Identification of Down-Expressed CRNN Associated with Cancer Progression and Poor Prognosis in Laryngeal Squamous Cell Carcinoma

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Abstract

Background: The prevalence of laryngeal squamous cell carcinoma (LSCC) is increasing, and it poses a significant threat to human health; therefore, identifying specific targets for LSCC remains crucial. Methods: Bioinformatics analysis was used to compare the different expression genes expressed in LSCC. Immunohistochemical assay and western blotting were used to analysis protein expression. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 5-ethyl-2’-deoxyuridine (EdU) assay. Flow cytometry was used to measure the cell cycle. Cell migration was measured by wound healing assay and transwell assay. Results: Our analysis revealed 36 upregulated and 65 downregulated differentially expressed genes (DEGs) when comparing LSCC tumors to adjacent tissues, with cornulin (CRNN) identified as a key hub gene connecting these DEGs. We observed a consistent downregulation of CRNN expression in LSCC cell lines and tissues and was associated with poor patient survival and the tumor microenvironment. CRNN overexpression was found to significantly inhibit cell growth, cell cycle progression, migration and invasion, while CRNN knockdown had the opposite effects. Additionally, in vivo experiments demonstrated that CRNN overexpression suppressed tumor growth in nude mice. Conclusions: CRNN functions as a potential tumor suppressor and regulates important aspects of LSCC, providing valuable insights into the role of CRNN in LSCC pathogenesis and potential for targeted therapeutic interventions.

Keywords: CRNN; laryngeal squamous cell carcinoma; poor prognosis; cancer progression

1. Introduction

Laryngeal squamous cell carcinoma (LSCC) originates from the epithelium of the laryngeal mucosa and ranks among the most prevalent malignant tumors in regard to head and neck malignancies. Annually, LSCC accounts for approximately 2.4% of all malignant tumor cases worldwide, with an estimated 95,000 fatalities attributed to laryngeal cancer in 2018 [1]. LSCC typically has an insidious onset with malignant symptoms, and roughly 60% of patients are diagnosed at an advanced stage (clinical stage III and IV) [2]. The high infiltration and early cervical lymph node metastasis make LSCC treatment challenging and greatly impact patient survival. Although surgical intervention remains the primary treatment modality for LSCC [3], patient survival rate has been declining in recent years. Over the past four decades, its 5-year survival rate has decreased from an initial 66% to 63% [4], and statistical data from 2012 indicated that its incidence and mortality rates continue to rise annually [4].

Currently, early diagnosis and treatment have led to improved clinical outcomes for LSCC using conventional treatment modalities such as surgery, radiotherapy, chemotherapy and others. However, for advanced LSCC cases, total laryngeal surgical resection remains the primary option [5–7]. In general, most patients lack distinct symptoms at the time of diagnosis, and those who exhibit noticeable symptoms have already reached advanced stages (III or IV), leading to challenges and uncertainties surrounding their treatment [8,9]. Additionally, research has suggested potential links between LSCC pathogenesis and various molecules, such as Shp2 ((Src homology-2 domain-containing protein tyrosine phosphatase-2)), HPV16, various forms of long non-coding RNAs, and microRNAs and their involvement in the modulation of signaling pathways such as Ras/Raf/Mek/Erk and PI3K/Akt [10]. This underscores the complex nature of LSCC’s pathogenesis, with the molecular mechanisms underlying its development and progression still requiring further elucidation. Therefore, there is a pressing need for additional research to uncover the molecular mechanisms of LSCC and identify new markers for early clinical screening and treatment.

The application of tumor molecular markers in the diagnosis, prognosis and treatment selection strategies plays a pivotal role in reducing cancer-related mortality [11–13]. In recent years, the emergence of microarrays and high-throughput technologies has significantly enhanced...
our comprehension of fundamental cancer biology and allowed us to identify novel biomarkers associated with tumor behavior and patient prognosis [14–16]. To identify molecular markers for LSCC, we conducted an analysis of Gene Expression Omnibus (GEO) microarray data and identified cornulin (CRNN) as one of the significantly down-regulated genes in LSCC tissues. CRNN was also previously reported to be down-regulated genes in esophageal cancer [17]. Subsequent high-throughput analyses of esophageal cancer confirmed the down-regulation of CRNN at the messenger RNA level [18]. Recent studies have also demonstrated that the loss of CRNN expression is linked to advanced tumor stage and poor survival in patients with esophageal squamous cell carcinoma [19]. Furthermore, down-regulation of CRNN expression in MSI (microsatellite instability)-positive oral squamous cell carcinoma has been associated with poor prognosis [20]. Nevertheless, reports on the clinical significance of CRNN expression remain limited, and the relationship between CRNN expression and the prognosis of LSCC patients has not been previously documented.

In this study, we screened for differentially expressed genes (DEGs) using GEO chip data, performed gene GO-KEGG (Gene Ontology-Kyoto Encyclopedia of Genes and Genomes) enrichment analysis, evaluated CRNN mRNA and protein expression levels in collected LSCC tumor tissues and cancer tissues, and examined the correlation between CRNN expression and patient survival prognosis. Our findings confirm the downregulation of CRNN expression as an indicator of poor prognosis in LSCC, providing valuable insights for potential targeted therapeutic interventions.

2. Methods and Materials

2.1 PCA, Volcano and Heatmap Analysis

The GEO dataset (GSE143224) (https://www.ncbi.nlm.nih.gov/) was used for differential gene expression analysis, and after batch correction, we conducted Principal Component Analysis (PCA) using the methodology outlined in a prior study [21] to identify patterns and relationships among the DEGs in LSCC and normal tissues. For volcano and heatmap analysis, genes meeting the criteria of |logFC| > 1 and p < 0.05 in both groups were plotted. Volcano plots were generated to visualize the significance and fold-change differences of DEGs between LSCC and normal tissues, with color-coded representations of significantly upregulated and downregulated genes. Heatmap analysis illustrated gene expression patterns, offering an overview of their clustering and levels across the samples.

2.2 GO-KEGG Enrichment Analysis and PPI Analysis

After screening the DEGs between normal and tumor specimens from volcano and heatmap analysis, the data was further analyzed using the online platform Metascape (Metascape Foundation, San Diego, USA) and R language (R Foundation for Statistical Computing, Vienna, Austria), and for protein-protein interaction (PPI) analysis, the proteins were analyzed using STRING.

2.3 Immunohistochemistry (IHC)

For this experiment, we obtained 97 pairs of HNSC (head and neck squamous cell carcinoma) tissues from patients based on pre-determined inclusion and exclusion criteria to ensure the relevance and reliability of the experimental samples. Patients diagnosed with HNSC were included and excluded those who had undergone chemotherapy, radiotherapy or any other treatment, as well as individuals with a history of recurrence. All tissue samples were embedded, sliced, incubated and assessed via microscopy [22]. In the IHC experiments, specific CRNN antibodies were used to validate its protein expression in LSCC tissues. The tissue sections were subjected to antibody incubation, and staining intensities were evaluated. Subsequently, we quantitatively analyzed the images to determine the relative expression levels of CRNN in both tumor and normal tissues to obtain insights into its protein-level regulation. The detailed information regarding the tissue specimens are shown in Table 1.

Table 1. Relationship between CRNN expression and clinical pathological parameters in LSCC.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>CRNN High expression</th>
<th>CRNN Low expression</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>15</td>
<td>36</td>
<td>0.912</td>
</tr>
<tr>
<td>≥60</td>
<td>14</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Drinking</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>11</td>
<td>28</td>
<td>0.765</td>
</tr>
<tr>
<td>No</td>
<td>18</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Smoking (yxp)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;400</td>
<td>17</td>
<td>43</td>
<td>0.668</td>
</tr>
<tr>
<td>≥400</td>
<td>12</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>T-category</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 + T2</td>
<td>8</td>
<td>42</td>
<td>0.002*</td>
</tr>
<tr>
<td>T3 + T4</td>
<td>21</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>9</td>
<td>42</td>
<td>0.006*</td>
</tr>
<tr>
<td>N1 or N2</td>
<td>20</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Well</td>
<td>17</td>
<td>32</td>
<td>0.297</td>
</tr>
<tr>
<td>Moderate-Poor</td>
<td>12</td>
<td>36</td>
<td></td>
</tr>
</tbody>
</table>

*p < 0.05. CRNN, cornulin; LSCC, laryngeal squamous cell carcinoma.
2.4 Real-Time qPCR and Western Blotting Assay

Total RNA was extracted as previously described, and then reverse transcription was performed for analyzing mRNA expression. The primers of CRNN (forward primer: 5′-AAACTGTAAGCCACGGAGGG-3′; reverse primer: 5′-GTCCTCCCGTACTGCTCT-3′) and GAPDH (forward primer: 5′-GTCTCCTCTGACTTCAACAGCG-3′; reverse primer: 5′-ACCACCTGTTGCTGTAAGCCAA-3′) were used in this experiment. Western blotting involved protein isolation using an EBC buffer (50 mM Tris pH 7.5, 120 mM NaCl, 0.5% NP-40), followed by electrophoresis, membrane transfer, blocking, incubation, and visualization using an ECL (Enhanced chemiluminescence) reagent.

2.5 Kaplan-Meier Plotter Analysis and Correlation Analysis

Kaplan-Meier curve analysis was conducted using an online platform (https://kmplot.com/analysis/). The correlation between CRNN and T-cell enrichment was analyzed following previous protocols [23].

2.6 Cells, Tissues, Reagents, and Animal Assay

Human laryngeal epithelial cells (HLEs) and LSCC cell lines (AMC-HN-8, Hep2, TU177, and TU686) were obtained from Procell (Wuhan, China) and cultured in DMEM supplemented with 10% FBS and Penicillin-Streptomycin. Routine screening for Mycoplasma infection was conducted using polymerase chain reaction (PCR) and
fluorescence microscopy. The authenticity of human cell lines was confirmed by analyzing their short tandem repeat (STR) profiles. Lentiviral RNA/siRNA vectors were prepared following established procedures. For cell transfection, the PEI reagent was used to introduce the specified plasmids. HNSC tissues were collected from Hangzhou Hospital of Zhejiang Medical and Health Group and manipulated under the investigation of the Hangzhou Hospital of Zhejiang Medical and Health Group ethics committee. Antibodies, including CRNN (1:1000, Santa Cruz, sc-514602, Dallas, TX, USA), E-cadherin (1:1000, Santa Cruz, sc-8426, USA), N-cadherin (1:1000, Santa Cruz, sc-8424, USA), Ki67 (1:1000, Santa Cruz, sc-23900, USA), and GAPDH (1:1000, Santa Cruz, sc-47724, USA), were acquired from Santa Cruz. For the subcutaneous tumorigenesis assay involving nude mice, 4-week-old mice were injected with the designated cell lines. After 5 weeks, tumor size and weight measurements were conducted. Ethical approval was obtained from the Ethics Committee of Hangzhou Hospital of Zhejiang Medical and Health Group.

2.7 MTT, Edu, and Flow Cytometry Analysis

We seeded 5000 HNSC cell lines into a 96-well plate and assessed cell growth rates at 24 hours, 48 hours and 72 hours using the MTT reagent. Additionally, cell lines were seeded and stained with Edu reagent after 24 hours, followed by microscopic assessment. For cell cycle analysis via flow cytometry, the cells were collected, fixed with 70% alcohol, stained with PI (propidium iodide), and analyzed using flow cytometry.

2.8 Wound-Healing Assay and Transwell Analysis

HSNC cell lines were seeded into 6-well plates and subjected to a wound assay using 10 µL tips to create wounds. Images were captured at the initial time point (0 h) and after 2 days (48 h) to compare the wound area. For the transwell assay, cells were seeded into the upper chamber with Matrigel treatment, and after 24 hours, the count of migrated cells was analyzed.

2.9 Statistical Analysis

Statistical analyses were conducted using the student’s t-test for continuous variables and the chi-square test for
3. Results

3.1 Analysis of DEGs in LSCC

Initially, bioinformatics methods were employed to analyze DEGs in LSCC using data from GSE143224. As illustrated in Fig. 1A,B, significant differences in gene expression were observed between the tumor and non-malignant groups. Subsequently, we utilized the R software “limma” package for further screening and analysis.
of the DEGs and the results are shown in the volcano and heatmap plots of Fig. 1C,D. Among these DEGs, there were 36 upregulated genes and 65 downregulated genes, and they were primarily associated with processes such as epidermis development, cell differentiation, cell adhesion and glutathione metabolism (Fig. 2A,B). The bubble plot also highlighted the significant roles of DEGs in regulating epidermis development and glutathione metabolism (Fig. 2C). Moreover, among the DEGs, CRNN was identified as a critical interaction gene (Fig. 2D).

### 3.2 CRNN Expression is Associated with Poor Prognosis in LSCC

Next, we further analyzed the expression of CRNN in HNSC (LSCC is a subtype of HNSC) (Fig. 3A,B), and the results indicated downregulation of CRNN expression in tumor samples compared to normal samples in HNSC. Moreover, CRNN expression was also decreased in LSCC compared with the normal specimens from GSE143224 (Fig. 3C). Additionally, we validated the downregulation of CRNN mRNA in LSCC patients’ tissues (Table 1) in com-
Fig. 5. CRNN inhibits cell growth in LSCC cells. (A) Western blotting showing the expression of CRNN in LSCC cell lines. (B) Expression of CRNN in TU177 and AMC-HN-8 cells after transfection with the indicated plasmids, as measured by western blotting. (C) Cell growth of CRNN-OE or knockdown TU177 and AMC-HN-8 cells assessed by MTT assay. (D) Edu assay was conducted to determine cell viability in AMC-HN-8 and TU177 cells with the indicated plasmids transduction. (E) Flow cytometry assay was performed to assess cell cycle in CRNN-OE or KD TU177 and AMC-HN-8 cells. Data represent three independent experiments. *p < 0.01. **p < 0.001. ##p < 0.05. ###p < 0.01. ####p < 0.001. * indicates CRNN group compared to NC group. # indicates shCRNN-1# group compared to Scramble group.

Comparison to matched adjacent normal tissues (Fig. 3D). The expression of CRNN protein was found to be consistently lower in the tumor group compared to the normal group, as indicated by both IHC (Fig. 3E) and western blotting (Fig. 3F). Survival analysis using Kaplan-Meier plots revealed that patients with high CRNN expression had longer...
survival rates compared to those in the low CRNN expression group (Fig. 3G,H). These findings suggest a positive correlation between CRNN downregulation and poor prognosis in LSCC.

3.3 The Correlation between CRNN and TME

We further investigated the relationship between CRNN expression and the tumor microenvironment. As shown in Fig. 4A, a significantly positive correlation was observed between CRNN expression and CD8+ T cells, while CRNN expression displayed a negative association with tumor purity and macrophage infiltration. Additionally, CRNN exhibited positive correlations with mast cells, B cells and neutrophils, and conversely, it displayed negative correlations with NK cells and macrophages (Fig. 4B). Furthermore, CRNN expression was positively correlated with T-cell enrichment (Fig. 4C). These findings collectively indicate that CRNN is closely associated with the immune profile of tumor cells. However, the underlying mechanisms through which CRNN regulates immune responses in LSCC warrant further investigation.

3.4 CRNN Inhibited Laryngeal Carcinoma Cell Lines Viability

To confirm the reduced expression of CRNN in LSCC, we compared CRNN expression between LSCC cell lines and human laryngeal epithelial cells. As shown in Fig. 5A, the expression of CRNN was significantly lower in AMC-HN-8, Hep2, TU177 and TU686 compared to HLEs. To investigate the functional role of CRNN in LSCC, we utilized CRNN overexpression and shCRNN plasmids to create CRNN-OE and KD cell lines in AMC-HN-8 and TU177, respectively (Fig. 5B). As illustrated in Fig. 5C, CRNN overexpression led to a decrease in cell growth in AMC-HN-8 and TU177 cell lines, while CRNN knockdown resulted in increased cell growth in these cell lines. Cell proliferation, assessed using Edu staining in the same cell lines, yielded similar changes (Fig. 5D), further confirming the role of CRNN in cell proliferation regulation. Moreover, CRNN overexpression inhibited cell cycle progression, while CRNN deficiency increased the proportion of cells in the G2 phase compared to control group cell lines (Fig. 5E). These findings collectively indicate that CRNN negatively regulates cell viability and cell cycle progression in LSCC cells.
Fig. 7. **CRNN overexpression suppresses tumor growth in nude mice.** (A) Nude mice were divided into four groups: NC, CRNN, scramble, and shCRNN-1#. Images of tumors in each group, as well as tumor volume and weight, are presented in (A). (B) IHC analysis of tumors with matched staining intensity from nude mice was conducted, as shown in Fig. 3E. **p < 0.01, ***p < 0.001. # represents the p < 0.05. ###p < 0.001. * represents the CRNN group compared to the NC group. # represents the shCRNN-1# group compared to the Scramble group.
3.5 CRNN Attenuated the Migration and Invasion of LSCC Cells

Subsequently, we assessed the impact of CRNN on cell migration and invasion using the same cell lines and found that CRNN overexpression led to reduced cell migration in both AMC-HN-8 and TU177 cell lines, while CRNN knockdown had the opposite effect, promoting cell migration in these cell lines (Fig. 6A). Additionally, CRNN overexpression inhibited cell invasion, whereas CRNN depletion increased cell invasion in both AMC-HN-8 and TU177 cell lines (Fig. 6B). Furthermore, CRNN overexpression increased E-cadherin levels and decreased N-cadherin levels in AMC-HN-8 and TU177 cell lines, whereas CRNN deficiency had the opposite effects (Fig. 6C), indicating that CRNN negatively regulates cell migration and invasion in LSCC cells.

3.6 Up-Regulation of CRNN Inhibits Tumor Growth in Vivo

Similarly, we introduced AMC-HN-8 cells transduced with CRNN plasmids into subcutaneous injections in nude mice. As shown in Fig. 7A, CRNN overexpression resulted in reduced tumor growth in nude mice, whereas knockdown of CRNN induced tumor growth. Furthermore, in tumors from nude mice with CRNN overexpression, we observed a decrease in the expression of Ki67 and N-cadherin, while E-cadherin expression increased (Fig. 7B). Conversely, CRNN deficiency led to an increase in Ki67 and N-cadherin expression, accompanied by reduced E-cadherin expression in tumors. Collectively, our data strongly suggest that CRNN negatively regulates tumor growth in vivo.

4. Discussion

LSCC originates from the mucosal epithelium of the larynx, representing one of the common malignant tumors in the head and neck region. It is characterized by high infiltration and easy metastasis, significantly impacting patient quality of life and survival rates. Currently, it is mainly treated by surgery, and the new morbidity and mortality rates continue to increase year by year. In this study, 36 upregulated and 65 downregulated DEGs were identified when comparing LSCC tumors with adjacent tissues, and CRNN was identified as a pivotal hub gene connecting various dysregulated genes in LSCC. We also observed CRNN downregulation in LSCC, and it was associated with poor patient survival. Functionally, CRNN negatively influences cell viability, cell cycle progression, cell migration and cell invasion, and in vivo experiments demonstrated that CRNN overexpression effectively suppresses tumor growth in nude mice.

Numerous prior investigations have examined the role of CRNN in various cancer types, such as esophageal squamous cell carcinoma (ESCC), oral squamous cell carcinoma (OSCC), and tongue squamous cell carcinoma (TSCC), consistently identifying it as a significant marker associated with tumor progression. Our findings align with these prior studies, emphasizing a parallel downregulation of CRNN in LSCC and reinforcing its association with unfavorable prognosis. Our bioinformatics analysis further suggests that CRNN is an important gene with potential key functions in LSCC. Previous research has demonstrated the downregulation of collagen in multiple epithelial squamous cell carcinomas, including those affecting the head and neck, esophagus, cervix and oral regions, and was linked to clinical tumor progression and poor prognosis. In addition, CRNN was downregulated in OSCC, showed high frequency of allelic imbalances at 1q21.3, and was identified as a marker for prognosis [24]. Thus, it has emerged as an important prognostic indicator of poor survival, with its deletion in ESCC being associated with advanced tumor progression. Additionally, its low expression in TSCC has been correlated with pathology grading and metastasis [25]. Saira Saleem et al. [19] have also reported reduced CRNN expression in the development of TSCC, and this downregulation has been linked to the grading of pathology and metastasis. Furthermore, CRNN has been reported to be downregulated in cutaneous T-cell lymphomas [26]. In our present study on LSCC, we have observed a similar downregulation of CRNN, consistent with its role as a potential tumor suppressor influencing both the development and progression of the disease.

Studies have shown that in psoriasis and ESCC, dysregulation of collagen was associated with cell cycle events such as the G1/S transition [20]. In our present study, further investigations revealed CRNN’s involvement in cell cycle regulation, influencing cells to remain in the G1 phase upon overexpression and promoting transition to the G2 phase upon deletion. Chen et al. [27] reported that CRNN functions as a tumor suppressor in ESCC, and consistently, we also speculated that CRNN functions as a tumor reducer in LSCC. While the precise mechanism of CRNN in LSCC remains unclear and requires further exploration, its critical role in cell proliferation, metastasis and tumor growth is evident. Similar to the tumor suppressor role of CRNN in ESCC, our data indicates a negative association between CRNN and E-cadherin, a biomarker for EMT, as well as Ki67, a marker of cell proliferation [27,28]. Our data showed that the expression of E-cadherin and Ki67 was negatively associated with CRNN in LSCC. However, the intricate molecular regulatory mechanisms underlying CRNN in LSCC warrant thorough investigation in future studies.

5. Conclusions

In summary, CRNN was found to be a downregulated gene in LSCC and exerted negative regulation on cancer cell growth, motility and tumorigenesis, thereby suggesting it as a tumor suppressor in LSCC.
Abbreviations

DEGs, differentially expressed genes; LSCC, laryngeal squamous cell carcinoma; CRNN, cornulin; PPI, protein-protein interaction; DMEM, Dulbecco’s modified Eagle’s medium; TME, tumor microenvironment.

Availability of Data and Materials

The datasets used during the current study are available from the corresponding author on reasonable request.

Author Contributions

FH designed the research study. XW performed the research. YB provided help, advice on the experiments and analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All procedures performed in studies involving human participants were in accordance with the standards upheld by the Ethics Committee of Hangzhou Hospital of Zhejiang Medical and Health Group and with those of the 1964 Helsinki Declaration and its later amendments for ethical research involving human subjects (Ethic approval number: 2017-012). All animal experiments were approved by the Ethics Committee of Hangzhou Hospital of Zhejiang Medical and Health Group for the use of animals (Ethic approval number: 2021-043) and conducted in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines. Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

Acknowledgment

Not applicable.

Funding

This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest.

References


