Loss of *CRNN* expression is associated with advanced tumor stage and poor survival in patients with esophageal squamous cell carcinoma

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Objective: *CRNN* gene expression is downregulated in esophageal squamous cell carcinoma (ESCC), although its clinical implications in esophageal cancer remain unclear.

Methods: We performed integrated microarray analysis and identified the *CRNN* gene as 1 of the major downregulated genes in ESCC. *CRNN* downregulation was validated at the nucleic acid level in 16 ESCC cases using complementary DNA microarray and at the protein level by immunohistochemical stains in an additional 220 ESCC cases. The clinicopathologic relevance and prognostic significance of *CRNN* expression in ESCC were explored.

Results: Downregulated *CRNN* expression was noted at the messenger RNA and protein levels. Immunohistochemical staining revealed negative and positive *CRNN* expression in 171 (77.7%) and 49 (22.3%) patients with ESCC, respectively. Patients with negative *CRNN* protein expression had an advanced tumor invasion depth ($P = .002$), advanced nodal involvement ($P = .014$), and longer tumor length ($P = .037$). Patients with negative *CRNN* expression (median survival, 14.0 months; 5-year survival rate, 20.5%) had poorer overall survival than those with positive expression (30.0 months and 40.3%, respectively; $P = .006$). On multivariate analysis, negative *CRNN* expression, nodal involvement, and distant metastasis remained significant prognostic factors for poor overall survival (negative vs positive *CRNN*, hazard ratio, 1.464; $P = .047$).

Conclusions: Our analysis has highlighted the clinical implications of *CRNN* expression in ESCC. Loss of *CRNN* expression correlated with advanced tumor length, greater tumor invasion depth, lymph node metastasis, and poor survival in patients with ESCC. (J Thorac Cardiovasc Surg 2014;147:1612-8)

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Esophageal squamous cell carcinoma (ESCC) is an aggressive malignancy with a dismal prognosis. Despite advances in multimodal treatment, the 5-year overall survival rate has been about 25% to 30% among patients

who have undergone curative surgical resection.¹⁻⁴ We have previously reported several clinicopathologic parameters as significant prognostic factors (eg, tumor invasion depth, tumor length, positive lymph node number, and disease recurrence) for the identification of high-risk patients.^{3,4} Many genetic changes and biomarkers have been reported to be involved in the development of esophageal cancer. For example, activation of cyclooxygenase 2 and overexpression of growth factors such as epidermal growth factor, fibroblast growth factor, and C-erb B2 (Her2/neu) can contribute to the progression from Barrett's esophagus to adenocarcinoma.⁵⁻⁷ Numerous genetic mutations, such as p53, p21, p16, and cyclin D, have also been identified in ESCC.⁷ With the advent of microarray and high-throughput technology, genome-wide expression profiling has contributed greatly to our understanding of the fundamental cancer biology and the identification of new biomarkers associated with tumor behavior and patient outcomes.⁸⁻¹¹ To explore the differentially expressed (in particular, downregulated) genes in ESCC, we analyzed 4 publicly available microarray data sets and identified *CRNN* (official full name "cornulin") as 1 of the major downregulated genes with a high normal/tumor ratio. First cloned and reported in 2000, the *CRNN* gene was identified as 1 of the downregulated genes in

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Abbreviation and Acronym

ESCC = esophageal squamous cell carcinoma

esophageal carcinoma.¹² Subsequent high-throughput analysis of esophageal cancer also confirmed downregulation of *CRNN* at the messenger RNA level.^{13,14} Pawar and colleagues¹⁵ performed quantitative proteomic analysis and identified *CRNN* as a fivefold downregulated protein in ESCC tumor tissue versus adjacent normal epithelium. A recent report also demonstrated downregulation of *CRNN* protein expression in 239 of 266 (89%) ESCC cases using immunohistochemical staining.¹⁶ However, reports on the clinical implications of *CRNN* expression in esophageal cancer have been limited. The relationship between *CRNN* expression and ESCC tumor aggressiveness and patient prognosis has not yet been reported. In the present study, the downregulation of *CRNN* expression was validated at the nucleic acid level using complementary DNA microarray and at the protein level using immunohistochemical staining in another 220 ESCC cases. Furthermore, we explored the clinicopathologic relevance and prognostic significance of *CRNN* expression in patients with ESCC.

METHODS

Experimental Design and Workflow

The present study was conducted in 3 consecutive phases: (1) an integrated microarray analysis-based discovery screen; (2) an initial validation of candidate biomarkers using complementary DNA microarray; and (3) clinical validation of the protein biomarkers using immunohistochemical staining in patients with ESCC who had undergone surgery and had complete clinicopathologic and follow-up data available.

Microarray Data Set Collection and Analysis

Four independent data sets (GSE5364, GSE20347, GSE23400, and GSE29001) were obtained from the National Center for Biotechnology Information Gene Expression Omnibus database (available at: <http://www.ncbi.nlm.nih.gov/geo/>).⁹⁻¹¹ The GSE20347 and GSE29001 data sets were obtained using Affymetrix HG U133A, version 2.0, chips, and the other data sets were derived from Affymetrix HG U133A chips. Quantile normalization was applied before analysis. Statistical analyses of the microarray data were conducted using BRB-ArrayTools (Biometrics Research Branch, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, Md).¹⁷ A class comparison using paired *t* tests with permutations was performed to identify differentially expressed genes in squamous cell carcinoma and matched normal esophageal epithelium. The threshold for significance was $P < .001$, determined from 10,000 random permutations.

Verification of Messenger RNA Expression by Complementary DNA Microarray

To validate the results from the publicly available data sets, paired tumor and adjacent normal tissues were collected from 16 patients with ESCC who had undergone primary resection without neoadjuvant treatment. The specimens were snap frozen in liquid nitrogen immediately after resection and stored in liquid nitrogen until use. Total RNA was extracted

from the frozen tissues using the RNeasy RNA extraction kit (Qiagen, Venlo, The Netherlands) plus DNase I treatment (Promega, Madison, Wis) and then processed for microarray hybridization on Affymetrix U133A GeneChips (Affymetrix Inc, Santa Clara, Calif) according to the manufacturer's instructions. The ".CEL" file format expression data were read using BRB-ArrayTools (National Cancer Institute, Bethesda, Md) with the guanine cytosine robust multi-array analysis methods, using the "gcrma" package from Bioconductor, to compute probe set summaries.

Verification of Protein Expression by Immunohistochemical Staining

Immunohistochemical stains were performed in a surgical cohort of 220 patients undergoing resection for ESCC at Taipei Veterans General Hospital from 1995 to 2000. Clinical data were obtained from the Cancer Registry Database at our hospital. Overall survival, defined as the interval from the date of diagnosis to the date of death or last known follow-up visit, was used as a measure of prognosis. The pathologic data were also collected and determined according to the American Joint Committee on Cancer TNM classification, 7th edition.¹⁸

Immunohistochemical staining was conducted in the tissue microarray format (Figure E1). After deparaffinization and rehydration, all sections were heated in boiling buffer (10 mM sodium citrate, pH 6.0) for 10 minutes to retrieve antigenicity, immersed in 3% hydrogen peroxidase for 10 minutes to block endogenous peroxidase activity, and incubated at 4°C overnight with primary antibodies (anti-*CRNN* rabbit polyclonal antibody, 1:200, 11799-1-AP, Proteintech Group, Chicago, Ill). Visualization was achieved by incubation with horseradish peroxidase-labeled secondary antibody (goat anti-rabbit immunoglobulin) for 1 hour at room temperature, followed by the addition of 3,3'-diaminobenzidine as a chromogen to detect antigen-antibody complexes (EnVision+ System-HRP, Dako, Carpinteria, Calif). The tissues were counterstained with hematoxylin and dehydrated. The evaluation was performed by a pathologist who was unaware of the patient's characteristics. Normal esophageal mucosal cells served as a positive control, because they are known to exhibit strong *CRNN* expression. The same procedure was followed, except the primary antibody was replaced with phosphate-buffered saline (Figure E2). Using adjacent normal mucosa, which stained intensely, as an internal control, the staining intensity was scored from 0 (no staining at all), 1 (weak), 2 (medium), to 3 (strong), and staining extent was scored from 0 (0%), 1 (0%-5%), 2 (6%-50%), to 3 (51%-100%), according to the percentage of positive cells. The tumors with strong intensity (intensity score 3) in $\geq 6\%$ of the tumor area (area score 2-3) were defined as exhibiting "positive" expression; otherwise, the tumor was defined as having "negative" expression. The patients were defined as *CRNN* expression-positive if ≥ 1 positive tissue core was noted.

Statistical Analysis

Pearson's chi-square test was used to compare the categorical variables and Student's *t* test to compare the continuous variables. The survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test. Multivariate analysis was performed using the Cox regression model. All calculations for clinical relevance were performed using Statistical Package for Social Sciences, version 17.0 (SPSS, Chicago, Ill), software, and $P < .05$ was considered significant.

Ethical Statement

The institutional review board of Taipei-Veterans General Hospital approved this study and granted a waiver of the informed consent process.

RESULTS

Analysis of Publicly Available Microarray Data Sets

A total of 95 cases from 4 independent data sets were included in our analysis (data sets GSE5364, GSE20347,

GS23400, and GSE29001 included 13, 17, 53, and 12 cases, respectively.). Using $P < .001$ as the threshold, 796, 1728, 3315, and 1443 genes were found to be upregulated in ESCC in the GSE 5364, GSE20347, GSE23400, and GSE29001 data sets, respectively. In addition, 712, 1797, 4147, and 1518 genes were downregulated in the corresponding tumor tissues. To identify a target for additional analysis, we first selected the top 50 genes with the greatest normal/tumor ratio (ie, the genes most strongly downregulated in the ESCC tumors) in each data set. Eighteen genes overlapped in all 4 data sets (Table E1). Although the “CRISP3” gene ranked first in all 4 data sets, it is a secretory protein, whose cellular component is “extracellular matrix” according to the Gene Ontology project. Therefore, we selected “*CRNN*,” which ranked second in 3 of the 4 data sets and third in the fourth, for additional study.

Verification of Messenger RNA Expression by Complementary DNA Microarray in a Validation Cohort

The mean age of the 16 patients with ESCC in the validation cohort was 62.6 ± 13.8 years; 15 were men. The cancer was stage T1-T2 and T3-T4 in 4 and 12 patients, respectively. Of the 16 patients, 10 presented with positive lymph node involvement; none had distant metastasis. In the analysis of the differentially expressed genes, 1989 target genes (1005 upregulated and 984 downregulated) were differentially expressed in the tumor and normal tissue samples using a threshold of $P < .001$ under 10,000 permutations. Regarding *CRNN* (220090_at) gene expression, the first quartile, median, and third quartile of log-transformed signal in the normal mucosa and tumor tissue was 13.07, 13.31, and 13.51 and 2.24, 2.28, and 3.18, respectively (Figure E3). *CRNN* expression in the tumor tissue was significantly lower than that in the normal mucosa (384.2-fold in normal vs tumor tissue, $P = 4 \times 10^{-7}$). Thus, the downregulation of *CRNN* transcripts in ESCC was validated in our own microarray data set.

Clinical Correlation of *CRNN* Protein Expression

The immunohistochemical staining results were similar to those of the gene expression profiles. Although the normal mucosa had uniformly intense *CRNN* expression, negative and positive *CRNN* expression was noted in 171 (77.7%) and 49 (22.3%) ESCC tumors, respectively. Representative views of the immunohistochemical stains are shown in Figure 1. The relationship between the clinical characteristics and immunohistochemical staining results are listed in Table 1. *CRNN* expression correlated significantly with the tumor invasion depth. Negative *CRNN* expression was noted in 9 of 20 (45.0%), 44 of 57 (77.2%), 98 of 120 (81.7%), and 20 of 23 (87.0%) patients with stage T1, T2, T3, and T4 disease, respectively.

FIGURE 1. Representative images of immunohistochemical stains in esophageal squamous cell carcinoma sections: A, no staining at all, defined as negative *CRNN* staining; B, little staining, defined as negative *CRNN* staining; and C, positive *CRNN* staining.

In contrast, 53 of 171 (31.0%) and 118 of 171 (69.0%) patients with negative *CRNN* expression had stage T1-T2 and T3-T4 tumors, respectively. A greater proportion of patients with positive *CRNN* expression had stage T1-T2 tumors (T1-T2, 49.0% [24 of 49]; T3-T4, 51.0% [25 of 49];

TABLE 1. Relationship between clinicopathologic features and immunohistochemical staining results

Variable	CRNN expression		P value
	Negative	Positive	
Patients (n)	171 (77.7)	49 (22.3)	
Age (y)	65.3 ± 10.6	64.1 ± 9.6	.491
Gender			.631
Male	161 (94.2)	47 (95.9)	
Female	10 (5.8)	2 (4.1)	
Depth of invasion (T stage)			.002*
T1	9 (5.3)	11 (22.4)	
T2	44 (25.7)	13 (26.5)	
T3	98 (57.3)	22 (44.9)	
T4	20 (11.7)	3 (6.1)	
Nodal status (N stage)			.014*
N0	65 (38.0)	31 (63.3)	
N1	50 (29.2)	10 (20.4)	
N2	37 (21.6)	4 (8.2)	
N3	19 (11.1)	4 (8.2)	
Distant metastasis (M stage)			.606
M0	165 (96.5)	48 (98.0)	
M1	6 (3.5)	1 (2.0)	
Stage			.002*
I	11 (6.4)	10 (20.4)	
II	58 (33.9)	23 (46.9)	
III	96 (56.1)	15 (30.6)	
IV	6 (3.5)	1 (2.0)	
Differentiation†			.141
Well	8 (7.8)	2 (5.9)	
Moderate	74 (72.5)	30 (88.2)	
Poor	20 (19.6)	2 (5.9)	
Location‡			.865
Upper third	18 (11.7)	6 (14.3)	
Middle third	91 (59.1)	25 (59.5)	
Lower third	45 (29.2)	11 (26.2)	
Tumor length (cm)‡	5.3 ± 2.3	4.5 ± 2.4	.037*

Data presented as mean ± standard deviation or n (%). * $P < .05$ was considered significant. †Data available for 136 patients. ‡Data available for 196 patients.

$P = .002$). *CRNN* expression also correlated significantly with lymph node involvement. Negative *CRNN* expression was noted in 65 of 96 (67.7%) and 106 of 124 (85.5%) patients with node-negative and node-positive disease. In contrast, 65 of 171 (38.0%) and 56 of 171 (32.7%) patients with negative *CRNN* expression had stage N0 and tumors with advanced lymph node involvement (stage N2-N3), respectively. A greater proportion of patients with positive *CRNN* expression had N0 tumors (N0, 31 of 49 [63.3%]; N2-N3, 8 of 49 [16.4%]; $P = .014$). In addition, *CRNN* expression correlated with tumor stage and tumor length. The patients with negative *CRNN* expression were more frequently diagnosed with stage III-IV than were those with positive *CRNN* expression (59.6% vs 32.6%, $P = .002$). Patients with negative *CRNN* expression also had longer tumor length than those with positive *CRNN* expression (5.3 ± 2.3 cm vs 4.5 ± 2.4 cm, $P = .037$).

TABLE 2. Relationship between clinicopathologic features and overall survival

Variable	Patients (n)	5-y Survival rate (%)	Median survival (mo)	P value
Age (y)				.080
≤66	109	27.5	19.0 (13.5-24.5)	
>66	111	22.1	14.0 (9.4-18.6)	
Gender				.870
Male	208	24.8	16.0 (12.7-19.3)	
Female	12	25.0	22.0 (5.1-39.0)	
Depth of invasion (T stage)				.005*
T1	20	43.3	45.0 (0.0-101.8)	
T2	57	29.6	24.0 (12.3-35.7)	
T3	120	21.6	15.0 (11.3-18.7)	
T4	23	13.0	9.0 (5.9-12.1)	
Nodal status (N stage)				<.001*
N0	96	38.8	29.1 (18.0-40.1)	
N1	60	26.1	14.2 (10.6-17.8)	
N2-N3	64	3.1	11.0 (8.4-13.6)	
Distant metastasis (M stage)				<.001*
M0	213	25.6	18.0 (14.1-21.9)	
M1	7	0.9	7.0 (3.2-10.8)	
Stage				<.001*
I	21	51.1	68.0 (0.0-154.9)	
II	81	37.8	29.0 (17.3-40.7)	
III	111	12.0	13.0 (10.1-15.9)	
IV	7	0.0	7.0 (3.2-10.8)	
Differentiation†				.465
Well	10	20.0	13.0 (0.0-30.0)	
Moderately	104	24.0	17.0 (11.7-22.3)	
Poorly	22	31.8	9.0 (0.0-19.1)	
Location‡				.700
Upper third	24	18.8	13.0 (4.6-21.4)	
Middle third	116	22.0	15.0 (10.2-19.8)	
Lower third	56	23.2	16.0 (10.5-21.5)	
Tumor length (cm)				.157
<5	88	25.7	17.0 (6.0-28.0)	
≥5	109	18.8	14.0 (10.5-17.5)	
CRNN expression				.006*
Negative	171	20.5	14.0 (11.1-16.9)	
Positive	49	40.3	30.0 (24.6-35.4)	

Data in parentheses are 95% confidence intervals. * $P < .05$ was considered significant by log-rank test. †Data available for 136 patients. ‡Data available for 196 patients.

Survival Effect of CRNN Protein Expression

In the survival analysis, the mean follow-up duration for all patients was 35.1 ± 41.0 months (median, 16.0; range, 0.0-161.0). The median survival and 5-year overall survival rate in the entire cohort was 17.0 months (95% confidence interval, 13.2-20.8) and 24.8%, respectively. In addition to the T, N, and M stage, *CRNN* expression was also a significant prognostic factor (Table 2). Patients with positive *CRNN* expression had a median survival of 30.0 months (95% confidence interval, 24.6-35.4) and a

Patients at risk	49	39	29	20	19	18	15	13	8	5	3
CRNN (+)	171	92	55	41	34	32	26	22	18	14	10
CRNN (-)											

FIGURE 2. Patients with positive *CRNN* protein expression (red line) had significantly better overall survival than those with negative *CRNN* expression (blue line).

5-year survival rate of 40.3%. However, patients with negative *CRNN* expression had a median survival of 14.0 months (95% confidence interval, 11.1-16.9) and a 5-year survival rate of 20.5%. The survival plot indicated that patients with positive *CRNN* expression had better survival than those with negative *CRNN* expression ($P = .006$; Figure 2). Survival analyses stratified by the T, N, and pathologic stage also showed a trend toward better survival for patients with positive *CRNN* expression than for those with negative *CRNN* expression, although the trend was not significant (Figure E4). On multivariate analysis, negative *CRNN* expression, along with nodal involvement and distant metastasis, remained an independent predictor for poor overall survival (*CRNN*, positive vs negative, hazard ratio, 0.683; $P = .047$; Table 3).

TABLE 3. Multivariate analysis for overall survival

Variable	HR	95% CI	P value
Depth of invasion			.140
T1/2	1	—	
T3/4	1.285	0.921-1.795	
Nodal status			.001*
Negative	1	—	
Positive	1.773	1.284-2.448	
Distant metastasis			.004*
M0	1	—	
M1	3.231	1.469-7.106	
<i>CRNN</i> expression			.047*
Negative	1	—	
Positive	0.683	0.469-0.995	

HR, Hazard ratio; CI, confidence interval. * $P < .05$ was considered significant by Cox regression model.

DISCUSSION

Microarray technology is commonly used in genome-wide expression analysis and provides novel insight into many complex human diseases. In the present study, we first selected the top 50 genes from each data set with the greatest normal/tumor ratio; 18 of these genes overlapped among the databases. To correlate gene expression with gene function, a Gene Ontology analysis was performed using the web-based toolkit, DAVID Bioinformatics Resources (National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, Md).¹⁹ The Gene Ontology enrichment analyses of these 18 genes indicated significant perturbation in the “epithelial cell differentiation”-related annotations ($P = 1.8e^{-7}$). In addition to the corresponding genes, which included FLG, KRT14, SCEL, SPINK5, TGM1, and TGM3, the target gene “*CRNN*” played a role in epidermal differentiation, indicating that a loss of gene expression in “epithelial cell differentiation” is a major process during ESCC development.

The *CRNN* gene is a member of the “fused gene family,” a group of genes present in the epidermal differentiation complex, a 2.5-Mbp region on chromosome 1q21 containing a high density of structurally and functionally related genes involved in terminal epidermal differentiation. The encoded product is a protein of 495 amino acids containing 2 EF-hand Ca^{2+} -binding domains in its N-terminus and 2 glutamine- and threonine-rich 60 amino acid repeats in its C-terminus.¹² Because of its expression in the upper layer of the squamous epithelium, it has been termed “cornulin” and has been suggested to have an important role in epidermal differentiation.²⁰ *CRNN* has also been referred to as squamous epithelial heat shock protein-53 and functions as a stress-responsive factor.²¹ Molecular studies of squamous epithelial heat shock protein-53 have shown that its expression helps maintain the barrier function in the squamous epithelium in response to injury and functions as a survival factor in mammalian cell lines. Darragh and colleagues²² observed that squamous epithelial heat shock protein-53 attenuates deoxycholic acid-mediated elevations in intracellular free calcium and deoxycholic acid-induced apoptotic cell death. They suggested it might function between the signal cascade initiated by exposure and the trigger that releases calcium, leading to apoptosis.²² Regarding the expression of *CRNN* in cancer tissue, its expression was high in the adjacent normal esophageal mucosa but was dramatically reduced or absent in the esophageal cancer cell lines and primary esophageal tumor tissue samples.¹²⁻¹⁶ In addition to esophageal cancer, *CRNN* expression is also downregulated in head and neck squamous cell carcinoma, tongue cancer, and cervical cancer.²³⁻²⁶ Imai and colleagues²⁷ reported that *CRNN* overexpression in oral cancer cells causes a significant

decline in cell proliferation by arresting cell cycle progression at the G₁/S phase, with downregulation of cyclin D1 expression. Their results suggested that *CRNN* plays a role in controlling cell cycle progression and that *CRNN* downregulation might be linked to tumor progression.²⁷ The results of these studies have suggested that the loss of *CRNN* expression might result in an abnormal response of the esophageal epithelium to environmental stimulation, possibly leading to cancer formation owing to deregulated cell cycle control. However, the mechanism of *CRNN* downregulation remains unknown. In the Catalogue of Somatic Mutations in Cancer database (available at: <http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/>), *CRNN* mutation was noted in only 1 of 12 ESCC samples (8.3%), which could not explain the frequency of weak protein expression in immunohistochemical staining.

The clinical relevance of *CRNN* expression, especially in esophageal cancer, remains largely unknown. Only limited reports could be found in the published data. Pawar and colleagues¹⁶ demonstrated downregulation of *CRNN* expression in 89% of ESCC cases, a greater rate than observed in our study (77.7%). This discrepancy might have resulted from differences in the antibodies used (HPA024343 from Human Protein Atlas vs 11799-1-AP from Proteintech) and the method of immunohistochemical scoring. Although staining was scored according to both the intensity and the area in our study, only the staining intensity was assessed in the study by Pawar and colleagues.¹⁶ In our study, tumors with strong intensity in $\geq 6\%$ of the tumor area were defined as “positive” and patients were grouped as having positive *CRNN* expression if any tissue core showed “positive expression.” Few reports have focused on the clinical relevance of *CRNN* gene expression. Zhang and colleagues²⁸ reported the association between polymorphisms of *CRNN* and the susceptibility to esophageal cancer. They found that subjects with the -1139CC genotype had a greater risk of developing ESCC than those with the -1139GG genotype.²⁸ In the expression profiling study by Luthra and colleagues,²⁹ the patients with high epidermal differentiation complex (IVL, *CRNN*, NICE-1, S100A2, and SPPR3) gene expression were more likely to achieve a pathologic complete response to neoadjuvant chemoradiotherapy, suggesting that the expression of the epidermal differentiation complex gene cluster, including *CRNN*, might be a predictor of chemoradiotherapy response.²⁹

In the present study, we have demonstrated the correlation between a loss of *CRNN* expression and longer tumor length, deeper tumor invasion depth, more advanced lymph node metastasis, and poorer survival. Our analysis has highlighted the clinical role of *CRNN* expression in ESCC. However, we do not know the mechanism of *CRNN* downregulation. Although *CRNN* expression can be a prognosticator and predictor for the treatment

response,¹⁶ no evidence is available to suggest that *CRNN* might be a therapeutic target. Additional investigation of the underlying mechanism of *CRNN* downregulation is critical to defining how *CRNN* contributes to ESCC carcinogenesis, and the clinical implications of *CRNN* expression should be validated in a larger clinical cohort.

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FIGURE E1. Tissue microarray sections.

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FIGURE E2. *Left*, Positive and, *right*, negative controls. Normal esophageal mucosal cells served as a positive control, because they are known to exhibit strong *CRNN* expression. The same procedure was followed, except that the primary antibody was replaced with phosphate-buffered saline.

FIGURE E3. *CRNN* expression in 16 patients with esophageal squamous cell carcinoma patient cohort. The *CRNN* expression in the tumor tissue was significantly lower than that in the normal mucosa ($P = 4 \times 10^{-7}$).

FIGURE E4. Survival analyses stratified according to A and B, T stage, C and D, N stage, and E and F, pathologic stage also showed a trend toward better survival in patients with positive *CRNN* (green lines) expression compared with those with negative *CRNN* expression (blue lines), although the trend was not significant.

TABLE E1. *CRNN* normal/tumor ratio and rank in each data set

Gene symbol	Description	Normal tissue/tumor ratio (rank)			
		GSE5364	GSE20347	GSE23400	GSE29001
CEACAM5	CEA-related cell adhesion molecule 5	10.71 (48)	16.04 (27)	4.20 (49)	64.46 (23)
CEACAM6	CEA-related cell adhesion molecule 6 (nonspecific cross-reacting antigen)	11.13 (42)	15.41 (28)	5.14 (26)	44.82 (32)
CLCA4	Chloride channel accessory 4	46.14 (3)	40.46 (7)	11.35 (5)	101.24 (11)
CRCT1	Cysteine-rich C-terminal 1	28.8 (10)	49.53 (4)	9.59 (7)	157.82 (7)
CRISP3	Cysteine-rich secretory protein 3	94.32 (1)	145.08 (1)	20.34 (1)	1260.46 (1)
<i>CRNN</i>	Cornulin	61.57 (2)	84.39 (2)	13.13 (3)	345.2 (2)
ENDOUP	26 Serine protease	20.79 (21)	23.85 (13)	7.18 (12)	162.87 (6)
FLG	Filaggrin	23.21 (15)	19.01 (19)	5.63 (20)	121.2 (9)
HPGD	Hydroxyprostaglandin dehydrogenase 15-(NAD)	12.61 (18)	20.91 (16)	6.00 (17)	95.05 (13)
KLK13	Kallikrein-related peptidase 13	8.77	20.21	7.4	35.78
KRT4	Keratin 4	26.62 (11)	33.98 (9)	6.19 (18)	70.18 (22)
MAL	Mal, T-cell differentiation protein	28.89 (9)	57.86 (3)	16.35 (2)	50.64 (29)
SCEL	Sciellin	34.38 (5)	41.05 (6)	12.18 (4)	88.73 (15)
SLURP1	Secreted LY6/PLAUR domain containing 1	30.31 (8)	32.55 (10)	8.14 (9)	73.7 (20)
SPINK5	Serine peptidase inhibitor, Kazal type 5	22.86 (16)	32.39 (11)	7.08 (14)	35.98 (40)
TGM1	Transglutaminase 1 (K polypeptide epidermal type I, protein-glutamine-gamma-glutamyltransferase)	23.4 (13)	10.97 (45)	4.51 (36)	42.77 (35)
TGM3	Transglutaminase 3 (E polypeptide, protein-glutamine-gamma-glutamyltransferase)	32.71 (7)	47.42 (5)	10.65 (6)	105.77 (10)
TMPRSS11E	Transmembrane protease, serine 11E	33.02 (6)	24.15 (12)	9.05 (8)	81.78 (17)

CEA, Carcinoembryonic antigen.