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Molecular discrimination of cutaneous squamous cell carcinoma from actinic keratosis and normal skin

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Actinic keratosis is widely believed to be a neoplastic lesion and a precursor to invasive squamous cell carcinoma. However, there has been some debate as to whether actinic keratosis is in fact actually squamous cell carcinoma and should be treated as such. As the clinical management and prognosis of patients is widely held to be different for each of these lesions, our goal was to identify unique gene signatures using DNA microarrays to discriminate among normal skin, actinic keratosis, and squamous cell carcinoma, and examine the molecular pathways of carcinogenesis involved in the progression from normal skin to squamous cell carcinoma. Formalin-fixed and paraffin-embedded blocks of skin: five normal skins (pooled), six actinic keratoses, and six squamous cell carcinomas were retrieved. The RNA was extracted and amplified. The labeled targets were hybridized to the Affymetrix human U133plus2.0 array and the acquisition and initial quantification of array images were performed using the GCOS (Affymetrix). The subsequent data analyses were performed using DNA-Chip Analyzer and Partek Genomic Suite 6.4. Significant differential gene expression (>2 fold change, $P<0.05$) was seen with 382 differentially expressed genes between squamous cell carcinoma and normal skin, 423 differentially expressed genes between actinic keratosis and normal skin, and 9 differentially expressed genes between actinic keratosis and squamous cell carcinoma. The differentially expressed genes offer the possibility of using DNA microarrays as a molecular diagnostic tool to distinguish between normal skin, actinic keratosis, and squamous cell carcinoma. In addition, the differentially expressed genes and their molecular pathways could be potentially used as prognostic markers or targets for future therapeutic innovations.

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Squamous cell carcinoma is the second most common cutaneous malignancy with over 250 000 cases diagnosed per year.[1] Although it is typically a straightforward diagnosis, there are many lesions, which may clinically and microscopically simulate cutaneous squamous cell carcinoma. Some of the lesions that can be the most difficult to differentiate, such as actinic keratosis and squamous cell carcinoma *in situ*, display varying different degrees of keratinocyte dysplasia and are typically considered neoplastic conditions.[2] Although actinic keratosis is widely regarded as a neoplastic lesion that is a precursor to squamous cell carcinoma, some believe actinic keratosis is actually squamous cell carcinoma and should be treated as such.[3,4] However, there is a consensus that differentiating between actinic keratosis and squamous cell carcinoma is important, as there are prognostic differences and treatment implications for the patient.

There have been only a few microarray studies to date that examined differential gene expression among cutaneous squamous lesions.[5–8] These studies have been hampered by limited sample sizes, poor reproducibility, and the use of cell lines.[5–8] We profiled and examined the gene expression of over 47 000 genes using the most comprehensive GeneChip microarrays available (human U133 plus

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2.0 array), and identified differentially expressed genes among normal skin, actinic keratosis, and squamous cell carcinoma using formalin-fixed and paraffin-embedded tissue. Our goal was to identify unique gene signatures that can discriminate between normal skin, actinic keratosis, and squamous cell carcinoma, and examine the molecular pathways of carcinogenesis involved in the progression from normal skin to squamous cell carcinoma.

Materials and methods

Sample Selection

Six actinic keratoses, six squamous cell carcinomas, and five normal skins (pooled) were identified from the PowerPath database and the slides and paraffin blocks (<6 months old) were retrieved. The slides were reviewed by two of the authors (SHR, SB) and their diagnosis confirmed. Normal skin demonstrating mild sun damage in the form of solar elastosis was retrieved from cosmetics procedures. The areas of interest were removed from the paraffin blocks with a sterile surgical scalpel.

RNA Isolation and Quality Control

Total RNA was isolated using the Ambion RecoverAll (Applied Biosystems/Ambion, Austin, TX, USA) kit according to the manufacturer's instructions. Briefly, formalin-fixed and paraffin-embedded samples were deparaffinized using a series of xylene and ethanol washes, and then subjected to a proteinase K digestion at 50°C for 16 h to release RNA from covalently linked proteins. Finally, total RNA was purified by capture on a glass fiber filter. After washing, the total RNA was eluted. RNA Integrity was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and purity/concentration was determined using a NanoDrop 8000 (NanoDrop Products, Wilmington, DE, USA). The RNA samples with RNA Integrity Number (RIN) ≥5 and 260:280 ratio ≥1.7 were selected for the microarray.

Target Preparation and Microarray Hybridization

Microarray targets were prepared using NuGEN WT-Ovation formalin-fixed and paraffin-embedded RNA Amplification System V2. This system offers the most efficient cDNA amplification powered by Ribo-SPIA technology. It is, therefore, ideal for global gene expression analysis with the small amount of degraded RNA derived from formalin-fixed and paraffin-embedded samples. Fifty nanograms of total RNA were used for the first-strand synthesis. After the second-strand cDNA synthesis, the double-stranded cDNA was purified using Agencourt RNAClean beads provided with the WT-Ovation kit, followed by SPIA cDNA Amplification. Amplified cDNA (5 μg) was fragmented and labeled using NuGEN's FL-Ovation cDNA Biotin Module V2 according to the instructions (NuGEN Technologies, San Carlos, CA, USA), and then hybridized to the Affymetrix GeneChip U133plus 2.0 Array (Affymetrix, Santa Clara, CA, USA) according to manufacturer's instructions. The arrays were washed and stained with streptavidin phycoerythrin in Affymetrix Fluidics Station 450 using the Affymetrix GeneChip protocol and scanned using an Affymetrix GeneChip Scanner 3000.

Data Analysis

The acquisition and initial quantification of array images were conducted using the AGCC software (Affymetrix). The subsequent data analyses were performed using Partek Genomics Suite Version 6.4 (Partek, St Louis, MO, USA). We first performed a one-way ANOVA to identify genes between groups at $P<0.05$, and then calculated relative difference in fold change (FC) between groups. The genes at ≥2 FC and $P<0.05$ were considered as differentially expressed between groups. Cluster analyses and principal component analysis were conducted with Partek default settings. The canonical pathway analyses were performed using Ingenuity Pathway Analysis Version 7.6 (Ingenuity Systems, Redwood City, CA, USA). Briefly, a differentially expressed gene list containing gene identifiers and corresponding FCs was first uploaded as an Excel spreadsheet into the software. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. These genes were then used as the starting point for pathway analysis. Canonical pathways analysis identified the pathways from the Ingenuity Pathways Analysis library of canonical pathways that were most significant to the data set. The significance of the association between the data set and the canonical pathway was measured in two ways: (1) ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway was displayed. (2) Fischer's exact test was used to calculate a P-value determining the probability that the association between the genes in the data set and the canonical pathway is explained by chance alone.

Quantitative Real-Time PCR Analyses

QRT-PCR confirmation was performed using NCode VILO miRNA cDNA synthesis Kit and NCode EXPRESS SYBR GreenER miRNA qRT-PCR Kit (Invitrogen) according to the manufacturer's instructions. The same RNAs for microarray hybridization were used for QRT-PCR confirmation. Applied Biosystems 7500 Real-Time PCR System was used for the analyses with the following primers:

S100A7: Left TGCTGACGATGATGAAGGAG;
Right ATGTCTCCCAGCAAGGACAG

S100A8: Left GAGCTGGAGAAAGCCTTGAA; Right AGACGTCTGCACCCTTTTTC
HOXC10: Left GCTGGTGTGTGTGTCAAACC; Right AACGATTCTGCCTGTGCTCT
C15orf48: Left AAGGGTGACCAAATGACGAG; Right TGCAGTTATTGCTGCACTCC
GAPDH was used as an internal control for normalization.

Results

The differential gene expression was compared between actinic keratosis and normal skin, squamous cell carcinoma and normal skin, and actinic keratosis and squamous cell carcinoma (>2 FC, $P<0.05$; Table 1). There were 382 differentially expressed genes between normal skin and squamous cell carcinoma with 290 downregulated and 92 upregulated. The most significantly upregulated genes (Table 2) included *MMP1, SPRR1A/SPRR1B, ABCG4, S100A8, S100A9, WNT5a*, and *KRT6A*. The most significantly downregulated genes included *DCD, HBA1/HBA2, PIP, SCGB1D2*, and *C7*.

There were 423 differentially expressed genes between normal skin and actinic keratosis with 326 downregulated and 97 upregulated. The most significantly upregulated genes (Table 2) included *IGFL1, SPRR1A/SPRR1B*, and *KLK6* and calcium-binding proteins *S100A7A, S100A8*, and *S100A9*. The most significantly downregulated genes included *DCD, CNN1, SCGB1D2, PIP, MYH11*, and *HBA1/HBA2*. There were nine differentially expressed genes between actinic keratosis and squamous cell carcinoma (Table 3). Upregulated genes included *HOXC4, HOXC6, ROBO4*, and *COL6A3*. Downregulated genes included *RPL13A, CASZ1, RTN4 (Nogo-A), NFIB*, and *RPS6*. Hierarchical cluster analysis using the list of the differentially expressed genes between squamous cell carcinoma and normal revealed a distinct genetic signature (Figure 1). To confirm the reliability of the results from microarray analysis, selected upregulated genes including *S100A7, S100A8*, and *C15orf48* and downregulated gene *HOXC10* were verified by QRT-PCR analyses (Figure 2).

The pathway analysis revealed 18 significantly enriched molecular pathways using the differentially expressed genes between squamous cell carcinoma and normal. The most significant enriched molecular pathways (Table 4 and Figure 3) included WNT/β-catenin signaling, calcium signaling, integrin signaling, TR/RXR activation, and NFAT activation.

Discussion

The normal skin keratinocyte can undergo DNA damage and mutations by excessive exposure to UV radiation leading to the formation of actinic keratosis and then squamous cell carcinoma.[9] It is widely believed that there is a stepwise progression from normal skin to actinic keratosis to squamous cell carcinoma.[10–12] Although most accept this model of multistep carcinogenesis, there are some that believe that actinic keratosis is the same as squamous cell carcinoma and not a precursor lesion.[3,4] This assertion is supported by one cDNA microarray study that showed no differentially expressed genes between actinic keratosis and squamous cell carcinoma.[7] However, our data using the most sophisticated DNA microarray to date profiling over 47 000 genes revealed that actinic keratosis and squamous cell carcinoma each has its own unique molecular signature and are therefore, distinct entities. Although actinic keratosis and squamous cell carcinoma displayed similar gene expression profiles and are closely related at the molecular level, there were nine genes in particular that were significantly differentially expressed (FC>2, $P<0.05$; Table 3).

Actinic keratosis and squamous cell carcinoma were distinguished by four upregulated genes and five downregulated genes. The upregulated genes *HOXC4, HOXC6*, and *ROBO4* have been implicated in tumorigenesis. *HOXC4* and *HOXC6* are part of the homeobox family of genes and encode transcription factors that have a key role in morphogenesis and cell differentiation during embryogenesis of animals. *HOXC6* has been found to modulate genes with both oncogenic and tumor suppressor activities, and has shown to be upregulated in prostate cancer and is correlated with its progression.[13,14] *ROBO4* is part of the roundabout family of receptors that are large, single-pass transmembrane cell surface receptors involved in directing cell migration in response to their cognate Slit ligands. They are best

Table 1 Summary of the differentially expressed genes in selected cutaneous squamous lesions

Sample	*Total # genes upregulated (>2 fold change and P<0.05)*	*Total # genes downregulated (>2 fold change and P<0.05)*	*Total # genes (>2 fold change and P<0.05)*
Normal skin *vs* SCC	92	290	382
Normal skin *vs* AK	97	326	423
AK *vs* SCC	4	5	9

Table 2 Signature genes distinguishing normal skin from AK and SCC (>2 fold change and $P<0.05$)

Normal skin vs squamous cell carcinoma			*Normal skin vs actinic keratosis*		
RefSeq	*Symbol/gene*	*FC*	*RefSeq*	*Symbol/gene*	*FC*
Upregulated genes					
NM_002421	MMP1: matrix metallopeptidase 1	48.51	NM_198541	IGFL1: IGF-like family member 1	61.53
NM_005987	SPRR1A /// SPRR1B: small proline-rich protein 1A /// small proline-rich protein 1B (cornifin)	7.13	NM_176823	S100A7A: S100 calcium-binding protein A7A	9.12
NM_022169	ABCG4: ATP-binding cassette, sub-family G (WHITE), member 4	6.95	NM_005987	SPRR1A /// SPRR1B: small proline-rich protein 1A /// small proline-rich protein 1B (cornifin)	6.12
NM_002965	S100A9: S100 calcium-binding protein A9	6.23	NM_001012964 /// NM_001012965 /// NM_002774	KLK6: kallikrein-related peptidase 6	6.11
NM_005554	KRT6A: keratin 6A	5.81	NM_002965	S100A9:S100 calcium-binding protein A9	5.4
NM_002988	CCL18: chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	5.29	NM_032563	LCE3D: late-cornified envelope 3D	5.31
NM_004004	GJB2: gap junction protein, beta 2	5.19	NM_002964	S100A8: S100 calcium-binding protein A8	4.69
NM_002964	S100A8:S100 calcium-binding protein A8	5.12	NM_004004	GJB2: gap junction protein, beta 2	4.39
NM_007315	STAT1: signal transducer and activator of transcription 1	4.68	NM_003486	SLC7A5: solute carrier family 7 (cationic amino acid transporter, y+system), member 5	4.35
NM_012397	SERPINB13: Serpin peptidase inhibitor, clade B (ovalbumin), member 13	4.47	NM_207373	C10orf99: chromosome 10 open-reading frame 99	4.3
NM_003392	WNT5A: wingless-type MMTV integration site family, member 5A	4.35	NM_005547	IVL: involucrin	4.06
Downregulated genes					
NM_053283	DCD: dermcidin	−1229.21	NM_053283	DCD: dermcidin	−1016.55
NM_000558	HBA1 /// HBA2: hemoglobin, alpha 1 /// hemoglobin, alpha 2	−202.77	NM_001299	CNN1: calponin 1, basic, smooth muscle	−140.77
NM_002652	PIP: prolactin-induced protein	−93.47	NM_006551	SCGB1D2: secretoglobin, family 1D, member 2	−121.49
NM_006551	SCGB1D2: secretoglobin, family 1D, member 2	−73.51	NM_002652	PIP: prolactin-induced protein	−104.3
NM_000587	C7: complement component 7	−46.95	NM_001040113 /// NM_001040114 /// NM_002474 /// NM_022844	MYH11: myosin, heavy chain 11, smooth muscle	−89.59
NM_003480	MFAP5: microfibrillar-associated protein 5	−44.48	NM_000558	HBA1 /// HBA2: hemoglobin, alpha 1 /// hemoglobin, alpha 2	−55.81
NM_000518	HBB: hemoglobin, beta	−41.39	NM_173833	SCARA5: scavenger receptor class A, member 5 (putative)	−31.62
NM_032411	C2orf40: chromosome 2 open-reading frame 40	−34.75	NM_000668	ADH1B: alcohol dehydrogenase 1B (class I), beta polypeptide	−29.87
NM_001040113 /// NM_001040114 /// NM_002474 /// NM_022844	MYH11: myosin, heavy chain 11, smooth muscle	−32.75	NM_003480	MFAP5: microfibrillar-associated protein 5	−28.15

known for their role in neuronal development, but are now implicated in tumor angiogenesis. Increased *ROBO4* expression has been demonstrated on tumor vessels of brain, colon, breast, kidney, and bladder neoplasms.[15,16] Although *COL6A3* has not been associated with tumorigenesis, alterations of extracellular matrix-binding proteins would seem necessary for progression and invasion of the dermis by squamous cell carcinoma.

The downregulated genes *CASZ1, RTN4 (Nogo-A),* and *NFIB* also have been associated with tumorigenesis. *CASZ1* is a zinc-finger transcription factor that demonstrates increased expression in cells of neural origin as well as mesenchymal origin when they are induced to differentiate. *CASZ1* was found to be expressed in a number of tumor cell lines of retinoblastoma, neuroblastoma, glioblastoma, and rhabdomyosarcoma origin.[17] *RTN4* is an axon regeneration inhibitor that was strongly expressed in the majority of oligodendrogliomas. It has also been found to be a helpful marker in distinguishing oligodendrogliomas from astrocytomas and ependymomas.[18] *RTN4* expression has also been negatively correlated with the malignancy grade of oligodendroglial tumors.[19] *NFIB* is a transcription factor gene that has been associated with *MYB–NFIB*

Table 3 Signature genes distinguishing AK from SCC (>2 fold change and $P<0.05$)

RefSeq	*Symbol/gene*	*Function*	*Fold change*
Upregulated genes			
NM_004503 /// NM_153693	HOXC6: homeobox C6	Sequence-specific transcription factors, which is part of a developmental regulatory system that provides cells with specific positional identities on the anterior–posterior axis	4.32
NM_014620 /// NM_153633	HOXC4: homeobox C4		2.87
NM_019055	ROBO4: roundabout homolog 4, magic roundabout (Drosophila)	Receptor for Slit proteins, at least for SLIT2, and seems to be involved in angiogenesis and vascular patterning. May mediate the inhibition of primary endothelial cell migration by Slit proteins	2.49
NM_004369 /// NM_057164 /// NM_057165 /// NM_057166 /// NM_057167	COL6A3: collagen, type VI, alpha 3	Extracellular matrix binding proteins	2.08
Downregulated genes			
NM_007008 /// zNM_020532 /// NM_153828 /// NM_207520 /// NM_207521	RTN4: reticulon 4	Reduces the anti-apoptotic activity of Bcl-xl and Bcl-2	−2.53
NM_001010	RPS6: ribosomal protein S6	May have an important role in controlling cell growth and proliferation through the selective translation of particular classes of mRNA	−2.31
NM_005596	NFIB: nuclear factor I/B	These proteins are individually capable of activating transcription and replication	−2.23
NM_001079843 /// NM_017766	CASZ1: castor zinc-finger 1	Probable transcription factor	−2.10
NM_012423	RPL13A: ribosomal protein L13a	Ribosomal protein that is a component of the 60S subunit	−2.02

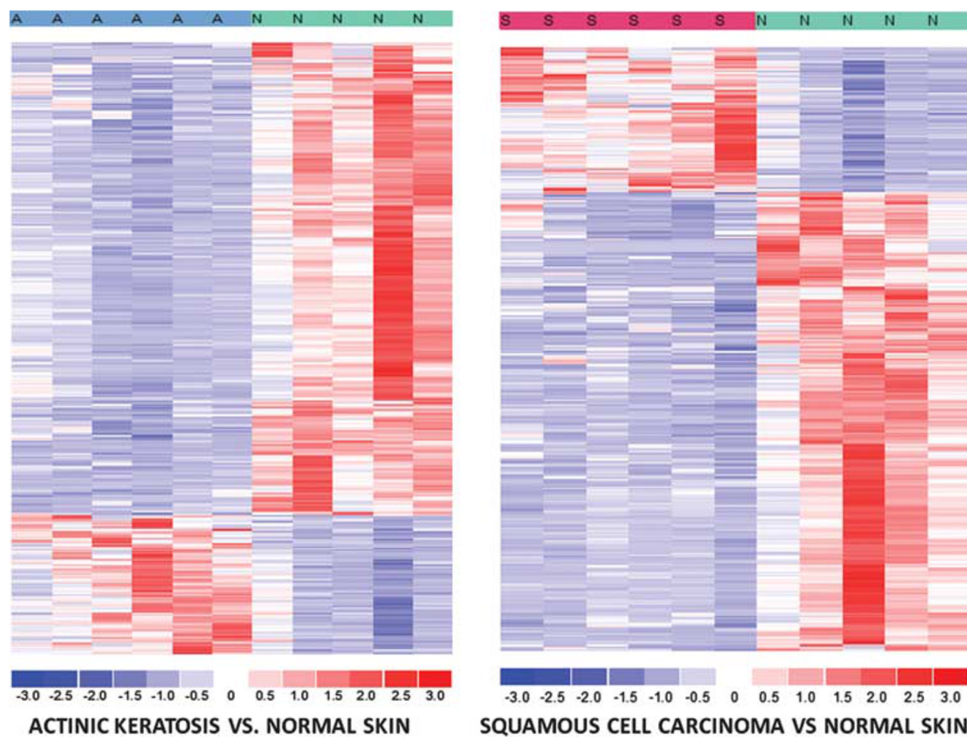

Figure 1 Hierarchical cluster analysis using the most significant differentially expressed genes (*y* axis) revealed that both actinic keratosis (A) *vs* normal skin (N) and squamous cell carcinoma (S) *vs* normal skin (*x* axis) demonstrated distinct genetic signatures. Downregulated genes are in blue and upregulated genes are in red.

fusion genes in adenoid cystic carcinomas of the breast and salivary glands and as a part of the *HMGA2-NFIB* fusion gene in lipomas.[20–22] The downregulated genes *RPS6* and *RPL13a* code for ribosomal proteins. Even though their roles in tumorigenesis remain unclear, upregulation of

ribosomal proteins may be needed for the increased transcription of proteins necessary for the proliferation of tumor cells.

Our data showed that the evolution of squamous cell carcinoma from normal skin involves the participation of many different molecular pathways (Figure 3 and Table 4). The p53 signaling pathway has been widely implicated in the tumorigenesis of cutaneous squamous cell carcinoma. Although there was deregulation of this pathway, the most significant alterations were in other pathways already implicated in carcinogenesis including WNT/β-catenin signaling, calcium signaling, and integrin signaling. A description of these signaling pathways and their relationship with tumorigenesis is discussed below.

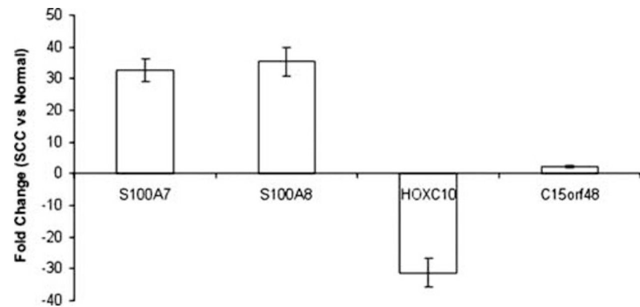

Figure 2 QRT-PCR analyses confirmed differential expression of the four representative genes between squamous cell carcinoma and normal samples. The *y* axis represents fold change (squamous cell carcinoma *vs* normal). The bar on the column stands for the s.d.

Wnt/β-catenin Signaling

The Wnt/β-catenin signaling pathway was the most significantly altered pathway in comparing squamous cell carcinoma to normal skin (−log (*P*-value) = 3.89). Wnt proteins are part of a large family of secreted glycoproteins that are involved in activating signal transduction pathways involved in numerous cellular processes including determination of cell fate, proliferation, migration, and polarity.[23,24] Wnt proteins can mediate signaling through several pathways, the best characterized of

Table 4 Significantly enriched molecular pathways in the differentially expressed genes between normal skin and squamous cell carcinoma (FC > 1.5 and $P < 0.05$)

Molecular pathway	*−Log (P-value)*	*Molecules*
Wnt/β-catenin signaling	3.89	TCF4, PPARD, PPP2CA, SOX10, TGFBR3, TLE1, FZD1, SOX13, TCF3, CSNK1E, TLE3, SFRP1, TCF7L2 (includes EG:6934), WNT5A
Calcium signaling	3.50	RAP2B, MYH10, TPM1, TRPC1, ITPR2, RCAN2, ACTA2, MEF2A (includes EG:4205), MAPK6, MYH11, RCAN1, ATF2, PRKAG2, ASPH
Integrin signaling	3.47	RAP2B, ARPC5L (includes EG:81873), PIK3R1, ACTA2, MAPK6, PTK2, MYLK, ARF6, ACTR3, RHOB, ARF3, ITGA9, PPP1R12A, ITGA7, ITGB5
TR/RXR activation	3.34	AKR1C1, LDLR, AKR1C3, SLC2A1, PIK3R1, RCAN2, NCOA1, THRA (includes EG:7067), DIO2
Role of NFAT in regulation of the immune response	3.12	BLNK, ITPR2, PIK3R1, RCAN2, GNA11, MEF2A (includes EG:4205), MAPK6, RCAN1, ATF2, CSNK1E, GNAI3, PLCB4, GNA15
Hepatic fibrosis/hepatic stellate cell activation	3.05	COL1A2, MYH10, LEPR, ACTA2, PDGFRA, MYH11, IGFBP5, STAT1, MMP1 (includes EG:4312), TIMP2, BCL2
IGF-1 signaling	2.98	YWHAQ (includes EG:10971), PTK2, NOV, PIK3R1, FOXO3, MAPK6, PRKAG2, IGFBP5, IRS2
GNRH signaling	2.80	PTK2, ADCY9, GNAI3, PLCB4, ITPR2, GNA11, MAPK6, PRKAG2, MAP2K3, ATF2
Corticotropin-releasing hormone signaling	2.52	ADCY9, GNAI3, IVL, ITPR2, PTCH1, MEF2A (includes EG:4205), MAPK6, PRKAG2, ATF2
Dopamine receptor signaling	2.40	ADCY9, GCH1, PPP2CA, PRKAG2, PPP1R12A, SMOX, MAOA
CXCR4 signaling	2.39	PTK2, ADCY9, GNAI3, PLCB4, RHOB, GNA15, ITPR2, CXCL12, PIK3R1, GNA11, MAPK6
Molecular mechanisms of cancer	2.37	RAP2B, ADCY9, TCF4, PIK3R1, GNA11, PTCH1, MAPK6, FZD1, TCF3, BCL2, PTK2, GNAI3, PLCB4, RABIF, RHOB, GNA15, PRKAG2, MAP2K3, WNT5A
Leukocyte extravasation signaling	2.34	PTK2, GNAI3, TIMP3, CLDN11, CLDN8, JAM3, CXCL12, PIK3R1, ACTA2, PECAM1, MMP1 (includes EG:4312), TIMP2
Xenobiotic metabolism signaling	2.32	PPP2CA, GSTM3 (includes EG:2947), PIK3R1, MAPK6, ALDH9A1, HMOX1, UST, CAT, NCOA1, SMOX, MAP2K3, NDST1, ALDH6A1, MAOA
Oncostatin M signaling	2.32	TIMP3, MAPK6, PLAU, STAT1, MMP1 (includes EG:4312)
Axonal guidance signaling	2.23	DPYSL2, SLIT3, ARPC5L (includes EG:81873), PIK3R1, GNA11, PTCH1, MAPK6, FZD1, NTN1, PTK2, GNAI3, PLCB4, NTRK2, ACTR3, GNA15, CXCL12, ABLIM3, PRKAG2, WNT5A
Cholecystokinin/gastrin-mediated signaling	2.15	PTK2, PLCB4, RHOB, ITPR2, MEF2A (includes EG:4205), MAPK6, MAP2K3, ATF2
p53 Signaling	1.34	SCO2 (includes EG:9997), PLAGL1, PIK3R1, CABC1, PERP, BCL2

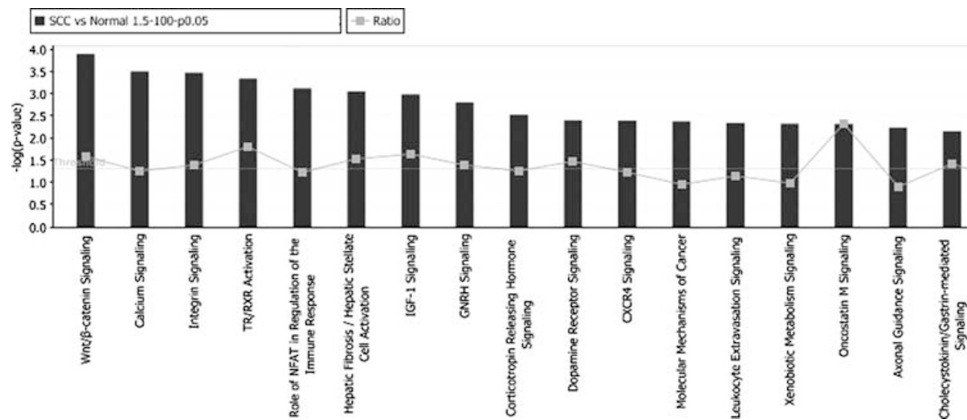

Figure 3 The most significantly deregulated molecular pathways (x axis) comparing squamous cell carcinoma *vs* normal skin (FC >1.5, $P<0.05$) utilizing the [$-\log$ (P-value)] on the y axis. The horizontal line represents the threshold for significance ($P<0.05$).

which is the β-catenin/Tcf-mediated pathway. Deregulation of the Wnt/β-catenin signaling pathway leads to continuous transcription of many target genes involved in cell proliferation such as *C-MYC* and *cyclin D1*.[23,24] Constitutive activation of this pathway has been identified in sporadic colon cancers and inherited familial adenomatous polyposis.[25] Abnormalities in this pathway have also been implicated in melanoma and carcinomas from the breast, liver, prostate, and stomach.[25,26]

Calcium Signaling

Calcium signaling was one of the most significantly altered pathways ($-\log(P\text{-value})=3.50$). Calcium is a universal ubiquitous intracellular messenger that has a vital role in many cellular processes. Calcium homeostasis is important in the orderly progression of the cell cycle, regulation of cell proliferation and growth, and apoptosis.[27] Deregulation of these intricate calcium signaling pathways has been implicating in tumorigenesis by evasion of apoptosis and the acquisition of self-sustaining growth signals.[27] The mechanisms of the deregulation of calcium signaling have been shown through T-type calcium channels, calcium pumps/exchangers, vitamin D, and calcium-sensing receptors.[27–33] Calcium and vitamin D pathways have been found to be especially important in activating the genetic pathways required for keratinocyte differentiation.[34,35] Bikle *et al*[35] showed that squamous cell carcinoma cell lines failed to respond to the prodifferentiating actions of vitamin D. Alterations of calcium signaling have also been linked to carcinomas of breast, colon, esophagus, liver, ovarian, and prostate origin.[27–29,33,36,37] These findings suggest a strong association between disruptions of calcium signaling and carcinogenesis and may provide targets for future therapeutic interventions by targeting of these pathways.

Integrin Signaling

Integrin signaling was another one of the most significantly altered pathways ($-\log(P\text{-value})=3.47$). Integrins are a family of transmembrane cell adhesion receptors that are integral to numerous intracellular signaling pathways that are vital to adhesion, migration, polarity, differentiation, and apoptosis of the cell.[38,39] Integrins transduce bidirectional signals from inside-out and outside-in the cell, thus representing the cellular link to the external environment. Integrins have been well studied in carcinogenesis and have been implicated in tumor cell proliferation and the inhibition of apoptosis through signal transduction pathways, such as Raf-ERK/MAPK, PI3K-Akt, nuclear factor-kappa B, and Jun.[38,39] Integrins have major roles in metastasis, invasion of the extracellular matrix, metastasis, and angiogenesis. The aberrant expression of certain integrins has been associated with decreased patient survival in breast carcinoma, cervical carcinoma, and colon carcinoma.[40–42] Lymph node and bone metastases have been associated with specific integrins in melanoma, breast carcinoma, prostate carcinoma, and pancreatic carcinoma.[43–47] The use of integrin antagonists in glioblastoma and colorectal carcinoma as a therapeutic modality has shown promise in clinical trials.[48,49] The finding of deregulated integrin signaling in our study is not surprising as the upregulation of different integrins has been previously demonstrated in cutaneous squamous cell carcinomas.[50,51]

P53 Signaling

The P53 signaling pathway has been widely implicated in the pathogenesis with *P53* mutations having been identified in 69% to over 90% of invasive squamous cell carcinomas.[52–54] *p53* encodes a tumor suppressor protein that functions as a

Table 5 All of the microarray studies to date comparing differential gene expression between SCC and normal skin

Study/year	*Samples*	*Genes examined*	*Differentially expressed genes*	*Upregulated genes*	*Downregulated genes*	*Significant upregulated genes*	*Significant downregulated genes*
Dooley *et al*[5]	5 SCC cell lines, 1 normal cell line, 4 SCC skin	7400; DermArray and PharmArray	96	96	N/A	FN1, HAPIP, G3P2, ANXA5, ZNF254	N/A
Nindl *et al*[7]	5 SCC skin, 4 AK skin, 6 normal skin	22 283; Affymetrix HG-U133A	118	42 (36%)	76 (64%)	CDHI, MAP4K4, IL-IRN, IL-4R, NMI, GRN, RAB31, TNC, *MMP1*	ERCCI, APR-3, CGI-39, NKEFB
Kathpalia *et al*[6]	5 SCC skin, 5 normal skin	12 627; Affymetrix U95A	73	37 (51%)	36 (49%)	BCL2A1, MUC4, PTPN11, FGF9	N/A
Haider *et al*[8]	8 SCC skin, 8 normal skin	>12 000; Affymetrix U95A	117	86 (74%)	31 (26%)	FZD6, PTN, CTSL2, *MMP1*, MMP10, MMP13, CDK7, CDC7, EREG, E2F3, *WNT5a*,	TIMP3, NFKB2, NFKBIA, VEGFC, CD69
Current study	6 SCC skin, 6 AK skin, 5 normal skin	>47 000; U133plus2.0 array	382	92 (24%)	290 (76%)	*MMP1*, *WNT5a* SPRR1A/SPRR1B, S100A8,S100A9, KRT6A, ABCG4	DCD, SCGB1D2, HBA1/HBA2, PIP, C7

DNA-binding transcription factor that is critically involved in the regulation of cell cycle control and apoptosis.[55] Upregulation of *p53* occurs in response to a variety of cellular stresses including DNA damage, oncogenic stimulation, hypoxia, oxidative stress, or telomere shortening.[54] Ultraviolet light is thought to have a major role in the carcinogenesis of squamous cell carcinoma by causing DNA damage, mutations in the *p53* gene, and immunosuppression.[54,55] Although our study revealed a statistically significant alteration of the *p53* pathway in squamous cell carcinoma compared with normal skin ($-\log(P\text{-value}) = 1.34$), many other pathways showed considerably more deregulation. In keeping with the concept of multistep carcinogenesis, the dysregulation of other molecular pathways in conjunction with or independent of the *p53* signaling pathway may be necessary for the evolution of squamous cell carcinoma from normal skin.

Significantly overexpressed genes distinguishing squamous cell carcinoma from normal skin included *WNT5a* and *MMP1*. These genes have also been found to be consistently overexpressed in other microarray and RT-PCR studies comparing cutaneous squamous cell carcinoma with normal skin.[7,8,56] *WNT5a* and *MMP1* have also been found to be overexpressed in squamous cell carcinoma from other anatomic sites, including the lip, oral cavity, head and neck, and lung. *WNT5A* is a member of the Wnt family of proteins (discussed previously) and was found to be significantly overexpressed in squamous cell carcinoma in comparison with normal skin in our study (FC = 4.35; Table 2). *WNT5a* was found to be overexpressed in another microarray study comparing cutaneous squamous cell carcinoma with normal skin.[8] Upregulation of *WNT5a* appears conserved in squamous cell carcinomas from different anatomic locations, including the lip, head and neck, and lung, and appears to be an important gene in the tumorigenesis of squamous cell carcinoma.[56–58] Upregulation of *WNT5a* has been identified in many different malignancies, including melanoma and carcinomas from the breast, esophagus, lung, pancreas, and stomach.[59–63]

MMP1 was found to be the most overexpressed gene in squamous cell carcinoma in comparison with normal skin (FC = 48.51; Table 2). *MMP1* is one of the proteins of the matrix metalloproteinase family that are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling.[64,65] Matrix metallopeptidases have been shown to have an important role in tumorigenesis by proteolytic destruction of extracellular matrix and basement membranes, facilitating tumor invasion and metastasis.[65,66] Matrix metallopeptidases have also been implicated in the early phases of tumor evolution, including stimulation of cell proliferation and modulation of angiogenesis.[65] *MMP1* is one of the rare genes consistently overexpressed in the different microarray studies examining cutaneous squamous cell carcinomas in comparison with normal skin.[7,8] Upregulation of *MMP1* appears to a conserved in squamous cell carcinomas from different anatomic locations including the head and neck and oral cavity.[67–70] Matrix metallopeptidases appear integral to tumorigenesis and have been targeted by inhibitors; however, clinical trials of advanced lung, prostate, pancreas, brain, and GI tract malignancies have shown little efficacy to date.[64,71] More data may

be needed before a successful clinical trial is completed.[64]

One surprising finding was that more differentially expressed genes were identified when comparing actinic keratosis with normal skin (423 genes) than comparing squamous cell carcinoma with normal skin (390 genes). Similarly, a study by Rehman *et al*[72] found that actinic keratoses showed higher rates of loss of heterozygosity of various chromosomes than squamous cell carcinoma. These results suggest that the relationship between the accumulation of genetic change, phenotype, and behavior for squamous cell carcinoma is not a straightforward progression from normal skin. It is possible that the presence of these additional altered genes/pathways in actinic keratoses is responsible for counteracting or delaying the incremental acquisition of additional deleterious mutations in the evolution of actinic keratosis to squamous cell carcinoma. An alternative explanation is that the initiation stage from the evolution of normal skin to actinic keratosis requires the involvement of various complex molecular signaling pathways, whereas the transformation from actinic keratosis to squamous cell carcinoma requires the involvement of more specific pathways. Clearly more work is needed in this area to clearly elucidate the genes/pathways leading to the formation of invasive squamous cell carcinoma.

Our microarray study revealed the greatest number of differentially expressed genes in comparison with previous microarray studies evaluating squamous cell carcinoma from normal skin (Table 5) and a unique gene signature distinguishing actinic keratosis from squamous cell carcinoma.[5–8] These discrepancies from previous studies are likely due to the type of tissues analyzed (cell line *vs* fresh tissue *vs* formalin-fixed and paraffin-embedded tissue) and advances in microarray technology including increasingly sophisticated RNA isolation techniques from formalin-fixed and paraffin-embedded tissue. We also utilized the most comprehensive microarray (Affymetrix U133plus2.0 array) to date that examined over 47 000 genes. With further advances in microarray technology and techniques to analyze the data, it is likely that more differentially expressed genes and their pathways will be identified.

Conclusion

The DNA microarrays were able to distinguish among normal skin, actinic keratosis, and squamous cell carcinoma. The differentially expressed genes between actinic keratosis and squamous cell carcinoma and their cancer-related functions demonstrate that they are distinct lesions and not the same entity. These differentially expressed genes may prove useful in the differentiation of these lesions in cases in which the histological diagnosis may be difficult because of sparse or superficial sampling and poor orientation of the specimen. We hope that some of the differentially expressed genes that we have identified may serve as potential biomarkers for disease prognosis or targets for future therapeutic interventions.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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