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A GENE SIGNATURE OF NON-HEALING VENOUS ULCERS: POTENTIAL DIAGNOSTIC MARKERS

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Abstract

Background—Venous leg ulcers are responsible for more than half of all lower extremity ulcerations. Significant interest has been focused on understanding the physiologic basis upon which patients fail to heal with standard therapy.

Objective—This study uses complementary DNA microarray analysis of tissue samples from healing and non-healing venous leg ulcers to identify the genetic expression profiles from these dichotomous populations.

Methods—Ulcer size and chronicity, factors that have been identified as prognostic indicators for healing, were used to distribute venous leg ulcers as healing versus non-healing. Punch biopsy samples were obtained from the wound edge and wound bed of all venous leg ulcers. The top fifteen genes with differential expression greater than twofold between the two populations of wounds ($p < 0.05$) were reported.

Results—Significant differences were demonstrated in the expression of a diverse collection of genes, with particular differences demonstrated by genes coding for structural epidermal proteins, genes associated with hyperproliferation and tissue injury, as well as transcription factors.

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Limitations—Small sample size may mitigate potential clinical implications of findings.

Conclusions—The genetic expression profiles displayed here may have implications for the development of novel therapies for chronic venous leg ulcers, and may also serve as prognostic indicators for wound healing.

INTRODUCTION

Venous leg ulcers are responsible for more than half of all lower extremity ulcerations, affecting over one million Americans annually with an annual cost upward of $2.5 billion dollars (1). The standard of care, multi-layered compression bandages, is effective in only one half to two thirds of patients (2-3). Significant interest has focused on understanding the population of patients who fail to heal with standard therapy. Several theories have suggested that the development of senescent cells within and at the wound edge, deficiency or lack of availability of cytokines or their receptors, and/or the presence of an abnormal wound bed matrix may be responsible for wound chronicity (4-7). Essential to our understanding of wound healing is knowledge of the genetic signals that lead to wound healing progression (8).

Studies have used high-density cDNA microarrays to delineate the gene expression of normal human skin (9-10) and early gene expression profiles of human skin following injury (11-13). Analysis of normal human skin found variability of genetic expression in 1.7% of the genes studied, these included genes coding for transport proteins, transcription, cell signaling proteins, and cell surface proteins. Little variability was identified in the wound matrix genes, growth factor genes and other groups of genes commonly thought to be involved in wound healing, suggesting that any newly identified expressed genes may be important to the wound healing process. Microarray analysis of injured human skin found expression of several genes historically unappreciated in the study of wound healing, specifically cytokine suppressor genes. Furthermore, microarray analysis has been performed to study the temporal analysis of gene expression in wound healing in an animal model, in which the temporal gene expression profile of wound healing was studied in the ear-punched tissue of mice (14). This study identified expression of genes principally related to the inflammatory stage that had not previously been associated with wound healing. Recent microarray study of biopsies of venous ulcers from two different wound edge locations (prior and post debridement) identified distinct wound region that may be better responsive to therapy (15). Finally, the study comparing biopsies obtained from a non – healing edges of venous ulcers to healthy, normal skin revealed de-regulation of keratinocytes activation and differentiation pathways in the epidermis deriving from a venous ulcers non – healing edges (16). In sum, the high-throughput analysis of gene expression by microarray technology in wound healing models has provided for the identification of novel genes that may play a critical role in non-healing.

In the present study we used high throughput cDNA microarray analysis to examine the patterns of local genetic expression to in order compare healing and non-healing chronic venous leg ulcers. Taking advantage of well-described prognostic indicators for wound healing in venous leg ulcers, ulcer size and duration; we grouped wounds into healing and

non-healing groups (17-18). Using biopsy samples obtained from these dichotomous populations of chronic venous leg ulcers, we analyzed genetic expression from keratinocytes, fibroblasts, and other cells, from within the tissue sampled using the microarray technique.

MATERIALS AND METHODS

Subjects

Patients were recruited according to a protocol approved by the University of Miami School of Medicine Institutional Review Board. Consent was obtained from all patients, and the Declaration of Helsinki protocols were followed. Five patients with non-healing and five patients with healing chronic venous leg ulcers as defined by previous prognostic models for wound healing in venous leg ulcers were studied (17-18). All ulcers were present for at least 4 weeks. Healing venous leg ulcers were defined as ulcers that were less than $5cm^2$ in surface area and present for less than 6 months, while non-healing ulcers were greater than $5cm^2$ and present longer than 6 months. Venous etiology of the leg ulcers was determined clinically and confirmed by vascular studies, if needed. Clinical parameters used to identify venous leg ulcers were the presence of a lower leg ulcer and at least two of the following: dermatitis, atrophie blanche, varicosities, hyperpigmentation, or lipodermatosclerosis. If these were not present, patients were studied with duplex venous Doppler ultrasound to document venous insufficiency. Patients with moderate to severe arterial disease were excluded, initially by an ankle brachial index (<0.9) and if needed, by pulse volume recordings.

Under local anesthesia, two 4-millimeter punch biopsy specimens were obtained from each venous leg ulcer: one from the wound margin, one from the center of the wound bed. All wounds were subsequently treated as per standard of care for chronic venous leg ulcers (19). Tissue samples were processed using a fixative based on polyethylene glycol and methanol (Universal Molecule Fixative) that preserves the tissue morphology as well as the integrity of the nucleic acids, including DNA and RNA (20).

RNA Extraction and Labeling

Total RNA extraction was performed by addition of Trizol reagent (GibcoBRL, Gaithersburg, MD) and subsequent homogenization using a Tissue Tearor (Biospec Products Inc, Bartlesville, OK). The RNA from homogenized tissue was extracted with chloroform followed by isopropyl precipitation on ice. The RNA pellets were re-suspended in 100 ml of diethylpyrocarbonate (DEPC)-treated water. Standard 1% agarose gel under denaturing condition with ethidium bromide was used to assess the integrity of RNA. In addition, RNA was run on an Agilent Technologies Bioanalyzer 2100 using RNA 6000 Nano Chips (Lindenhurst, NY) to determine RNA integrity and the ratio of ribosomal RNA. The quantity of the extracted RNA was determined by spectrophotometery (Ultraspec III, Pharmacia, Netherlands). RNA samples were analyzed at Expression Analysis Inc (Durham, NC) using Affymetrix (Santa Clara, CA) Human Genome U133 PLUS 2.0 Array and supporting kits and protocols for preparation of template and hybridization. Shortly, two micrograms of total RNA was used to synthesize double-stranded cDNA using One-Cycle cDNA Synthesis Kit

(Affymetrix) following manufacturer's instructions. In vitro transcription amplification and biotin labeling to prepare targets for arrays was performed using Affymetrix GeneChip IVT Labeling Kit following their protocol. Biotinylated cRNA was cleaned using GeneChip Sample Cleanup Module (Affymetrix). After hybridization for 16 hours, microarrays were washed and stained using Affymetrix standard protocol. Scanned images were processed and analyzed using Affymetrix GCOS software. Two group comparison was done on normalized expression values that were individually transformed using base 2 logarithm of the expression index. On log–transformed scale, the mean was calculated for every probe set within each group and a two sample, two-sided t-test was conducted (MS Excel)

Image Analysis

Following hybridizaton, an Axon GenePix 4000 scanner was used to scan slides and background-subtracted feature intensity was calculated. Quality criteria for inclusion in the data set for further analysis included signal to background > 3, no more than 20% missing or saturated pixels, and a minimum background subtracted signal level > 50. Intensity data was normalized and evaluated using GEMTools software.

Data Analysis

Duplicate hybridizations were performed on microarrays made by Affymetrix ® containing over 47,000 transcripts.

The difference ratios between the signals in healing and non-healing ulcer samples were then calculated for the subset of cases with complete and incomplete responses, identifying those genes that were most different between the two. Gene features that exhibited at least a twofold difference in intensity ratio with respect to the reference RNA in at least 2 wounds were selected for further analysis. A first pass analysis evaluated a 2-cluster classification, using k-means methods, on progressively smaller sets of genes to identify a set with maximum distance between groups. The list was narrowed based on the correlation response. Such analysis (e.g. bootstrapping and/or leave-one-out cross validation) was performed until an optimal group of genes was identified that correlated with wound chronicity or healing. Once duplicates were eliminated top 15 regulated (induced and down-regulated) are selected and presented in the tables. Disease associations and gene functions were obtained from several gene and genome search engines: http://smd.stanford.edu/cgi-bin/source/sourceResult; http://genome-www.stanford.edu/genecards/index.shtml; http://www.dsi.univ-paris5.fr/genatlas/

RESULTS AND DISCUSSION

Optimal wound healing depends upon the concerted interplay of thousands of genes. Of those identified to be differently expressed between these two groups of venous leg ulcers, several specific genes deserve special mention.

Gene Expression Profile of Healing versus Non-Healing Epidermal Wound Edge

Tables 1 and 2 demonstrate the top fifteen genes that were differentially expressed between the two groups of venous leg ulcers from the keratinocytes of the tissue sampled at the non-

healing wound edge. The most striking finding in this data set is the extent to which genes implicated in epidermal hyperproliferation and tissue repair were differentially expressed. Of additional interest is that the down- regulation (> 250.00) demonstrated in the non-healing wound edges was much greater than the up- regulation (>10.00) evidenced by the non-healing wounds.

The top activated gene in non-healing edges is secreted frizzled-related protein 4 (SFRP4), a mediator of Wnt signaling. Furthermore, branched chain aminotransferase 1 (BCAT1) was also found up-regulated, and this gene has been associated with c-myc induced tumors. These findings are consistent with previous findings of activation of b-catenin and c-myc in non-healing edges of venous ulcers (16). Another novel finding revealed up- regulation of cytochrome P450 (CYP1B1) and 17-beta-hydroxisteroid dehydrogenase VI (HSD17B6), genes associated with steroidogenesis . CYP1B1 has been found induced in keratinocytes upon challenge with UVB (21-22). Up- regulation of steroidogenesis associated genes suggests that steroid synthesis and metabolism may participate in the pathogenesis of non – healing ulcers . Secreted frizzled-related protein 4 have shown increased expression in disease processes associated with increased cell death (23), particularly those where apoptosis is occurring, such as heart failure and degenerative retinal disease and this propensity for cellular apoptotosis may have implications for impaired tissue regeneration in wound healing. Furthermore, adhesion molecules such as selectin E (SELE) have been shown to be expressed on activated endothelium and platelets at sites of vascular injury and inflammation. P and E selectin have been associated with microvascular dysfunction in chronic venous insufficiency, and studies have identified the importance of their combined roles in the process of wound healing (24). A study of mice deficient in both P- and E-selectins demonstrated markedly reduced recruitment of inflammatory cells and impaired wound clousure. Additionally, a wider epithelial gap was observed in the wounds of the P- and E-selectin-double-deficient mice 3 days after wounding indicating delayed keratinocyte migration.

Of the top twenty genes down- regulated more than 2 fold in the non-healing wound edge, majority genes express protein products that are considered crucial to epidermal structural integrity or are associated with epidermal injury, repair, hyperproliferation and or differentiation. Interestingly, the predominant gene group that is down- regulated are keratins. The gene that demonstrated the greatest downregulation in the non-healing epidermal wound edge was that which codes for keratin 16 (KRT16) (−258.78), a gene product that has been associated with cutaneous injury and timely epidermal repair (25).Two of its heterodimeric partners, keratin 6A (KRT6A) (−61.50) and 6B (KRT6B) (−43.98), which are thought to play an important role in epidermal regeneration and have been shown to be over-expressed in cutaneous injury and epithelial repair, demonstrated significant downregulation in the non-healing venous ulcer edges (26). Closely functionally related is also keratin 17 (KRT17) which is also participating in epidermal repair and may also play a role in contractility. Keratinocyte migration is also deficient in chronic wounds (16). Keratins K16, K6a, K6b and K17 play a role in epithelial migration and their down-regulation may contribute to lack of epithelial migration. Finally, keratins K14 and K1 were also found down- regulated. Keratin K14 is basal layer specific and demarcates the mitotically active compartment whereas keratin 1 demarcates differentiating keratins. Thus,

keratins marking all three epidermal phenotypes: basal, differentiating and activated (wound-healing like) were down- regulated, further suggesting that all three biological processes essential for maintenance of epidermis are affected. Furthermore, a cluster of differentiation associated genes that participate in stratification, cornification and desmosome formation (stratifin (SFN) , cornifin (CFN) , filaggrin (FIL) and desmoplakin (DSP)) were also found to be down- regulated, suggesting that differentiation process is not properly executed. This data are in agreement with our previous findings of de-regulation of differentiation in non-healing edges of venous ulcers when compared to healthy skin (16). Other genes that deserve attention in these top twenty down- regulated genes demonstrated in the non-healing wound edges are the skin-derived anti leukoproteinase(SKALP/elafin) (−126.11) , S100 calcium binding protein A7 (S100A7) (−120.44), and Aquaporin 3 (AQP3) (−48.01). The SKALP/elafin gene has been described as an epidermal proteinase inhibitor (27). It is absent in the normal epidermis, however it has been shown to be expressed following epidermal injury and is also present in inflammatory skin conditions such as psoriasis. The exact physiologic role of SKALP/elafin is unknown; however it has been associated with cutaneous homeostasis involved in the regulation of inflammation via neutrophilic regulation. The S100A7 gene expresses the protein products that have been proposed to be involved in keratinocyte differentiation. This is a family of calcium-activated signaling proteins that interact with target proteins to modulate skin disease and their levels are markedly elevated in psoriatic epidermis, suggesting a role in epidermal proliferation (28). The Aquaporin 3 gene expresses for aquaporins, which are a family of small water and/or glycerol transmembrane channels. Eleven mammalian aquaporins have been described so far. Specifically, AQ3 is an aquaglyceroporin with expression in the kidney collecting cells, red cells, dendritic and epithelial cells. Aquaporin 3 deficient mice demonstrate delayed wound healing with decreased epidermal water and glycerol content and decreased skin elasticity (29).

It is quite surprising that none of the genes that are classically thought to be involved in stimulating wound repair, such as those which encode for platelet-derived growth factor or keratinocyte growth factor, were significantly down- regulated in the non-healing epidermal wound edges.

Gene Expression Profile of Healing versus Non-Healing Dermal Wound Bed

Tables 3 and 4 demonstrate the top fifteen genes that were differentially expressed between the two groups of venous leg ulcers from the cells sampled at the wound bed. Interestingly, the extent of differential gene expression between the two groups of ulcers is much less than that seen in the wound edge cells. This piece of data may be indicative of the importance of proper epithelial migration for appropriate wound closure.

The tissue sampled from the non-healing wound bed also demonstrated a heterogeneous group of genes that were up- regulated greater than two-fold. The gene that demonstrated the greatest extent of up- regulation, properdin (BF) (+8.41), codes for a factor of the alternative pathway of complement activation known as complement factor B, implicating an association between immune function and optimal wound healing. In addition, strong properdin induction was noticed by turbulent flow and possibly associated with

atherosclerosis (30) Additionally, of the top twenty up- regulated genes from non-healing dermal wound bed cells, several code for proteins that have been directly associated with tissue injury, extracellular matrix formation, and the wound healing process. The extracellular matrix protein dermatopontin (DPT) (+7.77) has been shown to play a critical role in tissue elasticity and collagen accumulation necessary for collagen fibrillogenesis in *in vivo* murine wound healing models (31). Additionally, transforming growth factor-beta 1 can increase the expression of dermatopontin in normal cultured human skin fibroblasts indicating a potential association between dermatopontin and cytokines critical in the wound healing process (32). Another important group of extracellular matrix proteins important in the wound healing process are the fibulins. Fibulin 1 (FBLN1) (+3.50) has been found to be present in normal skin granulation tissue and wounds; but has not been shown to be distinctly up- regulated during the healing process of murine wounds (33). Two other genes that deserve special mention in this group of upregulated genes are thrombospondin 1 (THBS1) (+3.39) and platelet-derived growth factor receptor (PDGFRA) (+3.36). The protein encoded by thrombospondin 1 is a multifunctional extracellular matrix molecule that has been shown to be involved in re-epithelialization as well as dermal reorganization in murine wound models (34). Additionally, this matricellular glycoprotein has been associated with skin angiogenesis through its interactions with the cytokine vascular endothelial growth factor (35). Lastly, the association between platelet-derived growth factor (PDGF) and wound healing has been well described.

The tissue sampled from the wound bed demonstrated a diverse array of genes that were also down- regulated greater than two-fold. A number of these genes also deserve special mention. The gene that encodes for the protein kazrin (KIAA1026) (−2.75) may be important for epidermal repair and the wound healing process. Kazrin is a novel component of desmosomes that associates with periplakin (36). Interestingly, two tumor suppressor genes were significantly down- regulated in this non-healing population of wounds, retinoblastoma binding protein 6 (RBBP6) (−2.57) and SAM and SH3 domain containing 1 (SASH1) (−2.49), implicating a role of altered cell cycle regulation in normal wound healing. A novel macrophage expressed gene 1 (MPEG1) (−2.48) was found down-regulated in a wound bed of non – healing ulcers. Macrophages play an important role in the adult inflammatory response to wounding and are responsible for cellular debridement. They recruit other inflammatory and fibroblastic cells and influence cell proliferation and tissue remodeling as a source of growth factors and cytokines (37) . Interestingly, a disintegrin-like and metalloproteinase with trombospondin type 1 motif, 14 (ADAMTS14) (−2.27) was found down- regulated as well. Down- regulation of ADAMTS-14 in a wound bed of non-healing wounds may play a role in decreased collagen synthesis and consequently to improper wound bed formation. Lastly, of great importance, down- regulation was demonstrated in heparin-binding epidermal growth factor – like growth factor (HBEGF) (−2.40), a gene that has clearly been associated with appropriate wound healing. Studies of wound healing have revealed that wound closure is markedly impaired in keratinocyte-specific HB-EGF-deficient mice (38) and that ligand shedding of heparin-binding EGF-like growth factor is important for keratinocyte migration and proper wound epithelialization (39). Furthermore, HB-EGF has been shown to be a major growth factor component of wound fluid and, since it is mitogenic for fibroblasts and keratinocytes it plays an important

role in wound healing (40) Therefore down– regulation of HB- EGF in biopsies deriving from a wound bed of non – healing ulcers can partly explain phenotype of non – healing wounds. It is noteworthy that - SMART/HDAC1 associated repressor protein (SHARP), was also down- regulated indicating that perhaps histones may be acetylated and that chromatin changes may favor transcriptional activity.

The findings of the present study demonstrate a diversity of genetic expression associated with wound healing in chronic venous leg ulcers. Altered expression was seen in genes that code for structural factors, mediators of inflammation, and apoptotic pathways. While the significance of this information is yet to be determined, this study provides a unique understanding by demonstrating that healing and non-healing venous leg ulcers do display a unique and dichotomous genetic physiology. With this information, future studies may focus on topical growth factors and/or genetically modified tissue engineered skin that may optimize the wound environment for optimal healing. Furthermore, the factors demonstrated in this study may represent physiologic prognostic indicators of wound healing and may be useful for stratifying venous leg ulcers according to their potential to heal. This may help clinicians to identify venous leg ulcers that may require advanced wound healing treatment modalities in conjunction with compression therapy at the outset of treatment. Additionally, studies should be conducted to evaluate the information described herein for the development of improved therapeutic approaches. Techniques such as reverse transcriptase real-time polymerase chain reaction may be employed to study the gene expression of a similar population of large group of venous leg ulcers to help further investigate the significance of the present findings.

In conclusion, significant differences exist in the genetic expression between healing and non-healing venous leg ulcers. These findings should help to identify the aberrant physiologic processes associated with impaired tissue repair in this population of wounds. More importantly, the genetic expression profiles displayed here may have implications for the development of novel therapies for chronic venous leg ulcers, and may also serve as prognostic indicators for healing.

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Table 1

Top 15 up- regulated genes in the edge of non-healing ulcers compared to the edge of healing ulcers.

	Affymetrix Probe Set ID	P-Value	Raw Fold Change	Gene Descriptor	Unigene ID	Gene Symbol	Sequence Derived From	Function	Disease association
1	204051_s_at	0.0346	11.57	Secreted frizzled-related protein 4	Hs.105700	**SFRP4**	AW089415	Transcription	Inhibition of cancer progression
2	205941_s_at	0.0032	8.91	Collagen, type X, alpha 1	Hs.179729	**COL10A1**	AI376003	ECM	Schmid type metaphyseal chondrodysplasia
3	226517_at	0.0463	8.21	Branched chain aminotransferase 1, cytosolic	Hs.438993	**BCAT1**	AL390172	Catabolism amino acids	C-myc induced tumors
4	229271_x_at	0.0235	7.86	Collagen, type XI, alpha 1	Hs.439168	**COL11A1**	BG028597	ECM	Stickler syndrome type 2
5	212353_at	0.0130	6.64	Sulfatase 1	Hs.409602	**SULF1**	AI479175	Heparin signaling Ca2+ binding; hydrolase	All type of carcinoma, including SCC
6	202437_s_at	0.0061	6.05	Cytochrome P450, family 1, subfamily B, polypeptide 1	Hs.154654	**CYP1B1**	NM_000104.2	Steroidogenesis	Breast, colon, lung, esophagus, skin, lymph node, brain, and testis cancer, congenital glaucoma
7	222043_at	0.0154	5.99	Clusterin (complement lysis inhibitor)	Hs.436657	**CLU**	AI982754	Apoptosis	Prostate cancer
8	206211_at	0.0013	4.76	Selectin E (endothelial adhesion molecule 1)	Hs.89546	**SELE**	NM_000450.1	Inflammation	Tumors, vascular disease, psoriasis,
9	205422_s_at	0.0190	4.61	Integrin, beta-like 1 (with EGF-like repeat domains)	Hs.311054	**ITGBL1**	NM_004791.1	Adhesion	Unknown
10	236859_at	0.0256	4.58	Runt-related transcription factor 2	Hs.509746	**RUNX2**	AW469546	Transcription	Bone and cartilage diseases
11	37512_at	0.0035	4.53	17-beta-hydroxisteroid dehydrogenase VI	Hs.11958	**HSD17B6**	U89281	Steroidogenesis	Uterine fibroids, prostate cancer
12	209583_s_at	0.0040	4.38	Antigen identified by monoclonal antibody MRC OX-2	Hs.79015	**MOX2**	AF063591	Immune response	Spontaneous abortion
13	202833_s_at	0.0285	4.24	Serine proteinase inhibitor, clade A, member 1	Hs.297681	**SERPINA1**	NM_000295	Proteolysis	Lung disease
14	204174_at	0.0122	4.22	Arachidonate 5-lipoxygenase-activating protein	Hs.100194	**ALOX5AP**	NM_001629.1	Inflammation	Stroke, asthma, MI
15	210176_at	0.0252	4.22	Toll-like receptor 1	Hs.111805	**TLR1**	AL050262.1	Innate immunity	Response to microbial agents

Table 2

Top15 down- regulated genes in the edge of non-healing ulcers compared to the edge of healing ulcers.

	Affymetrix Probe Set ID	P-Value	Raw Fold Change	Gene Descriptor	Unigene ID	Gene Symbol	Sequence Derived From	Function	Disease association
1	209800_at	0.0013	−258.78	Keratin 16 (focal non-epidermolytic palmoplantar keratoderma)	Hs.432448	**KRT16**	AF061812.1	Cytoskeleton; structural integrity of epithelial cell	Pachyonychia congenita type 1; nonepidermolytic palmoplantar keratoderma;metaplasias; carcinomas of the uterine cervix; psoriasis vulgaris
2	205064_at	0.0018	−153.18	Small proline-rich protein 1B (cornifin)	Hs.1076	**SPRR1B**	NM_003125.1	Cornified envelope formation	Biomarker for squamous metaplasia in eye
3	215704_at	0.0030	−150.44	Filaggrin	Hs.73995	**FLG**	AL356504	Terminal keratinocyte differentiation	Ichthyosis vulgaris;atopic eczema; atopic dermatitis;
4	41469_at	0.0014	−126.11	Protease inhibitor 3, skin-derived (SKALP)	Hs.112341	**PI3**	L10343	An antiproteinase; elastase inhibitor	Psoriasis; squamous cell carcinoma of the lung
5	205916_at	0.0017	−120.44	S100 calcium binding protein A7 (psoriasin 1)	Hs.112408	**S100A7**	NM_002963.2	Chemotactic inflammatory protein for CD4+ T lymphocytes and neutrophils	Psoriasis; breast cancer progression
6	226926_at	0.0002	−96.77	Dermokine	Hs.417795	**DMKN**	AA706316	Epidermal morphogenesis	Inflammatory diseases
7	212236_x_at	0.0033	−94.47	Keratin 17	Hs.2785	**KRT17**	Z19574	Cytoskeleton; structural integrity of epithelial cell	Pachyonychia congenita type 2; steatocystoma multiplex; psoriasis
8	266_s_at	0.0000	−64.13	CD24 antigen (small cell lung carcinoma cluster 4 antigen)	Hs.375108	**CD24**	L33930	Modulates b-cell activation; embryonic vasculogenesis	Wound healing
9	209125_at	0.0051	−61.50	Keratin 6A	Hs.367762	**KRT6A**	J00269.1	Cytoskeleton; structural integrity of epithelial cell	Pachyonychia congenita type 1 nonepidermolytic palmoplantar keratoderma;metaplasias; carcinomas of the uterine cervix;psoriasis vulgaris
10	33323_r_at	0.0019	−57.40	stratifin	Hs.184510	**SFN**	X57348	Adapter protein; p53-regulated inhibitor of g2/m progression	Squamous cell carcinoma, melanoma progression; breast, ovarian and pancreatic cancer progression
11	200606_at	0.0018	−54.62	Desmoplakin	Hs.349499	**DSP**	NM_004415.1	Component of functional desmosomes	Cardiomyopathy; acantholytic epidermolysis bullosa;

	Affymetrix Probe Set ID	P-Value	Raw Fold Change	Gene Descriptor	Unigene ID	Gene Symbol	Sequence Derived From	Function	Disease association
									keratoderma, blisters, nail dystrophy
12	39248_at	0.0078	−48.01	Aquaporin 3	Hs.234642	**AQP3**	N74607	A water channel protein	corneal edema, renal disease
13	209351_at	0.0068	−46.13	Keratin 14	Hs.355214	**KRT14**	BC002690.1	Cytoskeleton; structural integrity of epithelial cell	Epidermolysis bullosa simplex, Dowling-Meara, Weber-Cockayne type and Koebner type
14	213680_at	0.0081	−43.98	Keratin 6B	Hs.432677	**KRT6B**	AI831452	Cytoskeleton; structural integrity of epithelial cell	Pachyonychia congenita type 2
15	203074_at	0.0040	−43.75	Annexin A8	Hs.87268	**ANXA8**	NM_001630.1	Anticoagulant, inhibitor of the thromboplastin-specific complex	Acute promyelocytic leukemia

Table 3

Top 15 up- regulated genes in the bed of non-healing ulcers compared to the bed of healing ulcers.

	Affymetrix Probe Set ID	P-Value	Raw Fold Change	Gene Descriptor	Unigene ID	Gene Symbol	Sequence Derived From	Function	Disease association
1	202357_s_at	0.0007	8.41	B-factor, properdin	Hs.69771	**BF**	NM_001710.1	Peptidase, a component of the alternative pathway of complement activation	Rheumatoid arthritis, atherosclerosis
2	213071_at	0.0319	7.77	Dermatopontin	Hs.80552	**DPT**	AL049798	ECM protein with possible functions in cell-matrix interactions and matrix assembly.	Myocardial infarction, keloids
3	229947_at	0.0362	4.85	Peptidase inhibitor 15	Hs.98558	**PI15**	AI088609	Peptidase, trypsin inhibitor	Glyoblastoma and neruoblastoma
4	202437_s_at	0.0401	4.63	Cytochrome P450, family 1, subfamily B, polypeptide 1	Hs.154654	**CYP1B1**	NM_000104.2	Steroidogenesis	Breast, colon, lung, esophagus, skin, lymph node, brain, and testis cancer, congenital glaucoma
5	202075_s_at	0.0232	4.21	Phospholipid transfer protein	Hs.439312	**PLTP**	NM_006227.1	Cholesterol metabolism	Atherosclerosis,hypertrigly ceridemia,insulin resistance
6	224840_at	0.0073	4.03	FK506 binding protein 5	Hs.7557	**FKBP5**	AI753747	Role in immunoregulation, binds steroid receptors in complex with Hsp 90 and Hsp70	Glucocorticoid resistance; prostate cancer; idiopathic myelofibrosis
7	202994_s_at	0.0474	3.50	Fibulin 1	Hs.445240	**FBLN1**	Z95331	Cell adhesion and migration along protein fibers within the extracellular matrix (ECM); ECM architecture	Breast and ovarian cancer
8	201108_s_at	0.0118	3.39	Thrombospondin 1	Hs.164226	**THBS1**	BF055462	Cell-to-cell and cell-to-matrix interactions. Can bind to fibrinogen, fibronectin, laminin, type v collagen and integrins alpha-v/beta-1, alpha- v/beta-3 and alpha-iib/beta-3	Wound healing, skin cancers, breast cancer
9	203131_at	0.0216	3.36	Platelet-derived growth factor receptor, alpha polypeptide	Hs.74615	**PDGFRA**	NM_006206.1	Receptor that binds both pdgfa and pdgfb and has a tyrosine-protein kinase activity; mitogen for cells of mesenchymal origin	Some cases of hypereosinophilic syndrome (hes)
10	229390_at	0.0443	3.28	Family with sequence similarity 26, member F	Hs.381220	**FAM26F**	AV734646	Unknown	Unknown
11	207761_s_at	0.0152	3.14	DKFZP586A0522 protein; synonyms: AAM B, UbiE	Hs.288771	**DKFZP586A0522**	NM_014033	Methyltransferase like 7A; gene expression	Unknown
12	1558881_at	0.0053	3.13	Hypothetical protein LOC145820	Hs.171000	**LOC145820**	AK057337	Unknown	Unknown
13	227525_at	0.0069	3.11	Glucocorticoid induced transcript 1	Hs.435806	**GLCCI1**	AA058770	Unknown	Unknown
14	219869_s_at	0.0452	2.95	Solute carrier family 39 (zinc transporter), member 8	Hs.284205	**BIGM103**	NM_022154	Involved in protein, nucleic acid, carbohydrate, and lipid metabolism, control of gene transcription, growth, development, and differentiation	Bladder cancer
15	204900_x_at	0.0345	2.73	Sin3-associated polypeptide, 30kDa	Hs.413835	**SAP30**	NM_003864	Active in deacetylating core histone octamers, but inactive in deacetylating nucleosomal histones	Melanoma

Table 4

Top 15 down- regulated genes in the bed of non-healing ulcers compared to the bed of healing ulcers.

	Affymetrix Probe Set ID	P-Value	Raw Fold Change	Gene Descriptor	Unigene ID	Gene Symbol	Sequence Derived From	Function	Disease association
1	205656_at	0.0181	−3.67	Protocadherin 17	Hs.106511	**PCDH17**	NM_014459.1	Cell- cell connections	Retinoblastoma
2	207134_x_at	0.0144	−3.45	Tryptase beta 2	Hs.405479	**TPSB2**	NM_024164	Major neutral protease present in mast cells and is secreted upon the coupled activation-degranulation response of this cell type	Asthma, allergic and inflammatory disorders
3	201490_s_at	0.0214	−3.45	Peptidylprolyl isomerase F (cyclophilin F)	Hs.381072	**PPIF**	NM_005729	Apoptosis and necrotic cell death; accelerates the folding of proteins	Myodegenerative disorders
4	222688_at	0.0100	−3.15	Phytoceramidase, alkaline	Hs.23862	**PHCA**	R12678	Ceramide metabolism	Unknown
5	201996_s_at	0.0439	−3.03	SMART/HDAC1 associated repressor protein	Hs.184245	**SHARP**	AL524033	Unknown	Unknown
6	213478_at	0.0368	−2.75	Kazrin	Hs.368823	**KIAA1026**	AB028949	Cell-cell conectins	Unknown
7	205178_s_at	0.0449	−2.57	Retinoblastoma binding protein 6	Hs.188553	**RBBP6**	NM_006910.1	Suppression of cellular proliferation	Inactivated in various cancers
8	41644_at	0.0398	−2.49	SAM and SH3 domain containing 1	Hs.166311	**SASH1**	4862579_RC	May have a role in a signaling pathway; could act as a tumor suppressor.	Breast tumor, colon cancer
9	212611_at	0.0229	−2.48	Macrophage expressed gene 1	Hs.62264	**MPEG1**	AV728526	Unknown	Fibrosis
10	221992_at	0.0138	−2.42	Similar to nuclear pore complex interacting protein	Hs.448833	**Unknown**	AI925734	Unknown	Unknown
11	38037_at	0.0240	−2.40	Heparin-binding epidermal growth factor-like growth factor	Hs.799	**HBEGF**	M60278	Cellular proliferation; mitogenic for fibroblasts and smooth muscle but not endothelial cells; binds egf receptors	Epithelial tumors, acute myeloid and acute lymphoid leukemia
12	206491_s_at	0.0001	−2.35	N-ethylmaleimide-sensitive factor attachment protein, alpha	Hs.75932	**NAPA**	NM_003827.1	Vesicular transport between the endoplasmic	Huntington's disease

	Affymetrix Probe Set ID	P-Value	Raw Fold Change	Gene Descriptor	Unigene ID	Gene Symbol	Sequence Derived From	Function	Disease association
								reticulum and the golgi apparatus	
13	224940_s_at	0.0432	−2.30	A specific BCL2 ARE-binding protein 2	Hs.440769	**ASBABP2**	BF107618	Unknown	Unknown
14	230167_at	0.0087	−2.27	A disintegrin-like and metalloprotease with thrombospondin type 1 motif, 14	Hs.352156	**ADAMTS14**	W60649	Collagen catabolism	Dupuytren's disease, Ehlers-Danlos syndrome dermatosparactic type or VIIC
15	231960_at	0.0123	−2.26	WD repeat domain 9	Hs.435904	**WDR9**	AA807344	Chromatin remodeling	Unknown