Exploring Potential Biomarkers, Ferroptosis Mechanisms, and Therapeutic Targets Associated with Cutaneous Squamous Cell Carcinoma via Integrated Transcriptomic Analysis

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Background. Cutaneous squamous cell carcinoma (cSCC) is the leading cause of death in patients with nonmelanoma skin cancers (NMSC). However, the unclear pathogenesis of cSCC limits the application of molecular targeted therapy. Methods. Three microarray datasets (GSE2503, GSE45164, and GSE66359) were downloaded from the Gene Expression Omnibus (GEO). After identifying the differentially expressed genes (DEGs) in tumor and nontumor tissues, five kinds of analyses, namely, functional annotation, protein-protein interaction (PPI) network, hub gene selection, TF-miRNA-mRNA regulatory network analysis, and ferroptosis mechanism, were performed. Results. A total of 146 DEGs were identified with significant differences, including 113 upregulated genes and 33 downregulated genes. The enriched functions and pathways of the DEGs included microtubule-based movement, ATP binding, cell cycle, P53 signaling pathway, oocyte meiosis, and PLK1 signaling events. Nine hub genes were identified (CDK1, AURKA, RRM2, CENPE, CCNB1, KIAA0101, ZWINT, TOP2A, and ASPM). Finally, RRM2, AURKA, and SAT1 were identified as significant ferroptosis-related genes in cSCC. Conclusions. By integrated bioinformatic analysis, the hub genes identified in this study elucidated the molecular mechanism of the pathogenesis and progression of cSCC and are expected to become future biomarkers or therapeutic targets.

1. Background

Cutaneous squamous cell carcinoma (cSCC) is a type of malignant tumor that originates from the epidermis or appendage keratinocytes, with an incidence only second to basal cell carcinoma (BCC), accounting for approximately 20% of all nonmelanoma skin cancers (NMSC) [1, 2]. Recent studies have shown that somatic mutations in cSCC are much more frequent than in other squamous cell carcinomas [3], indicating a complex genetic background of cSCC, and suggesting that its pathogenesis may involve diverse genes and pathways. P53, CDKN2A, NOTCH1, and NOTCH2 are the most commonly mutated genes [4, 5]. It has been confirmed that 54–95% of cSCC contains UV radiation-induced P53 mutations [6]. Immunohistochemistry (IHC) also revealed that the expression of P53 was closely related to the histological grade and TNM stage of cSCC [7]. Meanwhile, tumors with high P53 protein...
expression are more aggressive than tumors with low P53 protein expression [7]. Furthermore, cSCC often has heterozygous deletions or point mutations in the CDKN2A gene locus, and the deletion of p16INK4a is thought to be related to the progression of actinic keratosis (AK) to cSCC [8]. NOTCH is a direct target of P53, and more than 75% of cSCCs have NOTCH1 and NOTCH2 mutations [9]. However, the driving genes are not yet clear. It is thus significant to understand the exact molecular mechanisms underlying cSCC development, progression, and recurrence.

In recent years, gene expression analysis has provided an effective global method for elucidating the pathogenesis of many cancers, including skin cancer. However, the results of a single-chip data analysis are often unconvincing. In this study, therefore, three independent microarray datasets were downloaded from the GEO to obtain their common DEGs between cSCC and normal epidermis. Subsequently, we enriched the functions of these differential genes, constructed their PPI network, and selected the most important hub genes in the network. In summary, the DEGs and hub genes identified in this study may elucidate the molecular mechanism of the pathogenesis and progression of cSCC and are expected to become future biomarkers or therapeutic targets.

2. Methods

2.1. Raw Data Collection. GEO (https://www.ncbi.nlm.nih.gov/geo) [10] is a public database with free access to microarray data. We searched for related gene expression datasets using cutaneous squamous cell carcinoma as a keyword. The inclusion criteria were set as follows: the number of genes detected by the gene chip should be greater than 20,000 to obtain common DEGs, and the tested specimens included should be from humans. In addition, the number of genes detected in the validation set should be larger than that in the training set to prevent information about the hub genes from being unavailable. Three gene expression datasets (GSE2503 [11], GSE45164 [12] and GSE66359 [13]) were downloaded from it as the training set. In addition, the gene expression datasets (GSE53462 [14] and GSE7553 [15]) were downloaded as the validation set. Table 1 shows the details of the five datasets.

2.2. Identification of DEGs. The DEGs between cSCC and noncancerous samples were screened using GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r), which is an online tool for differential analysis of the original dataset based on the LIMMA software package [16]. A logFC (fold change) ≥ 1 and p value <0.05 were considered statistically significant.

2.3. Enrichment Analyses of DEGs. Gene Ontology (GO) analysis including biological processes (BP), cellular components (CC), and molecular functions (MF), was first performed to identify the unique biological characteristics of the DEGs. Then, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was used to explore the main pathways involved in the occurrence and development of cSCC. Both enrichment results of the GO function and KEGG pathway were obtained from DAVID 6.8 (https://david.ncifcrf.gov/), which is an online functional annotation tool [17]. A p value < 0.05 was considered statistically significant.

2.4. PPI Network Construction and Module Analysis. The STRING database (https://string-db.org/; version 11.0) was used to explore the interactions of the DEGs [18]. A comprehensive score > 0.4 was selected to construct a PPI network and was visualized with Cytoscape software (version 3.7.2). Then, the most important functional modules in the PPI network were obtained through the plug-in MCODE in Cytoscape using the parameters of MCODE scores >10, degree cutoff = 2, node score cutoff = 0.2, k-score = 2, and max depth = 100. Subsequently, the important biological pathways, that this, module participates in were obtained through FunRich, which is a free software package that can perform functional enrichment analysis of genes or proteins [19].

2.5. Hub Gene Selection and Analysis. The nine genes with the highest degree of connectivity in the above modules were selected as hub genes. Their pathway analysis was performed and visualized by ClueGO (version 2.5.4) and CluePedia (version 1.5.4). A p value < 0.05 was considered statistically significant. An interaction network between the hub genes and their coexpressed genes was created using GeneMANIA (https://www.genemania.org/) [20], which is a convenient web portal for analyzing gene lists and predicting gene function. The DGlDb database (https://www.dgidb.org/) can be used to generate hypotheses about how genes can be targeted for therapy or prioritized for drug development [21]. In this study, the gene-drug interaction relationship was obtained through DGlDb 3.0 and visualized by Cytoscape. The parameters were: preset filters; FDA approved; antineoplastic; all default.

2.6. Validation of Hub Genes in Other Databases and the Human Protein Atlas. To confirm the reliability of our results, hub gene expression was verified in the GSE53462 and GSE7553 datasets by Student’s t-test. A p value <0.05 was considered statistically significant. To further validate our findings, we searched the Human Protein Atlas (https://www.proteinatlas.org/) website for the immunohistochemical staining results of nine hub genes in normal skin and tumor tissue.

2.7. TF-miRNA-mRNA Regulatory Network Analysis. To further understand the regulatory mechanism of the hub genes, TF-target interactions were obtained through the Transcriptional Regulatory Relationships Unraveled by Sentence-based Text mining (TRRUST) [22], which is a database for the prediction of transcriptional regulatory networks, which contains the target genes corresponding to TFs and the regulatory relationships between TFs. In
addition, miRNA-target interactions were obtained by Mirwalk [23], which is a publicly available database that focuses on miRNA-target interactions. To improve the accuracy, the predicted miRNAs that had been verified by experiments and other databases were screened. Finally, miRNA-target interactions and TF-target interactions were integrated to construct the TF-miRNA-mRNA regulatory network by Cytoscape.

2.8. Identification and Validation of Ferroptosis-Related DEGs in cSCC. A total of 259 ferroptosis-related genes were obtained from the Ferroptosis Database (https://www.zhounan.org/ferrdb) [24], and we intersected these genes with the DEGs of cSCC to screen ferroptosis-related genes in cSCC. To ensure the rigor and accuracy of this study, we verified the expression of these genes in GSE53462 and GSE7553. The comparison between the cSCC and control sets of data was performed by the Student’s t-test, and p value < 0.05 was considered to be statistically significant. Based on the validation results, we removed the disqualified genes and finally obtained accurate ferroptosis-related genes involved in cSCC.

3. Results

3.1. Identification of DEGs in cSCC. The flow chart of this study is shown in Figure 1. After data standardization and differential expression analysis, the DEGs of each dataset were identified, with 1860 in GSE2503, 1649 in GSE45164, and 1990 in GSE66359. The volcano and heatmaps are shown in Figure 2. As shown in Figure 3(a), a total of 146 common DEGs, including 113 upregulated genes and 33 downregulated genes, were finally identified between cSCC tissues and normal tissues.

3.2. Enrichment Analyses of DEGs. The GO analysis results showed that for BP, the DEGs were significantly enriched in microtubule-based movement, negative regulation of cell growth, and positive regulation of apoptotic process (Figure 4(a)). Regarding CC, the DEGs were mainly concentrated in the nucleoplasm, extracellular exosome, and cytoplasm (Figure 4(b)). In terms of the MF, the DEGs mainly focused on ATP binding, ATPase activity, and microtubule motor activity (Figure 4(c)). KEGG pathway analysis showed that the DEGs were mainly concentrated

| Dataset   | Platform                        | No. of samples (cSCC vs. HC) |
|-----------|---------------------------------|------------------------------|
| GSE2503   | GPL96[HG-U133A] Affymetrix Human Genome U133A Array | 5, 6                         |
| GSE45164  | GPL571 [HG-U133A_2] Affymetrix Human Genome U133A 2.0 Array | 10, 3                        |
| GSE66359  | GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array | 8, 5                         |
| GSE53462  | GPL10558 Illumina Human HT-12 V4.0 Expression BeadChip | 5, 5                         |
| GSE7553   | GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array | 11, 4                        |
in the cell cycle, p53 signaling pathway, oocyte meiosis, and progesterone-mediated oocyte maturation (Figure 4(d)).

3.3 PPI Network Construction and Module Analysis. The PPI network contained 109 nodes and 617 interaction pairs (Figure 3(b)). The most significant module (score = 28.857) was aggregated from the PPI network (Figure 3(c)), including 29 nodes and 404 interaction pairs. Then, when it was put into FunRich for further functional analysis, all genes in this module were upregulated and the enriched biological pathway for the module showed that the DEGs were mainly enriched in PLK1 signaling events and polo-like kinase signaling events in the cell cycle (Figure 5).

3.4. Hub Gene Selection and Analysis. A total of nine genes with degrees ≥ 30 were identified as hub genes (details shown in Table 2). ClueGO revealed that the most involved pathways were the P53 signaling pathway, TP53 regulates transcription of cell cycle genes and TP53 regulates transcription of genes involved in G2 cell cycle arrest (Figure 6(a)). The interaction network between hub genes and their coexpressed genes is shown in Figure 6(b). These nine genes showed a complex DEG PPI network with coexpression of 72.69%, prediction of 22.58%, colocalization of 1.86%, physical interactions of 1.73%, a pathway of 1.12%, and genetic interactions of 0.02%. Based on the DGIdb database, we obtained 30 drug-gene interaction pairs, including four upregulated genes (AURKA, RRM2, CENPE, and TOP2A) and 29 drugs (Figure 6(c)).
3.5. Validation of Hub Genes in Other Databases and the Human Protein Atlas. Finally, the results of the independence testing analysis suggested that all hub genes were significantly increased in cSCC tumor tissue compared to normal skin tissue (Figure 7). By searching the Human Protein Atlas, we obtained immunohistochemically stained tissue images of six out of the nine hub genes in normal skin tissue and tumor tissue. The results indicated that the six hub genes were significantly differentially expressed between normal and tumor tissues (Figure 8).

3.6. TF-miRNA-mRNA Regulatory Network Analysis. Based on the TRRUST and Mirwalk databases, we found that seven TFs and 33 miRNAs may regulate the expression of these genes. Twenty-nine miRNA-mRNA pairs and 18 TF-mRNA pairs were integrated to construct a TF-miRNA-mRNA regulatory network (Figure 9).

3.7. Identification and Validation of Ferroptosis-Related DEGs in cSCC. DEGs in cSCC intersected with 259 ferroptosis-related genes and five genes, were screened. All five genes
were upregulated DEGs, including MAP3K5, SLC2A3, RRM2, AURKA, and SAT1. Subsequently, the expression of these genes was verified in GSE53462 and GSE7553. Especially, RRM2, AURKA, and SAT1 were determined to be significant ferroptosis-related genes in cSCC (Figure 10).

4. Discussion
cSCC shows the potential for recurrence and metastasis, making it the main cause of death in NMSC [25]. Previous reports have confirmed that mutations in P53, CDKN2A, RAS, NOTCH1, and NOTCH2 are closely related
to cSCC [6–9, 26]; however, the underlying molecular mechanisms behind the aggressive progression of cSCC subpopulations remain to be unveiled, which might account for the high mortality rate of cSCC in NMSC [27]. In such a context, both potential and efficient markers for diagnosis and treatment are urgently needed.

In the current study, through analysis of a large sample of cSCC and corresponding normal tissues, 146 DEGs were identified, including 113 upregulated genes and 33
TP53 Regulates Transcription of Genes Involved in G2 cell Cycle Arrest

Figure 6: KEGG pathway, coexpression network, and drug-gene interaction analysis of the hub genes. (a) The most significant pathway and related genes. The results show that these hub genes are mainly involved in the P53 signaling pathway, TP53 regulates transcription of cell cycle genes and TP53 regulates transcription of genes involved in G2 cell cycle arrest. (b) Hub genes and their coexpression genes were analyzed using GeneMANIA. (c) Drug-gene interaction diagram, the yellow circle indicates the differentially expressed gene and the blank square indicates the drug.

Figure 7: Hub genes expression in the GSE53462 and GSE7553 datasets. SCC stands for cutaneous squamous cell carcinoma tumor tissue and normal represented corresponding normal tissue. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.
downregulated genes. The upregulated DEGs were mainly enriched in the cell cycle, the P53 signaling pathway, and oocyte meiosis. According to previous studies, disorders of the cell cycle process play an important role in the development of tumors [28] and the P53 signaling pathway is closely related to the progression of cSCC [29]. In addition, the biological pathway for the most significant module showed that the DEGs were mainly enriched in PLK1 signaling events and polo-like kinase signaling events in the cell cycle. Previous studies have confirmed that by inhibiting cSCC keratinocyte PLK1 signaling in vitro, the cancer cells die first, emphasizing the indispensability of the PLK1 signaling pathway in the development of cSCC [30]. In this regard, our results were consistent with all of these theories.
A total of nine genes were identified as hub genes with degrees ≥ 30, namely, CDK1, AURKA, RRM2, CCNB1, KIAA0101, ZWINT, TOP2A, and ASPM. These genes were verified in the GSE53462 dataset. Among these genes, there are four druggable genes, including AURKA, RRM2, CENPE, and TOP2A. AURKA is one of three members of the highly conserved mitogen kinase family and it plays an essential role in regulating cell division, which is necessary for timely access to mitosis, centrosome maturation, and the assembly of bipolar spindles [31]. Previous studies have found that the expression levels of AURKA in squamous cell carcinoma and adenocarcinoma are significantly different [32]. In addition, Torchia et al. established a mouse model of AURKA overexpression, suggesting that AURKA has a clear role in the malignant progression of cSCC [33]. The overexpression of RRM2 significantly enhances the invasiveness of the cells and plays a key role in determining the degree of tumor malignancy [34, 35]. However, the role of RRM2 in the development of cSCC is unclear. The protein encoded by CENPE is a forward-directed kinesin belonging to the kinesin-7 subfamily, which has a critical role in mitosis [36]. Increasing evidence has shown that CENPE may be a useful drug target for several tumors without targeted therapy [37]. Recent studies have confirmed that CENPE is highly expressed in lung adenocarcinoma tissues and promotes lung adenocarcinoma cell proliferation [38]. Meanwhile, TOP2A encodes DNA topoisomerase and is involved in important cellular functions such as DNA replication, transcription, recombination, and mitosis. It is a sign of cell proliferation in normal and tumor tissues. High expression of TOP2A occurs most often in breast cancer, where it is strongly correlated with the patients’ disease-free survival and total survival, and thus it is regarded as a valuable prognostic biomarker for breast cancer [39–41]. In addition, high expression of TOP2A was related to the cell cycle, and targeting TOP2A is also considered to be an important method for treating human cancer [42]. In summary, these genes play significant roles in cSCC.

Studies have shown that CDK1 is overexpressed in breast cancer and liver cancer, causing tumor cell proliferation and development [43, 44]. In addition, CDK1 is a marker of the clinical prognosis of colon cancer [45]. CCNB1 is a member of the cyclin family. CCNB1 and CDC2 combine to form an M-phase promoting factor (MPF), which promotes cells from the G2 to the M phase [46]. Overexpression of CCNB1 damages the cell’s G2/M detection point and causes an increase in MPF. DNA damage cannot be detected, and mitosis still occurs, causing the proteasome to break down and recognize the MPFs only during the middle stage of division, resulting in the continuous proliferation and development of tumor cells [47]. Therefore, CCNB1 dysregulation allows cancer cells to proliferate and differentiate, and the new cancer
cells promote the expression of CCNB1 to increase further [48]. Moreover, previous studies have reported that KIAA0101 overexpression in mammalian cells can prevent UV-induced apoptosis, suggesting it has a protective effect in regulating DNA repair, cell proliferation, apoptosis, and cell cycle progression [49]. KIAA0101 is closely related to the invasion and metastasis of cancer cells [50]. However, no one has studied its role in cSCC. ZWINT is another centromere complex component required for mitotic spindle checkpoints, and it is involved in centromere function and cell growth [51]. Recently, ZWINT overexpression has been reported in ovarian cancer and hepatocellular carcinoma, and it is intimately linked to tumor progression and a poor prognosis [52, 53]. In addition, ASPM, as a cell cycle progression gene, is a key factor in mitotic spindle regulation [54]. Previous studies have shown that ASPM is highly expressed in ovarian, pancreatic, and prostate cancers and it is significantly associated with a poor prognosis [55–57]. According to recent findings, knockout of TPX2 in prostate cancer can induce cell cycle quiescence and apoptosis, reduce the ability of cells to invade, and inhibit cell proliferation [58].

Previous studies have mainly focused on the common DEGs between AK and cSCC tissues to confirm that AK is a precursor lesion of cSCC [59]. In addition, a group of epithelial-mesenchymal transition (EMT) and autophagy-related genes involved in cSCC was discovered, and the results showed that inhibition of autophagy and activation of EMT played important roles in the development of cSCC [59]. In this study, we identified and validated ferroptosis-related DEGs in cSCC to reveal the potential mechanism, which may provide a new direction for exploration. Ferroptosis is a novel iron-dependent type of programmed cell death different from apoptosis, necrosis, and autophagy [60]. Previous studies have reported that ferroptosis in squamous cell carcinoma is closely related to cancer progression [61–64]. However, the mechanism and role of ferroptosis in cSCC have rarely been reported in the literature. Among the ferroptosis-related hub genes, RRM2 was downregulated in cells treated with the ferroptosis inducer erastin, suggesting that ferroptosis may be inhibited in a GSH-dependent manner [65]. Similarly, inhibition of AURKA or reconstitution of miR-4715-3p inhibited GPX4 and induced cell death, suggesting a link between AURKA and ferroptosis [66]. Additionally, P53-mediated activation of SAT1 contributes to ferroptotic cell death in the presence of ROS stress. Knockdown of SAT1 partially rescued ROS-induced ferroptosis [67]. These studies suggest that the ferroptosis-related genes we identified may play an important role in the development of cSCC. However, a more in-depth study of the mechanism of ferroptosis in cSCC is urgently needed.

We would like to acknowledge the limitations of this research. First, this was a retrospective study. All of the data in this study come from publicly available databases. Second, further in vivo and in vitro experiments are required to confirm these results. Third, we must further study the underlying mechanism of signaling pathways in cSCC.

5. Conclusion

In summary, the purpose of this study was to explore the underlying molecular mechanism of cSCC. A total of nine hub genes were identified, including CDK1, AURKA, RRM2, CCNB1, KIAA0101, ZWINT, TOP2A, and ASPM. Among these genes, there are four druggable genes, including AURKA, RRM2, CCNB1, and TOP2A. In addition, RRM2, AURKA, and SAT1 were identified as significant ferroptosis-related genes in cSCC. The above findings provide potential research directions and drug targets for cSCC research. In conclusion, AURKA and RRM2 should be the focus of future research. Further mechanistic and drug development research on cSCC is necessary.

Abbreviations

cSCC: Cutaneous squamous cell carcinoma
IHC: Immunohistochemistry
AK: Actinic keratosis
GEO: Gene expression omnibus
DEGs: Differentially expressed genes
PPI: Protein-protein interaction network
BCC: Basal cell carcinoma
NMSC: Nonmelanoma skin cancers
FC: Fold change
GO: Gene ontology
BP: Biological processes
MF: Molecular functions
CC: Cell composition
MPF: M-phase promoting factor
EMT: Epithelial-mesenchymal transition.

Data Availability

In this study, mRNA microarray datasets were downloaded from the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo).

Conflicts of Interest

The authors declare no conflicts of interest.

Authors’ Contributions

This work was carried out in collaboration with all authors. JJ and YDJ defined research topics and directions. SWX, HB, ZQY, and HW analyzed the data, plotted the results, and explained their meaning. AL helped to collect data and references. GY helped to modify language expressions. All authors read and approved the final manuscript. Wening Su, Biao Huang, and Qingyi Zhang contributed equally to this work.

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