regulated. In Table SII[1] the top 20 up- and down-regulated genes are shown. In accordance with OLP, higher expression of *ASPRV1*, *CDSN* and several LCE genes could be seen. Also *S100A7A*, which is part of the EDC, and desmocollin (*DSC1*), a desmosomal cadherin showed higher expression. Among down-regulated genes, *KRT4* and *KRT8* were seen as well as cornulin (*CRNN*), a member of the EDC. DAVID identified the same biological processes to be enriched in OLP and GLP.

Table I. *The top 10 up- and top 10 down-regulated genes in normal genital epithelium compared to normal oral epithelium*

Top 10 up-regulated	Top 10-down regulated
IGLL1, ESR1, HOXA9, ISL1, HOXA5, LRMP, FOXA1, HOXD11, MAPK10, C12orf56	*SLC7A5, GAS1, PPARGC1A, MYADM, PTN, ISM1, TNNI2, GUCY2C, HS3ST3A1, ROR1*

Genital lichen planus compared to oral lichen planus epithelium

When comparing the 2 different gene lists for OLP and GLP, comprising genes differentially expressed ≥2 fold, 105 genes were common to both OLP and GLP. In Table II the top 20 up- and down-regulated genes in common are shown. Even if many individual genes do not overlap between OLP and GLP the same biological processes, concerning for example ectodermal development, keratinisation and epithelial cell differentiation (Table SII[1]), were enriched in both OLP and GLP.

Expression of corneodesmosin and keratin 4

Among the top 20 up-regulated and the top 20 down-regulated genes in OLP and GLP (Table SIII[1]) one up-regulated gene, *CDSN*, and one down-regulated gene, *KRT4*, were chosen for confirmation of array data.

In both OLP and GLP epithelium expression of KRT4 mRNA was decreased (Fig. 2a; OLP ($p=0.001$) and GLP ($p=0.1$)), as well as when looking at the whole

Table II. *The top 20 up- and top 20 down-regulated genes common between oral and genital lichen planus*

Top 20 up-regulated	Top 20 down-regulated
ASPRV1, LCE2B, LCE2A, LCE1B, LCE2D, LCE6A, LCE2C, LCE1C, CDSN, C6orf15, C1orf68, IL1F9, ALOXE3, KRT16, SERPINB7 WDR66, CD36, ADP2, CA2, FAM89A	*KRT4, RBM20, WNK4, MUC21 RHCG, FLJ40504, CLDN7, CLDN8, SCIN, TF, KRT8, TMPRSS2, SAMD5, OR7E37P, GPD1L, PGD, MAOA, RGMA, KCNJ2, FAM123B*

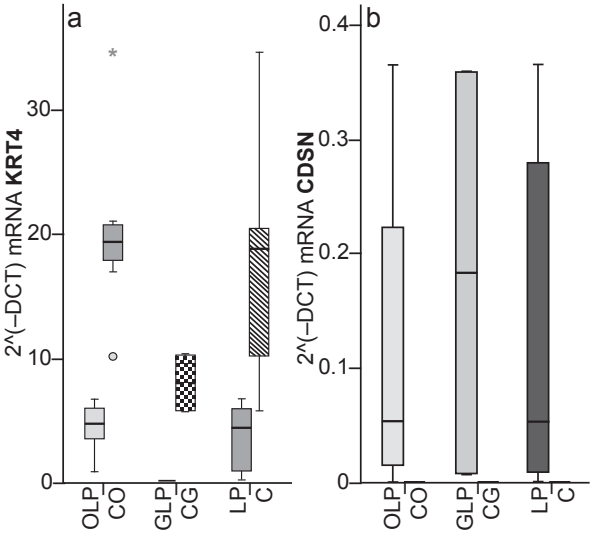

Fig. 2. mRNA expression levels of KRT4 and CDSN in LP. Lower levels of KRT4 were seen in a) both oral lichen planus (OLP) ($p=0.001$) and genital lichen planus (GLP) ($p=0.1$), as well as when looking at the whole group of samples ($p=0.001$) compared to controls (C). Levels of corneodesmosin (CDSN) were higher in both b) OLP ($p=0.002$) and GLP ($p=0.1$) as well as in the whole group of LP samples ($p<0.001$). 2^(-DCT) is the expression level of the gene of interest normalized to the reference gene. Results are displayed by the use of box plots, with the rectangle representing 50% of the cases and the whiskers going out to the smallest and largest value. The median value is displayed by the line inside the rectangle. In IBM SPSS statistics, outliers are defined as points extended more than 1.5 box length from the edge of the box.

group of LP samples ($p=0.001$). An increase in CDSN mRNA compared to normal controls was found (Fig. 2b; OLP ($p=0.002$) and GLP ($p=0.1$)), as well as in the whole group of LP samples ($p<0.001$). Regarding the absence of significance for the genital samples, the lack of material (only 2 LP and 2 controls) must be taken into consideration. The 2-ΔΔCT values for CDSN in OLP varied between 2.7–637 and in genital LP between 12–563. For KRT4 values in OLP varied between 0.047–0.335 and in GLP between 0.026–0.033. PCR results for *CDSN* and *KRT4* corresponded well with data from the array for each individual sample analysed.

KRT4 was expressed by most cells in the suprabasal layers in normal oral and genital epithelium, analysed by immunohistochemistry. In OLP, 6 samples showed no staining and 3 samples showed a few suprabasal cells expressing KRT4. Six GLP samples showed no expression of KRT4 and in 2 samples reduced expression was seen in the suprabasal layer (Fig. 3).

DISCUSSION

LP manifests itself in many different locations. Still, there is no consensus as to whether or not it is a systemic disease. Neither is the cause of this disease known, even if autoimmunity has been discussed, potentially caused by disturbances in differentiation of the keratinocytes (3, 17). In order to gain more insight into these issues, we performed whole genome expression analysis concentrating on the epithelium only. Normal epithelium originating from oral and genital mucosa were compared, showing a few genes involved in urogenital and reproductive systems and gland development to be differently expressed. Based on the differences in function of the oral and genital mucosa these findings seem plausible.

When comparing the gene lists between OLP and GLP 105 genes were in common. Although the majority of genes did not overlap, ontology analysis showed the most enriched biological processes to be the same in OLP and GLP, indicating that OLP and GLP lesions are manifestations of the same disease.

Among the differentially expressed genes in both OLP and GLP epithelium, several are part of the so-called epidermal differentiation complex. Increased expression of *LCE*, *CDSN*, *LOR*, *S100A7A* and *FLG* could be seen, most of these being expressed late during keratinocyte differentiation. This could be indicative of premature terminal differentiation of keratinocytes. The increased expression of *LCE* genes in LP is in contrast to the decreased expression of *LCE1*, *LCE2* and *LCE6* seen in psoriasis (18). Also in atopic dermatitis genes such as *LOR*, *CDSN*, *LCE*, and *FLG* are expressed at lower levels compared to normal skin (19). Down-regulation of genes involved in late differentiation of keratinocytes in psoriasis and atopic dermatitis has been interpreted as an impairment of the epithelial barrier (18–20), thus the increased expression seen in LP could be representative of a strengthened epithelial barrier.

Several keratins were differentially expressed in LP epithelium in accordance with previous reports (21). Keratins play an important role in the integrity

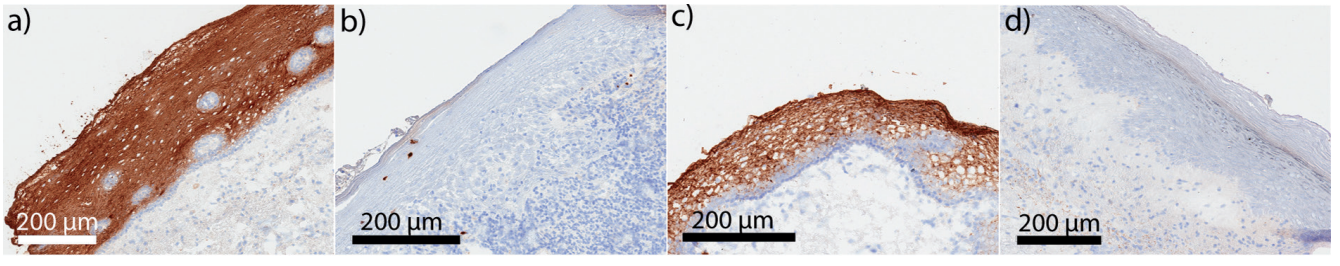

Fig. 3. Immunohistochemistry showed a higher expression of KRT4 in oral normal controls (a) compared to oral lichen planus (b) and in genital normal controls (c) compared to genital lichen planus (d).

of epithelial cells but also have regulatory functions in protection from stress, wound healing and apoptosis (22). In normal non-keratinised epithelium KRT4 is expressed in suprabasal layers and keratin 16 (KRT16) is expressed in suprabasal layers, sweat glands and wounds. In accordance with previous findings (21, 23) our results showed increased expression of KRT16, related to hyperproliferation, and down-regulation of the differentiation-specific KRT4 in both OLP and GLP epithelium. Theoretically, this could indicate increased proliferation in LP (24). The clinically visible changes in LP epithelium are thus likely to be caused by altered expression of genes involved in epidermal differentiation complexes and genes encoding different keratins.

The fact that many of the differentially expressed genes are in common between OLP and GLP and that the same biological processes are altered supports the fact that OLP and GLP are indeed manifestations of the same disease. Therefore it is important with a multidisciplinary treatment including both dermatologists and dentists with a special interest in the oral mucosa. The disturbances seen in differentiation together with our previous finding of decreased expression of the differentiation-related factor ELF-3 support the hypothesis that differentiation, at least in OLP, is altered. The issue whether this disturbance in differentiation is the cause of inflammation or whether inflammation *per se* causes changes in differentiation, however, still remains to be solved.

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