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Microarray Analysis of Cutaneous Squamous Cell Carcinomas Reveals Enhanced Expression of Epidermal Differentiation Complex Genes

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Abstract

Gene expression profiles were determined for 12 cutaneous squamous cell carcinomas (SCC) removed from sun-exposed sites on non-immunosuppressed patients. Gene expression in each SCC was compared to that in sun-exposed skin from the same patient using the Affymetrix HGU133 2.0 PlusGeneChip. We identified 440 genes with increased expression in SCC and 738 with decreased expression; overall we identified a large number of small changes in gene expression rather than a few marked changes that distinguished SCC from sun-exposed skin. Analyzing this robust data set according to biofunctional pathways using DAVID, transcriptional control elements using oPOSSUM, and chromosomal location using GSEA suggested genetic and epigenetic mechanisms of gene expression regulation in SCC. Some altered patterns of gene expression in SCC were consistent with regulation of spatially separated genes by a number of developmentally important transcription factors (forkhead, HMG, and homeo factors) that negatively regulated gene expression and to a few factors that positively regulated expression (Creb-1, NF κ B, RelA, and Sp-1). Enhanced expression of keratin 13 We also found that coordinately enhanced expression of epidermal differentiation complex genes on chromosome 1q21 was a hallmark of SCC. A novel finding in our study was enhanced expression of keratin 13 in SCC, a result validated by immunohistochemical staining of an SCC tumor tissue array.

Keywords

Skin neoplasms; Gene expression profiling; Gene expression regulation

INTRODUCTION

Neoplastic transformation is a complex multistep process, involving both genetic and epigenetic events (for example, [1,2]). These events are reflected in altered patterns of gene expression; alterations in expression arise as a result of mutations in individual genes that alter gene transcription levels, mRNA processing, or mRNA stability; changes in transcriptional control of individual genes or networks of functionally related genes; modifications of chromatinization leading to altered gene expression from large chromosomal regions; and numerical alterations in chromosomes or chromosome segments. Microarray techniques now allow us to examine the complex alterations in patterns of gene expression that distinguish neoplastic from non-neoplastic tissues (reviewed in [3–5]). Expression profiling is also able to distinguish subgroups among tumors with similar morphology; these molecularly defined subgroups may differ substantially in outcome. Thus, the expression signatures of tumors are being used increasingly to inform cancer diagnosis, prognosis, and therapeutics [6–8]. Furthermore, microarray studies can provide us with clues to the genetic and epigenetic events that underlie altered patterns of gene expression, allowing investigation of cancer pathogenesis [4,9–12]. However, effective use of this approach requires careful selection of homogeneous cancer samples, appropriate non-tumor control samples for comparison, and robust statistical analysis.

Such observations are particularly pertinent to cutaneous squamous cell carcinoma (SCC), for which multiple morphologic stages of development and associated molecular hallmarks have been identified [13–17]. Some of the genetic and epigenetic alterations that characterize SCC actually appear well in advance of the development of tumors, thus it may be difficult to identify those changes in gene expression associated specifically with malignant transformation [18–22]. In the present study, we compared gene expression patterns in sunlight-associated SCC and sun-exposed skin in 12 patients by microarray, using arrays representing over 50,000 cDNAs. Due to the large number of paired samples and the large numbers of transcripts examined, we were able to perform robust statistical analysis, thus identifying numerous small but significant differences in gene expression that distinguished SCC from sun-exposed skin. Using these data, we identified patterns of altered gene expression that suggested specific genetic and epigenetic mechanisms underlying neoplastic transformation, including individual gene regulation by transcription factors and coordinate regulation of chromosomal regions. Coordinate overexpression of genes in the epidermal differentiation complex (EDC) emerged as a gene expression signature of SCC.

EXPERIMENTAL PROCEDURES

Patients

Patients for this study were identified at the University of New Mexico Department of Dermatology. If clinical diagnosis by the dermatologist indicated SCC, and the recommendation was made for that lesion to be surgically removed, the patient was informed of the study and informed consent was obtained. The lesion was then surgically removed. Later, the patients returned for 4-mm punch biopsies of sun-exposed non-neoplastic skin from their forearms.

Tissue Samples

All of the tumors used for this study were at least 1 cm in diameter at the time of surgery, all were located on sun-exposed areas of the body, and all were classified histologically as cutaneous SCC showing intermediate differentiation. For none of the tumors was an unusual degree of inflammation or an unusually large stromal component noted in the original biopsy report. After tumor removal, an experienced Mohs surgeon (Dr. Padilla) carefully

dissected away any surrounding skin and dermal tissue from the sample to be submitted for array analysis. No tumor samples with grossly obvious areas of infection or necrosis were submitted for RNA isolation. Punch biopsy samples of sun-exposed non-tumor skin were also obtained by Dr. Padilla. He dissected away the dermis from these samples, leaving only the epidermis for RNA isolation.

RNA Isolation

Trimmed tissue was quickly frozen in liquid nitrogen. RNA was isolated from pulverized frozen tissue with the Qiagen (Valencia, CA) RNeasy Mini Kit and quantified using Nanodrop (Wilmington, DE) instrumentation. Agilent (Santa Clara, CA) RNA chip profiles of all RNA samples showed consistently excellent RNA quality.

Microarray Hybridization

Microarray studies were performed on all of the RNA samples with the Affymetrix HGU133 2.0 PlusGeneChip using standard protocols as recommended by the manufacturer (Affymetrix, Santa Clara, CA). Briefly, 3.0 µg of total RNA was used to generate double-stranded cDNA using an oligo-dT primer containing the T7 RNA polymerase promoter site and the One-Cycle Target Labeling Kit (Affymetrix, Santa Clara, CA). cDNA was purified via column purification using the GeneChip Sample Cleanup Module (Affymetrix, Santa Clara, CA), and biotinylated cRNA was synthesized by in vitro transcription using the GeneChip IVT Labeling Kit (Affymetrix, Santa Clara, CA). Biotin-labeled cRNA was purified with the GeneChip Sample Cleanup Module and the absorbance measured at 260 nm to determine yield. Twenty µg of the labeled cRNA was fragmented and quality was assessed using the Agilent 2100 Bioanalyzer and the RNA 6000 Nano Chip kit (Agilent, Santa Clara, CA). Labeled fragmented cRNA was hybridized to the Affymetrix GeneChip HGU133 2.0 array for 16 hours at 45° C using the recommended protocol. Washing and staining was performed on the Affymetrix 450 fluidics station using the antibody amplification protocol (Fluidics script: EukGE-WS2v5). Each GeneChip was scanned using the Affymetrix GeneChip Scanner 3000, a wide-field epifluorescent near-confocal microscope with a patented flying objective.

Bioinformatic and Statistics Analysis

Analysis was conducted using the original .cel files and processed using the Affymetrix Microarray Suite Version 5.0. Present/absent calls to filter data were not employed. A scaling normalization approach was applied to rescale the median of each array to be the same across the dataset. The paired sample t test was applied as test statistic on a gene-by-gene basis, in order to identify genes significantly differentially expressed in paired samples of SCC versus sun-exposed skin samples. For each gene, the p value was computed based on the paired sample t test. Since we carried out the test for each gene simultaneously we used a beta-uniform mixture model to adjust for multiple testing and to estimate the false discovery rate (FDR) [23,24]. When the FDR was set at FDR=0.1%, which corresponded to a p value of less than 0.0001, we identified almost 1600 probe sets that identified differentially expressed transcripts in paired samples of sun-exposed skin and SCC.

The Gene Set Enrichment Analysis (GSEA) algorithm was also applied. The purpose of using GSEA was to compare pre-defined gene sets collected from different molecular databases to our data in order to generate interesting hypotheses for further research. It is a popular tool and widely used. In the present case, we compared our data to a GSEA dataset that estimated the number of expressed genes in 268 individual chromosome segments. To identify significantly enriched gene sets, the FDR q value, an FDR-adjusted p value, was applied. Unlike the p value, which expresses the probability of a false-positive result for a single test, the FDR q value gives a conservative estimate of the proportion of results that

are false positive for the entire set of reported results. The q value is calculated based on p values, the number of tests performed, and the distribution of the p values among these tests. Detailed information of GSEA can be found in reference [25]. We used FDR q value <0.25 to identify significant gene sets, as suggested by the original authors. Such a value indicates that the result is likely to be valid 3 out of 4 times or, in other words, we expect not more than 25% of results to be false positive.

Genes that were significantly altered based on this analysis were categorized with the help of DAVID Bioinformatic Resources 2006 (National Institute of Allergy and Infectious Diseases, National Institutes of Health) [25,26]. Un-named genes were excluded from this categorization. DAVID provides gene-annotation category enrichment analysis to identify the most relevant annotation categories (e.g., GO terms, protein-protein interactions, protein functional domains, disease associations, bio-pathways, sequence general features, homologies, gene functional summaries, gene tissue expressions, literatures) within gene lists. DAVID Functional Annotation Clustering then applies a novel algorithm to cluster annotation terms into larger order categories based on shared genes. This reduces redundancy and facilitates biological interpretation. We used the Functional Annotation Clustering tool of DAVID to cluster differentially expressed genes into a total of 298 Annotation Clusters of related terms. From these clusters, we chose those terms with EASE (modified Fisher exact p value) scores of 0.05 or less. Similar terms from different clusters were combined into larger functional groupings of genes. Genes within each final functional grouping were edited to remove duplicates.

The oPOSSUM analysis system identifies over-represented transcription factor binding sites in sets of co-expressed genes (target gene set) by comparing the target gene set against a pre-compiled background set of genes [26–28]. Two measures of statistical significance, the Z score and the Fisher score, are then calculated. In general, a Z score of >10 and a Fisher score of <0.01 identify significantly over-represented transcription factor binding sites. We employed this system to identify transcription factors of interest in our studies.

Immunohistochemistry to Examine Keratin 13 Expression in Human SCC

A commercial tissue array that contained samples of 76 human cutaneous SCC (US Biomax, Rockville, MD) was employed. Samples were formalin-fixed paraffin-embedded sections 2 mm in diameter and 4–5 µm in thickness. Immunohistochemistry for keratin 13 was performed as follows: Slides were deparaffinized and rehydrated to water. Endogenous peroxidase activity was blocked with 3% H₂O₂ in water (10 minutes), antigen retrieval was performed by microwaving in 10 mM citrate buffer, pH 6.0 (10 minutes followed by a 20-minute cooling period), and blocking was performed in Biocare Blocking Reagent (Concord, CA) (10 minutes). Slides were incubated with anti-keratin 13 monoclonal antibody (Abcam, Cambridge, MA) diluted 1:200 at room temperature (1 hour), then with biotinylated anti-mouse F(ab) (Accurate Chemical, Westbury, NY) at a 1:250 dilution at room temperature (15 minutes), and finally with SA-HRP (BioGenex, San Ramon, CA) at room temperature (30 minutes); 3,3'-diaminobenzidine was used as chromagen and slides were counterstained with hematoxylin.

RESULTS

Patient and Tumor Data

A total of 12 patients participated in this study, including 10 males and 2 females (Table 1). Eleven patients described themselves as White; there was one Hispanic and no native American or African-American patient. The average age was 67.7 years (range, 51 – 84). None of the patients was receiving immunosuppressive therapy or was known to be

otherwise immunocompromised. The tumors were relatively homogeneous, as all had a histologic diagnosis of SCC with intermediate differentiation and all tumors were 1.0 cm or larger as measured at their greatest diameter.

Differences in Gene Expression Levels

We applied the two-sample t test on the array data obtained from matched SCC and sun-exposed skin samples from 12 patients (Supplemental Data 1). Our study included the largest number of clinical samples and the largest number of expressed sequences in SCC examined to date. Because of the multiple testing involved, the individual p values were not particularly meaningful. However, when we looked across the entire set of tests, the distribution of the p values (under the null hypothesis that no genes provide useful information) should have been uniform. If, on the other hand, some genes did provide useful information about predicting the response, we would have expected an overabundance of small p values. We captured this situation by modeling the distribution of the p values as a beta-uniform mixture.

Using this method, by setting FDR=0.1%, we identified almost 1600 probe sets that were significantly differentially represented in SCC and sun-exposed skin (Supplemental Data 2). These probes defined a total of 1178 individual named sequences. Expression of 440 genes was enhanced in SCC compared to sun-exposed skin, while expression of 738 genes was decreased. Expression of 19 genes was enhanced more than 2-fold, but expression of only a single gene was more than 2-fold decreased (Table 2). The 2-fold downregulated gene was an alcohol dehydrogenase. The group of 2-fold upregulated genes included several known to play roles in keratinocyte differentiation or skin cancer: genes encoding 2 S100 molecules, a small proline-rich protein (SPRR), keratin 13, parathyroid hormone-like hormone, fos-like antigen 1, and latent transforming growth factor- β binding protein. Interestingly, the S100 and SPRR genes are located within the EDC in chromosome band 1q21. In addition, 2 genes are located on chromosome 11q (fos-like antigen 1 and transcobalamin I) and 2 are located on chromosome 17q (keratin 13 and acidic hair keratin 4), suggesting that markedly upregulated genes were not distributed randomly within the genome.

Biofunctional Patterns of Gene Expression

To determine if genes with altered expression were clustered into functionally related groups, we analyzed our results with DAVID, a bioinformatics resource offered by the National Institute of Allergy and Infectious Diseases (Table 3) [26]. This program identifies the most relevant annotation categories within gene lists then clusters annotation terms into larger order categories to reduce redundancy and facilitate biological interpretation.

The most marked changes in expression levels were seen for genes encoding molecules important in epidermal differentiation, including 5 keratin genes (6A, 6B, 13, 16, and 17), 9 S100 genes (S100A2, 7, 7A, 8, 9, 10, 11, 12, 16), 5 SPRR genes (1A, 1B, 2B, 2C, and 3), 5 cornified envelope genes (involucrin, cornifelin, late cornified envelope 3D, stratifin, and transglutaminase 1), and 3 intercellular junction genes (alpha-like catenin 1 and connexins 26 and 30). All of these genes, except for alpha-like catenin 1, showed increased expression in SCC. Interestingly, many of these genes are located in the EDC, again suggesting non-random chromosomal distribution of genes with altered expression in SCC.

A large number of transcription factors had altered expression in SCC compared to sun-exposed skin. There were also alterations in expression of genes related to chromatin structure, RNA transcription, translation, and protein turnover. One prominently altered metabolic process was glycolysis; a total of 45 genes related to glycolysis, 35 of which were enzymes, had altered expression in SCC. Other metabolic alterations of potential interest in

SCC included altered expression of 11 genes related to the mTOR pathway and 10 genes related to metabolism of vitamins, including vitamins A and B12. According to DAVID algorithms, the pattern of altered gene expression in SCC was significantly similar to the findings in several other cancers, including acute myeloid leukemia, colorectal cancer, endometrial cancer, prostate cancer, and renal cell carcinoma. Five genes were upregulated in all of these tumors, as well as SCC: phosphoinositide-3-kinase, regulatory subunit I (p85 alpha); phosphoinositide-3-kinase, catalytic, alpha polypeptide; mitogen-activated protein kinase 1; protein kinase B, gamma; and growth factor receptor-bound protein 2. Interestingly, all of these genes are important in signal transduction. As observed for other cancers, SCC also exhibited changes in expression of genes involved in cell growth and division and apoptosis. However, for these genes, a pattern clearly consistent with enhanced cell proliferation and decreased apoptosis was not observed.

Transcriptional Control of Gene Expression

To determine if functionally clustered genes were coordinately controlled by common transcription factors, we performed oPOSSUM analysis of our results (Table 4) [27,28]. When both up and downregulated genes were considered together, 19 significantly over-represented transcription factor binding sites (Z score >10, Fisher score <0.01) were identified. Among these binding sites were a number recognized by transcription factors important in development and differentiation, including forkhead, HMG, and homeo factors. Analyzing up and down-regulated genes independently revealed that all 19 binding sites were associated with down-regulated genes. In addition, a number of other overrepresented recognition sites associated with down-regulated genes emerged, including bZIP, homeo, MADS, and TRP cluster factor binding sites. When upregulated genes alone were considered, over-represented transcription factor binding sites having both Z scores >10 and Fisher scores <0.01 were not identified. However, by relaxing the stringency of 10 our analysis to include genes with Z scores > 10 but Fisher scores above 0.01, we identified 6 over-represented sites in the promoters of upregulated genes. These sites included those recognized by Creb-1, NFκB, RelA, and Sp-1.

Overall, these findings suggested that recognition sites for over 20 transcription factors were significantly over-represented in genes down-regulated in SCC compared to sun-exposed skin, including forkhead, HMG, and homeo factors highly related to development and differentiation [29–31]. Transcription factor binding sites over-represented in up-regulated genes, on the other hand, included Creb-1, NFκB, RelA, and Sp-1, factors known to be important in skin cancer [32–36].

Regional Patterns of Gene Expression

Our initial observations suggested that genes with altered expression in SCC were not uniformly distributed throughout the genome. GSEA analysis was conducted, comparing the distribution of genes with altered expression over a total of 268 chromosomal segments [37]. This revealed that a number of the genes with altered expression were clustered in specific chromosomal regions (Table 5). Such nonrandom patterns of expression can be the result of coordinate gene regulation or regional changes in chromatin configuration. However, regional alterations in expression levels may also reflect karyotypic changes ranging from small deletions, amplifications, or rearrangements to duplications or deletions of entire chromosomes or chromosomal arms.

We identified a number of over or under-represented karyotypic regions consistent with numerical changes previously detected in SCC or actinic keratoses by cytogenetics, FISH, and comparative genomic hybridization [38,39]. These included +1q; +7, +7p, +7q; +11q; +14q; +17p, +17q; +20, +20q; +Xq; -4, -4q; -14; and -X. In our study, a number of genes

located at chromosome 1q21 were significantly over-represented among genes with increased expression. Previous studies have shown that the EDC located in 1q21 consists of over 40 genes that appear to be coordinately regulated [40]. Genes comprising the EDC include a number of S100, SPRR, and cornified envelope genes [40]. Our results showed that expression of 17 of these genes (9 S100, 6 SPRR, and 2 cornified envelope genes) was significantly enhanced in SCC.

Validation of Findings

Our analysis identified changes shared by the SCCs we examined, rather than alterations that distinguished among sub-categories of tumors. Thus, it was our prediction that this study would identify genes with expression levels altered in many SCC, not just in those SCC that we examined. One of the genes we identified as likely to have altered expression in many SCC was keratin 13. Immunohistochemistry for keratin 13 on an SCC array that included a total of 71 usable tumors, showed that 49% of the tumors were immunoreactive for keratin 13 (Figure 1). There was a suggestion that keratin 13 expression was reciprocally related to tumor grade, with 58% of the 45 well differentiated tumors, 43% of the 21 moderately differentiated tumors, and none of the 5 poorly differentiated SCC being keratin 13-positive. No keratin 13 immunoreactivity was seen in 4 sections of normal skin included on the array. Thus, our studies revealed the novel finding that keratin 13 was expressed *de novo* in approximately half of a random assortment of human cutaneous SCC, thereby fulfilling the prediction that our results would be generally applicable to cutaneous SCC.

DISCUSSION

In the present study, we performed pairwise array analysis of 12 individually matched tumor and non-tumor skin samples. Skin tumors were uniform with regard to level of differentiation and all tumors were relatively large, allowing isolation of abundant RNA. Gene expression was compared using gene arrays representing more than 50,000 transcripts. With this large number of paired samples and the many genes examined, we were able to perform robust statistical analysis of our results. Correction for multiple comparisons was possible to eliminate false positive findings and arbitrary cut-off levels were not required to identify transcripts of interest. Thus we were able to distinguish a large number of transcripts that had significantly altered levels in SCC compared to control skin even when differences in levels of expression were not marked. In addition, by comparing SCC with sun-exposed skin from the same individuals, we were able to identify genes specifically related to neoplastic transformation rather than to UVR exposure. Further analysis of this large number of altered transcripts using readily available software revealed statistically significant patterns of altered expression related to biofunctional pathways, transcription factor regulation, and chromosomal location.

A number of previous studies using array techniques to characterize the gene expression profile of cutaneous SCC have had intrinsic limitations, including use of skin cancer cell lines as sources of RNA, use of pooled tumor or non-tumor skin samples for analysis, lack of appropriately site-matched skin from individual patients as control tissue, use of arrays representing a limited numbers of genes, and use of tumor samples from immunosuppressed transplant recipients [41–45]. Thus these studies identified only a limited set of genes with fairly highly altered expression. Importantly, comparison of these studies did not yield a consistent pattern of gene expression characteristic of SCC [46]. In contrast, our studies comparing SCC to sun-exposed skin revealed many transcripts with relatively small changes in expression level. When compared with previous studies (Supplemental Data 3), our studies showed up to 35% concordance, defined as similar direction of significant expression change, with other studies for enhanced gene expression, but no more than 10% concordance for reduced gene expression.

The most highly enhanced gene expression in our study was seen for genes that play a role in cell differentiation (S100A7, keratin 13), the extracellular matrix (cysteine-rich secretory protein 3), growth factor activity (latent transforming growth factor- β binding protein), signaling (parathyroid hormone-like hormone), transcriptional regulation (fos-like antigen 1), and protease activity (serine protease 27, Kazal type 6 serine peptidase inhibitor), as well as transcobalamin I and vanin3, which play an undetermined role in differentiation. A number of these or highly related genes were identified as overexpressed in previous studies of human SCC, including keratin 6 and its binding partner keratin 16, S100A9, and fos-like antigen 1 [42,44,45]. Interestingly, in chemically induced murine SCC, keratin 13, keratin 16, S100aB, and S100a9 showed substantially increased expression [47]. There was less consistency when genes that showed reduced expression were compared across SCC microarray studies. Indeed, genes that were shown to have enhanced expression in some studies might have reduced expression in other studies, including insulin-like growth factor binding protein and S100A8 [42,45,47].

To validate the biological relevance of our findings, we looked for altered expression of keratin 13, a gene with relatively highly altered expression in our tumors, in a large panel of SCC from an independent source. We detected keratin 13 expression by immunohistochemistry in almost half of these tumors. This finding was novel, as keratin 13 expression was not previously believed to be altered in human SCC [48,49]. Indeed, for SCCs of the tongue, esophagus, and cervix in humans, loss of keratin 13 expression is associated with malignant conversion and increased risk of tumor recurrence [50–52]. On the other hand, the appearance of keratin 13 in chemically induced SCC of the skin in mice indicates a high risk for malignant conversion [53,54].

DAVID analysis revealed that multiple biofunctional pathways were altered in SCC compared to sun-exposed skin. Cellular processes involved included differentiation, proliferation, apoptosis, regulation of chromatin structure, transcription, RNA processing, translation, ubiquitination, and glycolysis. Coordinate regulation of multiple genes participating in a single biofunctional pathway may be accomplished by two important mechanisms: regulation of genes in multiple locations by common transcription factors and regional regulation of clusters of functionally related genes. In our study, we found evidence for both of these mechanisms.

oPOSSUM analysis identified that altered patterns of gene expression in SCC could be attributed to a large number of transcription factors we identified that negatively regulated gene expression and a smaller number of factors that positively regulated expression. The majority of transcription factors that negatively regulate gene expression were forkhead, HMG, and homeo proteins important in controlling development and differentiation. This was consistent with our other findings indicating marked alterations in expression of differentiation-related genes in SCC compared to sun-exposed skin. Transcription factors implicated in coordinately upregulating gene expression in SCC included several previously identified as playing a role in skin differentiation and carcinogenesis, including Creb-1, NF κ B, RelA, and Sp-1 [32–36].

In our studies, many genes with altered expression clustered in specific areas of the genome. This suggested to us that we might be able to use our results to identify common cytogenetic alterations in SCC, as accomplished by other investigators [55,56]. SCC of the skin exhibit very complex karyotypes with a large number of diverse chromosomal alterations, including deletions, amplifications, and translocations [38,57,58]. It has been suggested that genomic instability may be a “driving force” in skin cancer development, as SCC have substantially more karyotypic abnormalities than actinic keratoses, precursors of SCC, or than keratoacanthomas or basal cell carcinomas, other tumor types derived from basal

keratinocytes [57,59–61]. Moreover, well-differentiated SCC show distinctly fewer chromosomal aberrations than poorly or moderately differentiated SCC [62]. We found that the chromosomal regions with alterations in gene expression, including +1q; +3q; +7, +7p, +7q; +8q; +11q; +14q; +17p, +17q; +20, +20q; +Xq; -3p; -4, -4q; -8p; -9p; -13q; -14; and X, correlated with structural alterations previously revealed by cytogenetics, comparative genomic hybridization, and FISH studies [38].

Based on analysis by several techniques, changes in expression of genes belonging to the EDC located at 1q21 emerged as particularly important in distinguishing SCC from sun-exposed skin. Several genes located in the EDC were expressed at particularly high levels in SCC compared to sun-exposed skin, DAVID biofunctional analysis of genes with significantly altered expression clustered multiple functionally related genes within this region, and GSEA analysis revealed that genes within this cytogenetic region were significantly overrepresented among genes with altered expression in SCC. A previous study also noted that the preponderance of differentially expressed genes in SCC were located on chromosome 1, including 1 gene in the EDC; however, the validity of this observation was not tested statistically [43]. Other reports also suggest an important role for the EDC in cutaneous SCC. For instance, SAGE analysis of gene expression in human actinic keratoses showed strongly upregulated expression of the EDC gene cluster [63]. In addition, increased expression of genes within the murine EDC has been shown to enhance susceptibility to chemically induced skin cancers in the mouse [64].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

EDC	Epidermal Differentiation Complex
GSEA	Gene Set Enrichment Analysis
SCC	squamous cell carcinoma
SPRR	small proline-rich protein
TGF-β	transforming growth factor- β

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Figure 1. Immunohistochemical staining for keratin 13 in (A) human SCC and (B) normal human skin. Note that staining in the tumor is heterogeneous and that normal skin is keratin 13-negative.

Table 1

Patient information

Patient Characteristics				Lesion Characteristics		
Patient	Sex	Age	Ethnicity	Location	Size	Grade
1	M	74	White	Ear	3.0 × 2.0 cm	Intermediate
2	M	59	White	Forehead	3.0 × 4.0 cm	Intermediate
3	M	74	White	Mid chest	3.0 × 2.5 cm	Intermediate
4	F	84	White	Upper arm	1 × 0.5 cm	Intermediate
5	M	55	Hispanic	Lower lip	1.9 × 1.4 cm	Intermediate
6	M	74	White	Scalp	4.5 × 3.5 cm	Intermediate
7	M	65	White	Lower lip	1.6 × 1.4 cm	Intermediate
8	F	80	White	Dorsal hand	1.1 × 0.8 cm	Intermediate
9	M	53	White	Temple	3.2 × 2.4 cm	Intermediate
10	M	82	White	Forehead	1.7 × 1.8 cm	Intermediate
11	M	61	White	Temple	1.3 × 1.2 cm	Intermediate
12	M	51	White	Forehead	4.0 × 2.8 cm	Intermediate

Table 2

Genes with most highly altered expression in SCC compared to sun-exposed skin

Gene Symbol	p value	t score	Gene Title	Location	SCC/sun-exposed
ADH1B	0.0000002	-11.595	alcohol dehydrogenase 1B (class I), beta polypeptide	4q21-q23	-2.22
RHCG	0.0000021	9.0136	Rh family, C glycoprotein	15q25	2.02
KRTHA4	0.0000167	7.2354	keratin, hair, acidic, 4	17q12-q21	2.02
TMPRSS11D	0.0000006	10.144	transmembrane protease, serine 11D	4q13.2	2.05
DEFB4	0.0000037	8.4959	defensin, beta 4	8p23.1-p22	2.08
CHAC1	0.0000032	8.6159	ChaC, cation transport regulator-like 1 (E. coli)	15q15.1	2.09
CNTNAP2	0.0000039	8.4483	contactin associated protein-like 2	7q35-q36	2.15
ST00A12	0.0000031	8.6482	S100 calcium binding protein A12 (calgranulin C)	1q21	2.16
CRISP3	0.0000301	6.7847	cysteine-rich secretory protein 3	6p12.3	2.20
FOSL1	0.0000052	8.1865	FOS-like antigen 1	11q13	2.24
KRT13	0.0000616	6.2616	keratin 13	17q12-q21.2	2.32
VNN3	0.0000539	6.3563	vanin 3	6q23-q24	2.94
LTBP1	0.0000703	6.1677	latent transforming growth factor beta binding protein 1	2p22-p21	2.35

Table 3

DAVID Functional grouping of differentially expressed genes ^a

Functional Group	Number of Genes	Specific Terms Included
Differentiation	261	Ectoderm development, epidermal morphogenesis, keratinocyte differentiation, keratinization, Wnt receptor signaling pathway, hemidesmosome
Cell division/cell growth	200	Anatomical structure morphogenesis, cell growth, cell cycle, microtubule organizing center, G1/S transition
Apoptosis	255	Programmed cell death, cell death, apoptotic mitochondrial change
Transcription	437	Regulation of transcription, transcription factor, nucleic acid binding, RNA biosynthesis
Chromatin	40	Chromosome organization, chromatin assembly, DNA packaging, histone modification
RNA	74	Heterogeneous nuclear ribonucleoprotein complex, mRNA binding, mRNA metabolism, RNA 3'-end processing,
Translation	27	Ribonucleoprotein, ribosome
Ubiquitin	61	Ubiquitin cycle, protein ubiquitination, ubiquitin ligase
Glycolysis	45	Carbohydrate metabolism, monosaccharide metabolism, hexose metabolism
Alcohol dehydrogenase	5	Alcohol dehydrogenase
mTOR	11	mTOR signaling
Vitamin	10	Vitamin metabolism, retinol metabolism
Cancer	38	Acute myeloid leukemia, colorectal cancer, endometrial cancer, prostate cancer, renal cell carcinoma, CXCR4 signaling
Pigmentation	32	Melanocyte differentiation, pigmentation, tyrosine metabolism

^a All genes were included in terms that had EASE (modified Fisher exact p value) scores of 0.05 or less.

Table 4

oPOSSUM analysis of over-represented transcription factor binding sites

TF	TF Class	Target genes	Up and Down		Down Only		
			Z-score	Fisher score	Target genes	Z-score	Fisher score
Down and up-regulated genes together							
Foxa2	Forkhead	643	21.82	2.93E-09	455	29.73	3.97E-16
FOXD1	Forkhead	568	12.69	1.18E-04	414	20.49	5.61E-12
Foxd3	Forkhead	643	26.82	1.37E-07	463	38.5	1.39E-16
FOXF2	Forkhead	279	12.2	6.44E-07	231	24.54	3.76E-17
FOXJ1	Forkhead	637	23.51	4.55E-08	457	31.9	1.84E-16
Foxq1	Forkhead	460	17.08	1.95E-09	344	22.15	9.15E-18
Sox5	HMG	782	22.3	3.24E-05	540	32.48	5.63E-12
SOX9	HMG	648	11.68	6.86E-07	452	15.78	7.35E-12
SRY	HMG	783	29.93	7.01E-08	540	42.35	5.47E-15
Lhx3	Homeo	618	19.27	4.10E-11	459	31.22	9.28E-25
Lhx3	Homeo	387	17.91	6.20E-11	292	22.61	2.79E-18
Nkx2-5	Homeo	853	29.97	3.51E-07	569	42.18	2.23E-10
NKX3-1	Homeo	602	17.79	3.84E-08	448	26.22	1.70E-20
Nobox	Homeo	745	20.51	5.21E-06	513	30.19	2.20E-11
Pdx1	Homeo	834	23.63	2.71E-06	567	37.21	1.77E-12
Prrx2	Homeo	814	25.99	1.37E-06	560	39.98	2.40E-14
TBP	TATA-box	616	15.65	4.59E-09	427	21.35	7.48E-13
IRF1	TRP-Cluster	361	11.96	2.38E-05	274	16.47	1.55E-11
Gfi	ZN-finger, C2H2	707	10.71	1.41E-05	482	12.35	6.47E-09
Down-regulated genes only							
Cebpa	bZIP	443	17.09	6.14E-1			
NFIL3	bZIP	265	10.38	1.09E-16			
HNFI1A	Homeo	167	11.56	1.72E-09			
MEF2A	MADS	324	11.21	3.47E-15			
IRF2	TRP-Cluster	50	13.29	3.76E-05			

TF	TF Class	Target genes	Up and Down		Down Only		
			Z-score	Fisher score	Target genes	Z-score	Fisher score
Up-regulated genes only							
CREB1	bZIP	129	10.44	5.56E-02			
NF-kappaB	Rel	147	11.28	5.77E-02			
RELA	Rel	111	11.45	3.62E-01			
MZF1_1-4	ZN-finger, C2H2	298	15.64	1.28E-01			
SP1	ZN-finger, C2H2	211	10.07	2.33E-01			
Statf	ZN-finger, C2H2	40	13.8	3.21E-02			

Table 5

GSEA analysis of over-represented chromosome segments*

Expression	Chromosome	Adjacent Bands	Location	Expressed Genes	Genes of Interest
Increased	1	P35, P36	Telomere	21 up/6 down	None
		Q21		22 up/5 down	5 SPRR genes (up) 9 S100 genes (up) involucrin (up) late cornified envelope 3D (up)
	2	P12		0 up/0 down	None
		Q13		0 up/2 down	None
		Q21		1 up/0 down	None
		Q35		0 up/4 down	None
	4	Q21		1 up/9 down	Alcohol dehydrogenase 1B (down)
	7	P12		1 up/0 down	None
	11	P15	Telomere	4 up/6 down	WEE homolog (down)
		Q11	Centromere	1 up/0 down	Transcobalamin I (up)
		Q14		0 up/2 down	Tyrosinase (down)
	13	Q34	Telomere	0 up/3 down	None
	14	Q32	Telomere	3 up/3 down	None
	16	P13		9 up/3 down	2 serine proteases (up)
		Q24	Telomere	0 up/3 down	None
	17	Q12		7 up/2 down	4 keratin genes (up)
		Q25	Telomere	7 up/3 down	None
	18	Q21		2 up/4 down	2 serpin peptidase inhibitors (up)
	20	P13	Telomere	3 up/0 down	None
		Q11, Q12, Q13	Whole arm	9 up/9 down	None
	22	Q11 Q12, Q13	Whole arm	14 up/6 down	None
	X	Q28	Telomere	1 up/1 down	None
Decreased	4	Q23		0 up/3 down	Alcohol dehydrogenase 1B (down)
	8	Q21		0 up/8 down	None
	12	Q14		2 up/5 down	None

Expression	Chromosome	Adjacent Bands	Location	Expressed Genes	Genes of Interest
	14	Q21		1 up/2 down	None
	X	Q13, Q21, Q22		2 up/12 down	3 transcription elongation factors A (down)

* FDR q value <0.25