Identification of genes and signaling pathways associated with squamous cell carcinoma by bioinformatics analysis

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Received December 18, 2014; Accepted November 2, 2015

DOI: 10.3892/ol.2015.4051

Abstract. The present study aimed to investigate the genes and signaling pathways associated with squamous cell carcinoma (SCC) by bioinformatics analysis. For this purpose, the GSE2503 was downloaded from the Gene Expression Omnibus database, and the differentially expressed genes (DEGs) between 6 normal skin and 5 SCC samples were analyzed using the Linear Models for Microarray Data package. Gene Ontology (GO) and pathway enrichment analysis of DEGs were performed, followed by functional annotation and construction of a protein-protein interaction (PPI) network. Subnetwork modules were subsequently identified and analyzed. A total of 181 DEGs, including 95 upregulated and 86 downregulated DEGs, were identified, in addition to 20 GO biological processes terms enriched by upregulated DEGs and 14 enriched by downregulated DEGs. The upregulated DEGs were enriched in 18 pathways, and the downregulated DEGs were enriched in 7 pathways. Following functional annotation, three upregulated transcription factors (TFs), including hypoxia inducible factor 1, alpha subunit (HIF1A), and six downregulated TFs were identified. In the PPI network and subnetwork, matrix metalloproteinase 1 (MMP1), also known as interstitial collagenase, and interleukin 8 (IL8) were the hub genes with the highest degree of connectivity (degree =8). Integrin alpha (ITGA)6 and 2 were enriched in several pathways, including focal adhesion and extracellular matrix-receptor interaction. DEGs of SCC were primarily enriched in pathways associated with cancer and cell adhesion. Therefore, DEGs such as IL8, MMP1, HIF1A, ITGA6 and ITGA2 may be potential targets for the diagnosis and treatment of SCC.

Introduction

Squamous cell carcinoma (SCC) is a histologically distinct type of cancer (1). It arises from the uncontrolled proliferation of epithelial cells or cells exhibiting cytological or tissue architectural characteristics of SC differentiation, including the presence of keratin, tonofilament bundles or desmosomes, which are structures involved in cell-cell adhesion (1). SCC occurs in numerous tissues, including the lips, mouth, esophagus, lungs, urinary bladder and prostate (2). Of all the cases of SCC, 2.5% become metastatic and lead to substantial morbidity; which constitutes a considerable economic burden to the healthcare system (3). The incidence of SCC has notably increased worldwide over the last decade (4). Thus, an improved understanding of the underlying molecular mechanisms and gene networks involved in the development and progression of skin SCC is required.

Numerous studies on the mechanisms and therapeutic strategies for the treatment of SCC have been reported to date (5-8). Exposure to ultraviolet radiation, a potent mutagen and DNA-damaging agent, is considered to be a significant risk factor for the development of SCC (5,6). Carcinogenesis is a multistep process. During tumor progression, multiple genes experience up- or downregulation (7). A number of genes and signaling pathways involved in the progression of SCC have been previously identified (3,6-9). Streit et al (8) reported that thrombospondin-1 (TSP-1) was an effective inhibitor of angiogenesis and tumor growth in carcinomas of the skin. In addition, the authors observed that the expression of TSP-1 was downregulated in patients with SCC. Previous studies have established that the transforming growth factor β-mothers against decapentaplegic homolog 4 (SMAD4) signaling pathway is required for effective epithelial wound healing, and a conditional deletion of SMAD4 in the epidermis causes defects in skin wound healing, which are accompanied by spontaneous skin inflammation and SCC (6). Additionally, talin 1 and laminin alpha 3, which participate in signaling pathways associated with adhesion and migration, have been previously observed to be overexpressed in SCC (9). Padilla et al (3) and Nindl et al (7) selected differentially expressed genes (DEGs) between SCC and normal skin (NO) samples using the microarray expression profile dataset GSE2503. However, the interactions
between the carcinogenic genes that lead to SCC remain to be elucidated.

In the present study, GSE2503 was downloaded and used to identify the DEGs between SCC and NO samples, in order to investigate the underlying molecular mechanisms of SCC. Subsequently, functional enrichment analysis and functional annotation were performed. In addition, protein-protein interaction (PPI) networks and subnetworks were constructed and analyzed, in order to study and identify target genes for the diagnosis and treatment of SCC. The results of the present study may facilitate the understanding of the mechanisms responsible for the carcinogenesis and development of SCC. Furthermore, the genes identified in the present study may serve as biomarkers for the diagnosis and prognosis of SCC.

**Materials and methods**

*Affymetrix microarray data.* The microarray expression profile dataset GSE2503 (3,7), which is based on the Gene Expression Omnibus (GEO) Platform 96 GeneChip® Human Genome U133A 2.0 Array (Affymetrix, Inc., Santa Clara, CA, USA), was downloaded from the National Center of Biotechnology Information GEO database (http://www.ncbi.nlm.nih.gov/geo/). The dataset contained 15 samples, including six NO, four actinic keratosis and five SCC. In the present study, the SCC and NO samples were analyzed by bioinformatics methods.

*Data preprocessing and differential expression analysis.* The original array data were converted into expression measures. Background correction, quartile data normalization and probe summarization were performed using the Robust Multi-array Average (RMA) algorithm in the R affy package (https://www.biogeo.org/packages/release/bioc/html/affy.html). Paired t-test based on the Linear Models for Microarray Data package (https://www.biogeo.org/packages/release/bioc/html/limma.html) (11) in R (https://www.r-project.org/) was used to identify DEGs between SCC and NO samples. Multiple testing correction was performed with the Benjamini-Hochberg method (12) to obtain the adjusted P-value. Subsequently, log2 fold change (log2 FC) was calculated. Only those genes exhibiting |log2 FC|>1.0 and adjusted P<0.05 were regarded as DEGs.

*Gene ontology and pathway enrichment analysis.* Gene Ontology (GO; http://geneontology.org/) (13) is a tool for unification of biology that collects structured, defined and controlled vocabulary for large-scale gene annotation. The Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/) (14) knowledge database is a collection of online databases regarding genomes, enzymatic pathways and biological chemicals. The Database for Annotation, Visualization and Integrated Discovery (DAVID; https://david.ncifcrf.gov/) (15) contains a comprehensive set of functional annotation tools that have been developed for associating functional terms with lists of genes via clustering algorithms. In order to analyze the identified DEGs at the functional level, GO enrichment and KEGG pathway analysis were performed using the DAVID online tool. P<0.05 was set as the threshold value.

*Functional annotation of DEGs.* Functional annotation analysis was performed to determine whether the identified DEGs functioned as transcription factors (TFs). The Tumor Suppressor Gene Database (TSGene; http://bioinfo.mc.vanderbilt.edu/TSGene/) (16) integrates TSGs with large-scale experimental evidence to provide a comprehensive resource for the investigation of TSGs and their implication in the molecular mechanisms of cancer. The Tumor Associated Gene (TAG; http://www.binfo.ncku.edu.tw/TAG/GeneDoc.php) database (17) contains information regarding genes involved in carcinogenesis. In the present study, all the oncogenes and TSGs known to date were extracted from the TAG and TSGene databases.

*PPI network construction.* The Search Tool for the Retrieval of Interacting Genes (STRING; http://string-db.org/) database (18) is a precomputed global resource designed to evaluate PPI information. In the present study, the STRING online tool was used to analyze the PPI of DEGs, and those experimentally validated interactions with a combined score >0.4 were selected as significant.

The majority of the PPI networks in the biological network constructed were observed to obey the scale-free attribution (19). Thus, the degree of connectivity was statistically analyzed in networks using cytoscape (www.cytoscape.org) (20), to obtain the significant nodes or hub proteins (21) in the PPI networks.

*Subnetwork identification and functional enrichment analysis.* The BioNet package (https://www.bioconductor.org/packages/release/bioc/html/BioNet.html) (22) provides a comprehensive set of methods for the integrated analysis of gene expression data and biological networks. The GeneAnswers (23) package (https://www.bioconductor.org/packages/release/bioc/html/GeneAnswers.html) facilitates the understanding of the associations between a list of genes and any relevant annotations.

In the present study, the BioNet package was used to identify the subnetworks in the constructed PPI networks, with a threshold false discovery rate (FDR)<0.001. Subsequently, the GeneAnswers package based on Entrez Gene ID (http://www.ncbi.nlm.nih.gov/gene) was used to identify over-represented GO terms with an FDR<0.05, and significantly enriched pathways with P<0.05. Subsequently, data integration and network visualization were performed to obtain heat maps and association networks of the results derived from the enrichment analysis and the corresponding genes.

*Results*

**Identification of 181 DEGs.** Following data preprocessing, a total of 181 genes that were differentially expressed in SCC compared with NO were identified. These DEGs included 95 upregulated and 86 downregulated genes.

*GO and pathway enrichment analysis.* A total of 20 GO biological processes (BPs) terms enriched by the upregulated DEGs (including cell adhesion) and 14 GO BP terms enriched by the downregulated DEGs (including oxidation-reduction processes)
were identified by GO and pathway enrichment analysis. The most significant GO BPs terms are presented in Table I.

Table I also contains the most significantly enriched pathways of the upregulated and downregulated DEGs revealed by KEGG analysis. The upregulated DEGs were observed to be enriched in 18 pathways, while the downregulated DEGs were enriched in 7 pathways.

**Table I. GO functional and KEGG pathway enrichment analyses for the most significantly up- and downregulated DEGs.**

| Category         | Term            | Description                                      | Degree of connectivity | P-value     |
|------------------|-----------------|--------------------------------------------------|------------------------|-------------|
| **Upregulated DEGs** |                |                                                  |                        |             |
| BP               | GO:0007155      | Cell adhesion                                    | 18                     | 1.5x10^-5   |
| BP               | GO:0006954      | Inflammatory response                            | 12                     | 5.69x10^-5  |
| BP               | GO:0050900      | Leukocyte migration                              | 10                     | 4.37x10^-6  |
| BP               | GO:0030216      | Keratinocyte differentiation                     | 7                      | 3.74x10^-6  |
| BP               | GO:0032602      | Chemokine production                             | 5                      | 2.27x10^-3  |
| **Downregulated DEGs** |          |                                                  |                        |             |
| BP               | GO:0055114      | Oxidation-reduction                              | 10                     | 4.33x10^-4  |
| BP               | GO:0042391      | Regulation of membrane potential                 | 7                      | 1.23x10^-3  |
| BP               | GO:0006898      | Receptor-mediated endocytosis                    | 4                      | 5.09x10^-3  |
| BP               | GO:0006805      | Metabolism of xenobiotics                        | 4                      | 9.58x10^-3  |
| BP               | GO:0003215      | Cardiac right ventricle morphogenesis            | 3                      | 7.73x10^-3  |
| **Upregulated DEGs** |                |                                                  |                        |             |
| KEGG             | 5200            | Signaling pathways in cancer                     | 8                      | 9.37x10^-4  |
| KEGG             | 4810            | Regulation of actin cytoskeleton                 | 5                      | 1.14x10^-2  |
| KEGG             | 4640            | Hematopoietic cell lineage                       | 4                      | 2.36x10^-3  |
| KEGG             | 5219            | Bladder cancer                                   | 3                      | 2.41x10^-3  |
| KEGG             | 5412            | Arrhythmogenic right ventricular cardiomyopathy  | 3                      | 1.18x10^-2  |
| **Downregulated DGEs** |           |                                                  |                        |             |
| KEGG             | 480             | Metabolism of glutathione                        | 3                      | 3.14x10^-3  |
| KEGG             | 330             | Metabolism of arginine and proline               | 3                      | 3.91x10^-3  |
| KEGG             | 980             | Metabolism of xenobiotics by cytochrome P450     | 3                      | 8.41x10^-3  |
| KEGG             | 982             | Metabolism of drugs by cytochrome P450           | 3                      | 9.08x10^-3  |
| KEGG             | 310             | Lysine degradation                               | 2                      | 2.79x10^-2  |

GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; DEGs, differentially expressed genes; BP, biological process.

**Functional annotation of DEGs.** Upon analyzing the differential expression pattern of TFs and TAGs in SCC and NO samples, the present study identified a number of TFs, including hypoxia inducible factor 1, alpha subunit (HIF1A), aryl hydrocarbon receptor nuclear translocator-like 2 and paired-like homeodomain 1, which were significantly upregulated, in addition to six TFs, including nuclear receptor subfamily 3, hepatic leukemia factor and zinc finger protein 83, which were significantly downregulated in SCC. Among the upregulated DEGs, 11 genes were identified as TAGs. Of these, four were oncogenes and five were TSGs. The function of the remaining two genes identified in the analysis remains to be elucidated. Among the downregulated DEGs, five genes were identified as TSGs, including low-density lipoprotein receptor-related protein 1B and proline dehydrogenase (oxidase) 1.

**PPI network construction.** Based on the information contained in the STRING database, 104 protein pairs were identified (Fig. 1). A total of 14 nodes were selected as hub proteins (degree ≥5) in the PPI network, including matrix metalloproteinase 1 (MMP1) (also known as interstitial collagenase), keratin 6A (KRT6A) and interleukin 8 (IL8), which presented a degree of connectivity of 8 (Table II).

**Subnetwork identification and functional enrichment analysis.** As represented in Fig. 2, 43 nodes, 75 protein pairs and 9 hub proteins with a degree ≥6 were identified in the subnetwork. The hub proteins, including IL8, MMP1 and KRT6A, are summarized in Table II.

**Discussion**

SCC is characterized by a high rate of proliferation and nodal metastasis (24). Therefore, early detection or prevention of
this disease may be the most effective approach to improve patients' prognosis. In the present study, a total of 181 DEGs that were differently expressed in SCC vs. NO samples were identified via the gene expression profile contained in GSE2503. The upregulated DEGs were enriched in BPs terms in the subnetworks. These results suggested that the above genes and signaling pathways may participate in the progression of SCC.

Cell adhesion is a common event in BPs (23). Alterations in the expression levels of cell-cell adhesion molecules have been previously proposed to contribute to the progression of malignant tumors (25). In the present study, IL8, one of the hub genes with the highest degree of connectivity, was observed to be enriched in the BP of cell adhesion. IL8 is a proinflammatory cytokine that promotes chemotaxis and degranulation in neutrophils (26). Overexpression of IL8 or its receptors has been previously observed in cancer cells, endothelial cells and tumor-associated macrophages, suggesting a regulatory function for IL8 within the tumor microenvironment (26). Notably, IL8 has been previously proposed to contribute to the progression of SCC.

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Table II. Statistical analysis of the degrees of connectivity corresponding to the most significant hub genes identified in the protein-protein interaction network and subnetwork.

| Gene      | Degree of connectivity | Adjusted P-value |
|-----------|------------------------|------------------|
| MMP1      | 8                      | 0.012            |
| KRT6A     | 8                      | 0.028            |
| IL8       | 8                      | 0.005            |
| SPRR1B    | 7                      | 0.031            |
| KRT16     | 7                      | 0.001            |
| SPRR1A    | 6                      | 0.012            |
| IVL       | 6                      | 0.015            |
| S100A9    | 6                      | 0.028            |
| S100A2    | 6                      | 0.014            |

IL8, interleukin 8; IVL, involucrin; KRT6A, keratin 6A; KRT16, KRT 16; MMP1, matrix metallopeptidase 1; S100A2, S100 calcium-binding protein A2; S100A9, S100A 9; SPRR1A, small proline-rich protein 1A; SPRR1B, SPRR B.
the pathogenicity of oropharyngeal SCC by promoting cell growth (28). Therefore, \textit{IL8} and the signaling pathways associated with cell adhesion appear to be closely connected with SCC, and they may be used as potential targets for the treatment of SCC.

Using the PPI networks and subnetworks constructed in the present study, \textit{MMP1} was identified as one of the hub genes exhibiting the highest degree of connectivity. Additionally, \textit{MMP1} was observed to be enriched in signaling pathways associated with cancer. \textit{MMP1} belongs to the MMP family, and participates in a variety of BPs, including cell proliferation, differentiation, migration, apoptosis and host defense (29). \textit{MMP1} has been previously associated with cancer invasion and metastasis, since it degrades fibrillar collagens, thus enabling the tumor to traverse the extracellular space (29). Notably, \textit{MMP1} is frequently detected in various types of cancer, and may be associated with advanced stages of the disease (30). For example, \textit{MMP1} appears to be overexpressed
in skin cancer, according to the studies by Nindl et al (7). Taken together, these data support the hypothesis that MMP1 is a candidate molecular marker associated with SCC.

In the present study, the TF HIF1A was identified to be overexpressed in SCC. HIF1A functions as a TF in response to cellular hypoxia, and participates in BPs associated with tumor angiogenesis and pathophysiology of ischemic disease (31). It has been previously reported that HIF1A may be a predictor of disease progression in esophageal SCC (32). Fillies et al (33) suggested that the overexpression of HIF1A may be an indicator of favorable prognosis in SCC of the oral cavity. Accordingly, HIF1A may be an important TF associated with SCC.

The present study also revealed that ITGA6 and ITGA2 were enriched in several GO BPs terms, including regulation of cell adhesion and migration, and KEGG signaling pathways of focal adhesion and ECM-receptor interaction. The protein product of the ITGA6 gene is the integrin alpha chain alpha 6 (34). Integrins have a significant role in cell adhesion and migration (34), and different combinations of integrins act as receptors of certain ECM proteins (35). Integrins participate in a number of BPs (including cell adhesion, cell migration, blood clotting and tissue organization) and cancer processes (including cell migration, metastasis and invasion) (34). ITGA6 interacts with the ECM protein laminin, and is involved in the regulation of cell adhesion, growth
and migration (36). The role of ITGA6 in cancer development has been widely documented. Friedricks et al (37) observed that the overexpression of ITGA6 was associated with unfavorable prognosis in patients with breast cancer. In addition, previous studies have reported that ITGA6 is highly expressed in esophageal SCC tissues and participates in the tumorigenesis of esophageal SCC (38). Therefore, ITGA6 may be a potential target gene for the treatment of SCC. Notably, ITGA2 encodes a cell adhesion molecule termed α2β1 integrin receptor, which enables the interaction of the cells with the ECM and mediates the signaling events occurring within the ECM (39). Recent studies have indicated that the ITGA2 gene is associated with various types of cancer, including colorectal (40) and breast cancer (41). In addition, Beaulieu (42) reported that ITGA2 was expressed in colon cancer cell lines, and participated in the proliferation and migration of these cells. There are limited studies on the effects of ITGA2 on SCC thus far (42). However, it may be speculated that ITGA2 may be a key gene, along with ITGA6, in the progression of SCC.

In conclusion, the results of the present study provide a comprehensive bioinformatics analysis of DEGs that may be involved in SCC. The results of the current study may contribute to understand the underlying molecular mechanisms that lead to SCC. Furthermore, the DEGs identified in the present study, including ITGA2, ITGA6, ITGA2, and certain signaling pathways associated with focal adhesion and ECM-receptor interaction, may be potential targets for the diagnosis and treatment of SCC.

However, the present study has a number of limitations. Thus, the size of the sample employed in the microarray analysis was small, which may generate a high number of false positive results. Additionally, the present study lacked experimental verification. Therefore, further genetic and experimental studies with larger sample sizes are required to confirm the findings of the present study.

Acknowledgements

The authors would like to thank FengHe (ShangHai) Information Technology Co., Ltd. (Shanghai, China) for their advice and support.

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