Elevated neuregulin-1 expression modulates tumor malignancy and autophagy in esophageal squamous cell carcinoma

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Abstract. The 5-year survival rate of patients with esophageal squamous cell carcinoma (ESCC) is <20%, highlighting the need for the development of novel therapeutic targets. Neuregulin-1 (NRG1), a transmembrane protein involved in cell proliferation and survival signaling, has unclear biological functions and clinical value in ESCC. The present study investigated the association between NRG1 expression and ESCC by analyzing data from both patients with ESCC and The Cancer Genome Atlas database. Reverse transcription-quantitative PCR and immunohistochemistry staining were used to determine the levels of gene and protein in the tissue. The findings revealed that NRG1 gene and protein levels were significantly higher in tumor tissues compared with the normal tissues. Elevated expression of NRG1 was associated with poor outcomes, particularly in patients with advanced ESCC. Silencing NRG1 decreased both its mRNA and protein levels, disrupting key signaling pathways, such as phosphorylated (p-)AKT and cellular rapidly accelerated fibrosarcoma (p-cRAF), which led to decreased cancer cell proliferation, migration and tumor sphere formation, along with increased cell death. High expression levels of NRG1 and cRAF were significantly associated with poor prognosis. Additionally, silencing NRG1 promoted autophagosome and autolysosome formation, decreasing LC3B levels. The use of the autophagy inhibitor chloroquine significantly enhanced cell death induced by NRG1 silencing, suggesting that autophagy functions as a survival mechanism in ESCC cells in which NRG1 is silenced. Furthermore, high co-expression of NRG1 and LC3B was associated with a worse prognosis. On the whole, the present study demonstrated that targeting NRG1 with autophagy inhibitors may serve as a potential therapeutic strategy for ESCC.

Introduction

Esophageal cancer (EC) is among the top 10 leading causes of cancer-related mortality worldwide, particularly among Asian males (1). Of note, there were 0.51 million new cases of EC and 0.44 million related deaths worldwide in 2022, respectively (1). EC primarily manifests as two subtypes: Esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) (2). ESCC accounts for ~90% of global EC cases and incidence and mortality rates associated with ESCC are expected to increase in 2030 and 2040 compared to 2020 (3). Major risk factors for ESCC include alcohol consumption and tobacco use (4). Although alcohol is often identified as the primary risk factor, combining smoking with alcohol consumption can have a synergistic effect, significantly elevating the relative risk (4). For example, the relative risk for patients who heavily use both tobacco and alcohol is 35.4 for Caucasian males and 149.2 for males of African origin, compared with that of non-smokers or to those who do not consume alcohol of

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the same ethnicity (5). The pathogenesis appears to be associated with inflammation of the squamous epithelium, leading to dysplasia and malignant changes *in situ* (6). Observational studies indicate a 40-50% reduction in the risk of developing ESCC and EAC with aspirin or non-steroidal anti-inflammatory drug treatment (7,8). EC is a common malignant tumor worldwide, often presenting clinical symptoms of dysphagia when the disease has advanced, causing obstruction or metastasis in the esophageal cavity. Treatment of EC remains contentious, primarily due to high incidence of distant metastasis in ≥50% of patients (9). Achieving a favorable therapeutic outcome is challenging, whether through surgery, radiation therapy or chemotherapy. Following surgical intervention, the overall 5-year survival rate for patients with EC is <20% (9), suggesting that ESCC cells may develop survival mechanisms to survive under conditions of stress, such as hypoxia and use of anticancer drugs (10). Therefore, identification of novel biomarkers and potential therapeutic targets for ESCC is key.

Neuregulins (NRGs) belong to the group of transmembrane proteins encoded by four genes (NRG1-4). Among these, NRG1 is upregulated in several types of cancer, such as prostate, lung cancer, pancreas cancer, suggesting NRG1 is essential for cell proliferation and differentiation. NRG1 encompasses six distinct protein types (I-VI) and ≥31 isoforms (11-13). All NRG1 protein types share a conserved EGF-like domain that distinguishes them from other EGF family members. This EGF-like domain is sufficient to induce biological activity (14-17). The EGF-like domain of NRG1 can bind human epidermal growth factor receptor (HER) 3, inducing HER2-HER3 heterodimer formation and subsequent activation of the PI3K/AKT and RAS/RAF signaling pathways (12). NRG1 expression is increased in patients with head and neck squamous cell carcinoma (HNSCC) (18). NRG1 expression is induced in patients with breast cancer with diabetes, potentially through epigenetic regulation of hyperglycemia on the NRG1 enhancer region (19). NRG1 is upregulated in patients with gastric cancer (20). Although HER3 is not associated with poor prognosis, NRG1 serves as an independent prognostic marker in gastric cancer (20). Moreover, NRG1 is a potential tumor promoter, either through being a target of chromosome translocations or via activation by fusion or promoter insertion in breast cancer (21-25). The Cancer Genome Atlas (TCGA, cancer.gov/ccg/research/genome-sequencing/tcga) and MSK-IMPACT (cbioportal.org/study/summary?id=heme_msk_impact_2022) databases have identified NRG1 rearrangements with novel fusion partners in multiple types of cancer, including breast, head and neck, renal, lung, ovarian, pancreatic, prostate and uterine cancer (25). To the best of our knowledge, no monoclonal antibodies or inhibitors have yet been developed to directly target NRG1. Current therapies targeting NRG1 primarily focus on creating monoclonal antibodies against its binding receptors, HER2 and HER3 (26). Afatinib, an irreversible pan-HER inhibitor, inhibits cell proliferation and metastatic features of ESCC cells (27). Based on the oncogenesis role of NRG1 signaling, Kim *et al* (28) investigated the effects of zenocutuzumab, a bispecific antibody for HER2 and HER3, in patients with cancer that contain NRG1 gene fusions (trial no. NCT02912949).

Autophagy is a key cellular survival mechanism essential for various biological functions, including development, maintaining cellular equilibrium and immune responses (29). The dysregulation of autophagy leads to a range of diseases, such as cancer, neurodegenerative diseases, cardiovascular disorder, diabetes, autoimmune disease and aging (30). Autophagy involves cellular self-digestion, allowing cells to break down damaged organelles and misfolded proteins, particularly in response to nutrients and oxygen-deprivation conditions, such as starvation and hypoxia, which are common features in tumors prior to angiogenesis (31). The role of autophagy in cancer is complex (32). Elevated levels of autophagy have been observed in tumor cells; autophagy is also induced in tumor metastasis and during cancer treatments (33). Autophagy activators or inhibitors modulate epithelial-mesenchymal transformation-associated proteins, inhibiting cancer cell migration and invasion (34). Moreover, targeting autophagy-associated proteins enhances cancer suppressive effects of anti-cancer drugs (35-39). Conversely, sorafenib, a clinical drug used in treatment of hepatocellular carcinoma, induces excessive autophagy, triggering autophagic cell death in renal cancer cells (38). These results suggest that the role of autophagy in cancers depends on cancer types, stages and treatment.

The principal regulator of autophagy is mTOR and its activity is negatively modulated by downstream signals from PI3K and AKT (40). mTOR is a central regulatory factor governing cell proliferation and metabolism (40). During periods of nutrient scarcity, mTOR is inhibited, thereby activating autophagy (41). In the context of autophagy, two notable markers are ubiquitin-binding protein p62 and LC3-II (31). p62 functions as an autophagic receptor, binding LC3-II to facilitate the delivery of ubiquitinated proteins to autophagosome and lysosome for degradation (42). As autophagy is activated, there is an increase in p62 protein degradation (43). However, the role of NRG1 in autophagy and regulation in ESCC remains unclear. Thus, the present study aimed to investigate the role of NRG1 signaling in modulating biological functions of cancer cells, such as cell proliferation, mobility and survival, as well as the potential association between NRG1 and its downstream signaling components with clinical outcomes in patients diagnosed with ESCC.

Materials and methods

Cell culture. The human EC cell lines CE48T/VGH (cat. no. 60165), CE81T/VGH (cat. no. 60166) and CE146T/VGH (cat. no. 60167), derived from well-differentiated ESCC, were obtained from the Bioresource Collection and Research Center. DMEM (cat. no. 12100-046, Thermo Fisher Scientific, Inc.) supplemented with 10% (v/v) FBS (cat. no. SH30071.03, Cytiva), 100 U/ml penicillin, 100 mg/ml streptomycin and non-essential amino acids (cat. no. 11140-050, Thermo Fisher Scientific, Inc.) was used to culture ESCC cell lines as reported previously (39,44) in a humidified atmosphere containing 5% CO_2 at 37˚C.

Cell viability assay. Pooled small interfering (si)RNA was obtained from Dharmacon, consisting of 3-4 individual siRNAs with chemical modifications, which has been shown to achieve stable gene silencing *in vivo* for ≥1 week (45). ESCC cells were seeded in a 96-well plate (cat. no. 655083, Greiner Bio-One

International GmbH) and transfected at 37˚C for 72 h with 5 nM scrambled siRNA (cat. no. D-001810-10-05) or a pool of siRNA targeting NRG1(cat. no. L-004608-02-0005, both Dharmacon) using RNAiMAX (cat. no. 13778-150; Thermo Fisher Scientific, Inc.). Target sequences for scramble siRNA were 5'-UGGUUUACAUGUCGACUAA-3', 5'-UGGUUUACAUGUUGUGUGA-3', 5'-UGGUUUACAUGUUUUCUGA-3' and 5'-UGGUUUACAUGUUUUCCUA-3'. The target sequences for siNRG1 were 5'-UUUCAAACCCCUCGAGAUA-3', 5'-UUGUAAAAUGUGCGGAGAA-3', 5'-GGGGAGUGCUUCAUGGUGA-3' and 5'-ACAUCCACCACUGGGACAA-3'. CellTiter-Glo (cat. no. G7573, Promega Corporation) was added to the cells and luminescence was quantified using a Fluoroskan Ascent FL reader (Thermo Fisher Scientific, Inc.). ATP levels were considered to indicate viability. Furthermore, cell viability was monitored with an impedance-based instrument system (iCELLigence, ACEA Biosciences, Inc.) for live cells. In brief, ESCC cells ($2x10^4$ cells/well) were transfected with 5 nM scrambled siRNA or pooled siNRG1 in electronic plates (E-Plates L8, ACEA Biosciences, Inc.) containing 400 μl DMEM with 10% FBS. The cellular impedance was measured every 15 min for 96 h. Alternatively, cells were fixed in 70% ethanol at -20˚C overnight and stained with propidium iodide (50 μg/ml, MilliporeSigma) at room temperature in the dark for 30 min. The stained cells were analyzed and quantified with NovoExpress flow cytometry software (version 1.6.2) in an NovoCyte benchtop flow cytometer system (Agilent Technologies, Inc.; version 1.6.2).

Western blot analysis. Transfected cells were lysed in RIPA buffer [1% NP40, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.25% sodium deoxycholate, 1% SDS, protease inhibitor cocktail and phosphatase inhibitor]. The proteins were quantified with bicinchoninic acid assay and separated by 10-12% SDS-PAGE (20 μg of protein loaded per lane) and transferred onto nitrocellulose membranes. The membranes were blocked with 5% BSA (cat. no. A5611; Sigma-Aldrich; Merck KGaA) at room temperature for 3 h and incubated with 1,000-fold diluted primary antibodies against NRG1 (cat. no. ab191139, Abcam), phosphorylated (p-)AKT (cat. no. 4060), AKT (cat. no. 4691), p-cellular rapidly accelerated fibrosarcoma (p-cRAF; cat. no. 9427), cRAF (cat. no. 53745), β-actin (cat. no. 3700; all Cell Signaling Technology, Inc.), LC3B (cat. no. ARG55799) and p62 (cat. no. ARG55040; both Arigo Biolaboratories Corp.) at 4˚C overnight. The proteins were probed with 1:5,000 HRP-conjugated secondary antibody (cat. nos. sc-2004 and sc-2005, Santa Cruz Biotechnology, Inc.) at room temperature for 1 h and the proteins on the membranes were visualized by enhanced chemiluminescent (ECL) kit (TB-ECL-250 ECL, TOPBIO) using Multi-Function Gel Image System (cat. no. MGIS-21-C2-6M, TOPBIO). The protein levels were quantified using ImageJ software (National Institutes of Health).

Autophagy flux assays. LC3B-II turnover in cells were calculated with Western blot. Briefly, the cells were treated with or without chloroquine (CQ, 20 μM) at 37˚C incubator for 3 h prior harvesting. The proteins were extracted and used to measure autophagic flux as previously reported (46). Alternatively, autophagosome dye (DAP, 0.1 μM) or autolysosome dye (DAL, 0.5 μM) were used to stain cells in the culture medium at 37˚C for 30 min to monitor autophagy activity, respectively, as previously reported (47). The autophagosomes and autolysosomes were observed by confocal microscopy.

Clonogenic assay. The cells were plated in 12-well plates at a density of $5x10^3$ cells/well and transfected with scramble siRNA or siRNA against NRG1. Subsequently, cells were cultured in DMEM (cat. no. 12100-046, Thermo Fisher Scientific, Inc.) supplemented with 10% (v/v) FBS (cat. no. SH30071.03, Cytiva), 100 U/ml penicillin, 100 mg/ml streptomycin and non-essential amino acids (cat. no. 11140-050, Thermo Fisher Scientific, Inc.) at 37˚C, which was refreshed every 3 days for 2 weeks. The cell colonies were fixed with 2% paraformaldehyde at room temperature for 15 min and stained with 20% ethanol containing 0.25% crystal violet (cat. no. C0775, Merck KGaA.) at room temperature for 20 min. The stained cells were washed with PBS three times and colonies >1 mm in diameter were counted and quantified with Image J software (version 1.54; National Institutes of Health) in ≥3 independent experiments.

Clinical samples and reverse transcription-quantitative PCR. Human ESCC and normal adjacent (distance, >2 cm) tissue was obtained from 120 patients who underwent esophageal resection at the Department of Surgery of Kaohsiung Veterans General Hospital (Kaohsiung, Taiwan) between October 2002 and October 2018. The age range of participants was 35 and 75 years old. The sex distribution was predominantly male, with over 90% of patients being male. Patients who had received neoadjuvant treatment were excluded from the study. Only those who underwent esophagectomy with gastric conduit reconstruction with/without adjuvant treatment were included. The present study was approved by the institutional review board of Kaohsiung Veterans General Hospital (approval nos. VGHKS 95-CT3-21 and VGHKS 15-CT12-10). Written informed consent was obtained from all subjects. Total RNA from the 120 paired tissues was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.). RNA was precipitated using 0.5 ml isopropanol. The concentration, purity and quantity of total RNA were assessed using a Nanodrop 1000 spectrophotometer (Nanodrop Technologies, Inc.). A total of 2 μg RNA was extracted by RNA extraction kit (Invitrogen; Thermo Fisher Scientific, Inc.), then reverse-transcribed with oligo-dT primers and SuperScript III Reverse Transcriptase according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific, Inc.) at 50˚C for 50 min, followed by enzyme inactivation at 85˚C for 5 min. The resulting cDNA was used for quantitative PCR analysis with gene-specific primers as follows: NRG1 forward, 5'-CCACTGGGACAAGCCATCTT-3' and reverse, 5'-TTCACCATGAAGCACTCCCC-3' and β-actin forward, 5-'AGCGAGCATCCCCCAAAGTT-3' and reverse, 5'-GGGCACGAAGGCTCATCATT-3'. Thermocycling conditions were as follows: Initial denaturation at 3 min at 95˚C, followed by 40 cycles of 15 sec at 95˚C and 1 min at 60˚C. The gene expression was detected using SYBR Green I assay (Applied Biosystems; Thermo Fisher Scientific, Inc.). The relative abundance of NRG1 mRNA was assessed using the StepOnePlus system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The $2^{-\Delta\Delta}$Cq method was used for quantification of relative changes in gene expression (48).

Invasion and migration assay. For the invasion assay, $3x10^5$ ESCC cells were seeded into the upper chamber, which was pre-coated with 50 μl 0.5% Matrigel in DMEM containing 1% FBS at 37˚C for 30 min (47,48). The lower chamber was supplemented with 500 μl DMEM containing 10% FBS. Cells were allowed to pass through the Matrigel-coated chamber at 37˚C for 24 h, followed by fixation in 2% paraformaldehyde at room temperature for 15 min and staining with 0.25% crystal violet at room temperature for 30 min. The images were captured using a light inverted microscope (magnification, x10). The migration assay was conducted using Culture-Insert 2 Wells (Ibidi) designed for 24-well plates. A total of $1x10^6$ cells were seeded with 70 μl DMEM containing 10% FBS at 37˚C for overnight, after which the culture insert was removed for 24 h. The cells were fixed with 2% paraformaldehyde at room temperature for 15 min to measure the open area and images were captured using a light inverted microscope (magnification, x10).

Tumor sphere viability. ESCC cells were plated at a density of $4x10^3$ cells/well in an ultra-low attachment 96-well plate (Costar®; Corning, Inc.) using DMEM with 10% FBS and cultivated at 37˚C for 7 days to promote formation of spheroid cells. Viability of these spheroid cells was assessed by staining with Calcein AM (1 μM) and ethidium homodimer-1 (EthD-1, 2 μM) using the LIVE/DEAD® Viability/Cytotoxicity kit (Thermo Fisher Scientific, Inc.) at 37˚C for 30 min. Fluorescence microscopy (magnification, x10) was used to capture images of live (green) and dead (red) spheroid cells, which were quantitatively analyzed with a Fluoroskan Ascent FL reader (Thermo Fisher Scientific, Inc.) at excitation and emission wavelengths of 485 and 530 nm for Calcein AM and 645 nm for EthD-1, respectively.

Immunohistochemistry (IHC). Tissue microarray (TMA; cat no. ES701) purchased from SuperBiochips was analyzed through IHC staining of protein of the TMA blocks (50,51). The blocks were immersed in sodium citrate buffer (10 mM, pH 6.0), boiled at 125˚C in a pressure boiler for 10 min for antigen retrieval and then blocked with 3% hydrogen peroxide at room temperature for 30 min. The tissue sections were stained with anti-NRG1 (1:100, cat. no. ab191139, Abcam) at 4˚C overnight, Following the TBS-T (0.5% Tween-20) washes, staining was carried out with 1:3000 diluted secondary antibody conjugated with HRP) polymer at room temperature for 30 min using the Epredia UltraVision™ Quanto Detection System (TA-125-QHDX, Thermo Fisher Scientific, Inc.). The washed slides were stained with hematoxylin (Sigma-Aldrich; Merck KGaA) at room temperature for 5 sec. The slides were allowed to dry, then mounted with a coverslip, subsequently examined under a light microscope at the appropriate magnification (x20). Intensity and percentage of NRG1 staining was scored. The score was calculated as the total value of staining intensity (0, negative; 1, weak expression; and 2, moderate expression) and percentage of cells staining at each intensity level [0 (<5%), 1 (5-25%), 2 (26-50%), 3 (51-75%), and 4 (>75%)] (49).

Statistical analysis. The TCGA (The Cancer Genome Atlas) dataset for gene expression levels and clinical outcome of ESCC patients were downloaded from Xena platform (xenabrowser.net/). The gene and protein levels of NRG1 in tumor and adjacent normal tissue were evaluated by SPSS Statistics 28.0 (IBM Corporation) using Wilcoxon signed-rank test. The cutoff value is for high and low levels of NRG1 protein (3) depends on the receiver operating characteristic (ROC) curve. The cutoff values of NRG1 gene expression levels were 10.8322 for overall survival, progression-free interval, disease-specific survival, and 9.15815 for disease-free interval. To assess the association between NRG1 and clinicopathological factors, Student's unpaired t-test, Mann-Whitney U test and Kruskal-Wallis were applied. Cumulative survival rates were assessed using Kaplan-Meier curves, with significance determined using log-rank test. The association between protein levels and survival outcomes, including overall, progression-free interval, disease-specific and disease-free interval survival, was adjusted for cell differentiation (moderate + poor vs. well) and American Joint Committee on Cancer pathological stage (stage III + IV vs. stage I + II) using a multivariate Cox regression model. $P<0.05$ (two-sided) was considered to indicate a statistically significant difference. For cell culture experiments, results were derived from ≥3 independent replicates, with significance assessed using a non-parametric two-tailed Student's unpaired t-test. One-way ANOVA followed by Tukey's post hoc test was used for comparisons between >2 groups. The data were analyzed by GraphPad Prism 5.0 (Dotmatics). Data are expressed as the mean ± SD.

Results

NRG1 expression is elevated in tumor tissue and associated with poor prognosis of patients with ESCC. To investigate the potential association between NRG1 and patient survival, analysis of NRG1 mRNA expression was conducted in individuals with ESCC. Quantitative PCR was employed to assess NRG1 expression in 120 paired primary ESCC and adjacent normal tissue samples from Kaohsiung Veterans General Hospital. There was significantly elevated NRG1 gene expression in tumor tissue of patients with ESCC compared with adjacent normal tissue (9.36±24.90 vs. 3.27±7.32; Table IA; Fig. 1A). To determine NRG1 expression at the protein level, IHC was employed (Fig. 1B). NRG1 protein levels were significantly higher in ESCC compared with corresponding tumor-adjacent normal tissue cores (Fig. 1C). NRG1 levels were higher in tumor compared with those in adjacent normal tissue (Table IB). However, the association between NRG1 protein expression and overall and disease-specific survival was not significant (Tables SI and SII). To monitor the association between clinicopathological characteristics and NRG1, TCGA database was used to analyze the effect of high and low expression of NRG1 using the receiver operating characteristics curve (Fig. 1D-G). Higher expression of NRG1 was associated with unfavorable overall survival of patients with poor differentiation (Fig. 1D), advanced stage (AJCC stage III and IV; Fig. 1E) and lymph node invasion (Fig. 1G). Following adjustments for cell differentiation and AJCC pathological stage for adjusted hazard ratio (AHR) with multiple Cox regression analysis, mortality risk was significantly higher in patients with high expression of NRG1, particularly in male patients (AHR, 4.98; 95% CI,

Table I. Neuregulin-1 expression in tumor and adjacent normal tissue from patients with esophageal squamous cell carcinoma.

A, mRNA

Adjacent normal (n=120)		Tumor (n=120)		
Mean expression	Median expression	Mean expression	Median expression	P-value
3.27±7.32	0.41	9.36±24.90	1.46	0.004

B, Protein[a]

Adjacent normal (n=120)		Tumor (n=120)		
Mean expression	Median expression	Mean expression	Median expression	P-value
2.68±1.32	3.00	3.66±1.14	4.00	<0.001

[a]Z-score, 3.704.

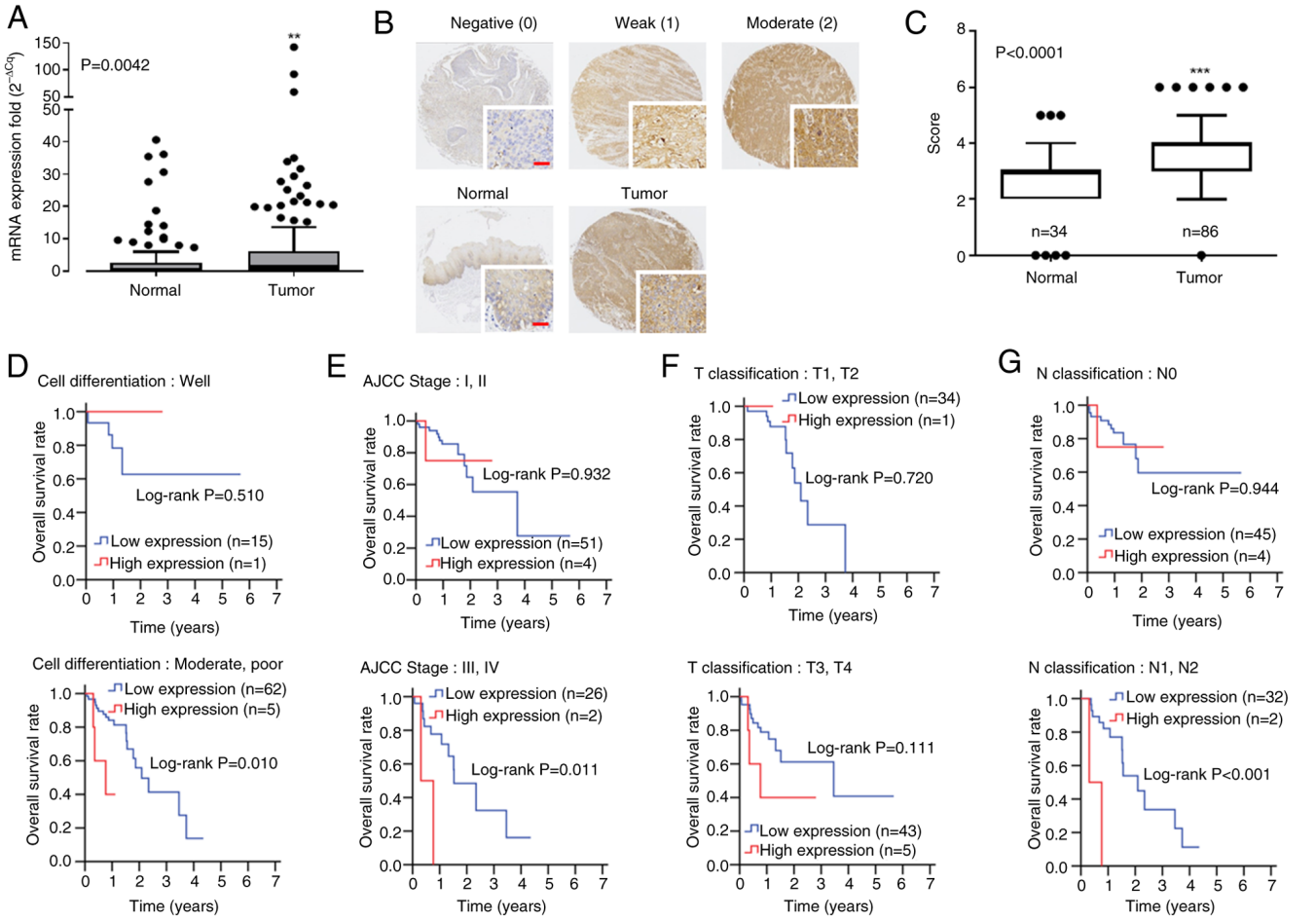

Figure 1. Association between NRG1 expression and clinical outcome of human ESCC. (A) NRG1 mRNA levels in tumor and adjacent normal tissue from 120 patients with ESCC were determined by quantitative PCR. (B) NRG1 protein levels in tumor tissue were examined with immunohistochemistry and imaged under light microscope with x20 magnification. Scale bar, 100 μm. (C) NRG1 protein levels between normal and tumor tissue. (D) Association between NRG1 gene expression and overall survival of patients with ESCC and well and moderate/poor differentiation was analyzed with Kaplan-Meier method. (E) Association between NRG1 expression and overall survival in early or advanced AJCC stages stratified by (F) tumor size (T classification) and (G) lymph node invasion (N classification). **P<0.01, ***P<0.001 vs. normal. NRG, neuregulin-1; ESCC, esophageal squamous cell carcinoma; AJCC, American Joint Committee on Cancer; yrs, years.

1.34-18.46, Table II) or patients with poor differentiation of ESCC (AHR, 5.03; 95% CI, 1.37-18.54), advanced stage of disease (stage III and IV; AHR, 7.00, 95% CI, 1.32-37.17) and poor nodal status (N1-2 vs. N0; AHR, 12.02; 95% CI,

Table II. Impact of NRG1 expression on overall survival of patients with esophageal squamous cell carcinoma.

Variable	NRG1 expression	n (%)	CHR (95% CI)	P-value[a]	AHR (95% CI)	P-value[b]
Sex						
Female	Low	10 (90.9)	1.00		1.00	
	High	1 (9.1)	0.04 (0.00-4.58x10^{11})	0.837	1.00 (0.00-2.67x10^{30})	1.000[c,d]
Male	Low	67 (93.1)	1.00		1.00	
	High	5 (6.9)	4.29 (1.20-15.32)	0.025	4.98 (1.34-18.46)	0.016[c,d]
Age, years						
≤60	Low	48 (92.3)	1.00		1.00	
	High	4 (7.7)	2.33 (0.52-10.46)	0.270	3.59 (0.73-17.68)	0.117[c,d]
>60	Low	29 (93.5)	1.00		1.00	
	High	2 (6.5)	2.63 (0.31-22.00)	0.373	3.64 (0.37-35.43)	0.266[c,d]
Cell differentiation						
Well	Low	15 (93.8)	1.00		1.00	
	High	1 (6.3)	0.41 (0.00-1.01x10^{5})	0.671	Incalculable	0.995[d]
Moderate, poor	Low	62 (92.5)	1.00		1.00	
	High	5 (7.5)	4.73 (1.29-17.30)	0.019	5.03 (1.37-18.54)	0.015[d]
AJCC pathological stage						
I, II	Low	51 (92.7)	1.00		1.00	
	High	4 (7.3)	1.09 (0.14-8.51)	0.932	1.25 (0.16-10.01)	0.836[c]
III, IV	Low	26 (92.9)	1.00		1.00	
	High	2 (7.1)	6.49 (1.25-33.78)	0.026	7.00 (1.32-37.17)	0.022[c]
T classification						
T1, T2	Low	34 (97.1)	1.00		1.00	
	High	1 (2.9)	0.05 (0.00-3.98x10^{9})	0.812	Incalculable	0.992[c,e]
T3, T4	Low	43 (89.6)	1.00		1.00	
	High	5 (10.4)	2.71 (0.76-9.76)	0.126	3.28 (0.87-12.38)	0.079[c,e]
N classification						
N0	Low	45 (91.8)	1.00		1.00	
	High	4 (8.2)	1.08 (0.14-8.43)	0.944	1.10 (0.13-8.99)	0.930[c,f]
N1, N2	Low	32 (94.1)	1.00		1.00	
	High	2 (5.9)	11.62 (2.10-64.17)	0.005	12.02 (1.99-72.60)	0.007[c,f]

CHR, crude hazard ratio; CI, confidence interval; AHR, adjusted hazard ratio; AJCC, American Joint Committee on Cancer. [a]Cox's regression; [b]multivariate Cox's regression; adjusted for [c]differentiation, [d]AJCC stage and [e]N and [f]T classification.

1.99-72.60). Therefore, it was hypothesized that NRG1 functions as an oncogene.

Silencing NRG1 decreases cancer cell proliferation. To determine the role of NRG1 in ESCC, siRNA was used to knock down NRG1 in CE48T, CE81T and CE146T cells. To minimize off-target effects of siRNA, a siRNA pool was used. NRG1 mRNA levels exhibited a decrease in the presence of siNRG1 (Fig. 2A), accompanied by a corresponding attenuation in protein expression (Fig. 2B and C). The phosphorylation levels of downstream regulators in NRG1/HER signaling, AKT and cRAF, were consistently decreased (Fig. 2B and C). To validate the effect of siNRG1 on cell viability, cellular ATP levels were used. Viability of ESCC cells decreased with increasing concentrations of siNRG1 (Fig. 2D). The use of 10 nM siRNA against NRG1 significantly suppressed viability of three ESCC cell lines, whereas 1 nM siRNA significantly deceased cell viability only in CE48T and CE146T (Fig. 2D). ESCC cell lines were cultured in electronic plates to monitor cell viability, with impedance plots showing cell indexes (CI), revealing a significant decrease in CI in NRG1-silencing compared with control cells (Fig. 2E). Colony formation assay was used to evaluate the effect of siNRG1 on anchorage-independent proliferation of ESCC cells (51). There was a significant decrease in number of colonies of ESCC cells (CE48T, CE81T, and CE146T) following 1-week treatment with siNRG1 (10 nM; Fig. 2F), suggesting a decrease in proliferative capacity. Thus, siNRG1 exhibited cytotoxicity against ESCC cells, effectively decreasing cell viability and proliferation.

Silencing NRG1 decreases cancer cell mobility and viability of tumor sphere. The present study explored the effect of

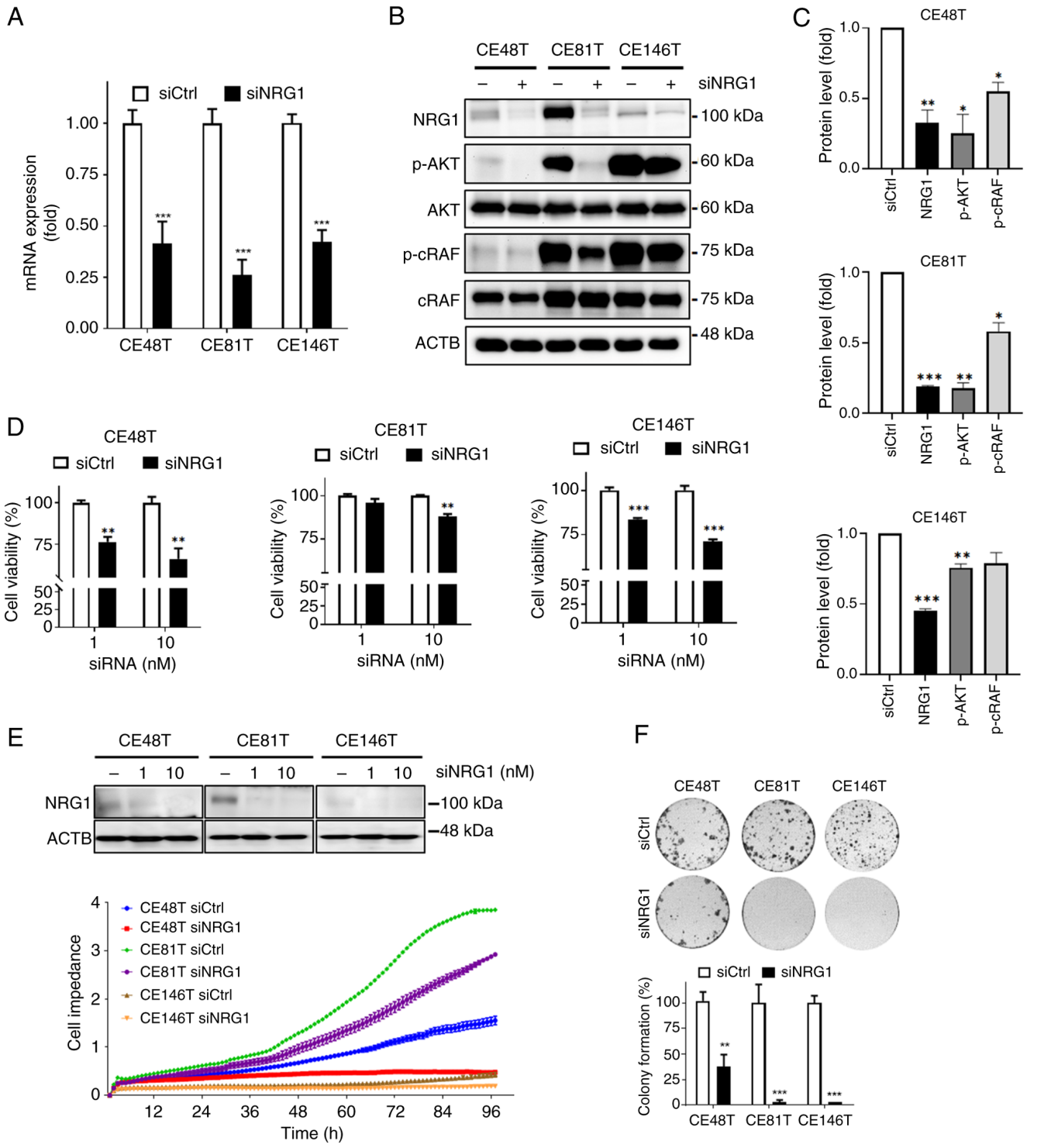

Figure 2. Effects of NRG1 silencing on esophageal squamous cell carcinoma cell signaling and cell proliferation. (A) NRG1 mRNA levels were examined by quantitative PCR. (B) Protein levels of NRG1 and its downstream regulators (AKT and cRAF) in transfected cells were determined by immunoblotting to verify the silencing efficiency of siNRG1. (C) Levels of protein. (D) Cells were transfected with 1 or 10 nM siRNA for 72 h and viability was assessed by CellTiter-Glo. (E) Cell impedance following silencing. (F) Colony formation of NRG1-silenced cancer cells. *P<0.05, **P<0.01, ***P<0.001 vs. siCtrl. NRG, neuregulin-1; cRAF, cellular rapidly accelerated fibrosarcoma; si, small interfering; Ctrl, control; p-, phosphorylated; ACTB, β-actin.

NRG1 on cell migration and invasion. Silencing NRG1 with 1 nM siRNA inhibited migration in all three ESCC cell lines (Fig. 3A and B). Additionally, silencing NRG1 significantly diminished the invasive ability of CE81T and CE146T cells (Fig. 3C). Furthermore, the sphere is a three-dimensional structure that possesses fewer nutrients and oxygen supply within its core compared with surface cells (52). This characteristic mimics the growth conditions of cancer cells within a tumor. Sphere formation assay indicated that transfection with siNRG1 led to smaller sphere volumes of ESCC cells compared with control (Fig. 3D). Live (green)/dead (red) staining was employed to determine whether silencing NRG1 affected the ratio of live and dead cells within tumor sphere. The results demonstrated a significant decrease in number of

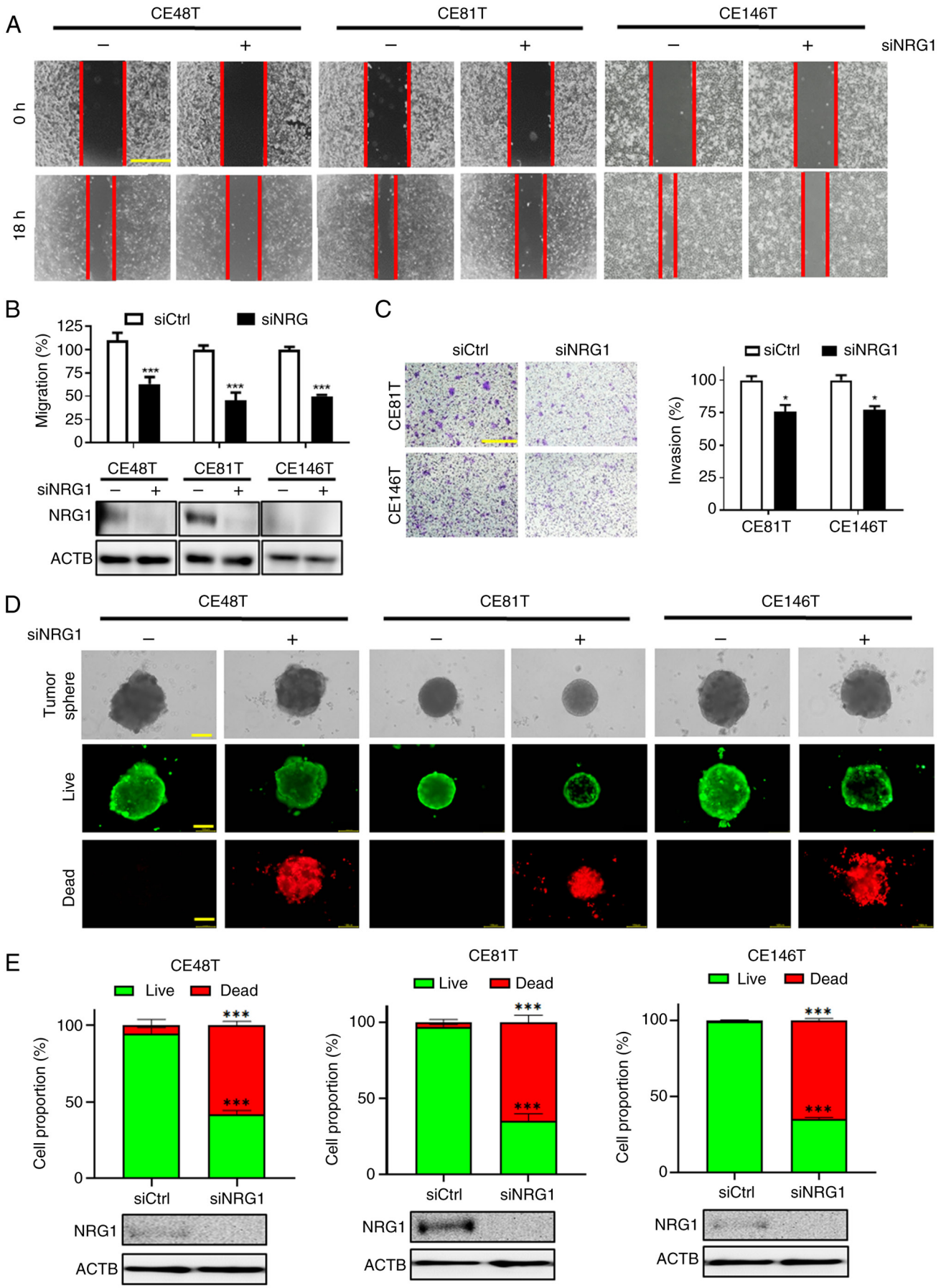

Figure 3. Effects of silencing NRG1 on mobility and tumor sphere viability of ESCC cells. Human ESCC cancer cells were transfected with 10 nM scramble siRNA or siNRG1 to assess (A) Gap closure assay. (B) Migration ability. (C) invasion ability. Scale bar, 100 μm. (D) Tumor spheres were stained to determine live (green) and dead (red) cells. Scale bar, 100 μm. (E) Viable and dead ESCC cells were quantified. *P<0.05, ***P<0.001 vs. siCtrl).NRG, neuregulin-1; ESCC, esophageal squamous cell carcinoma; si, small interfering; Ctrl, control; ACTB, β-actin.

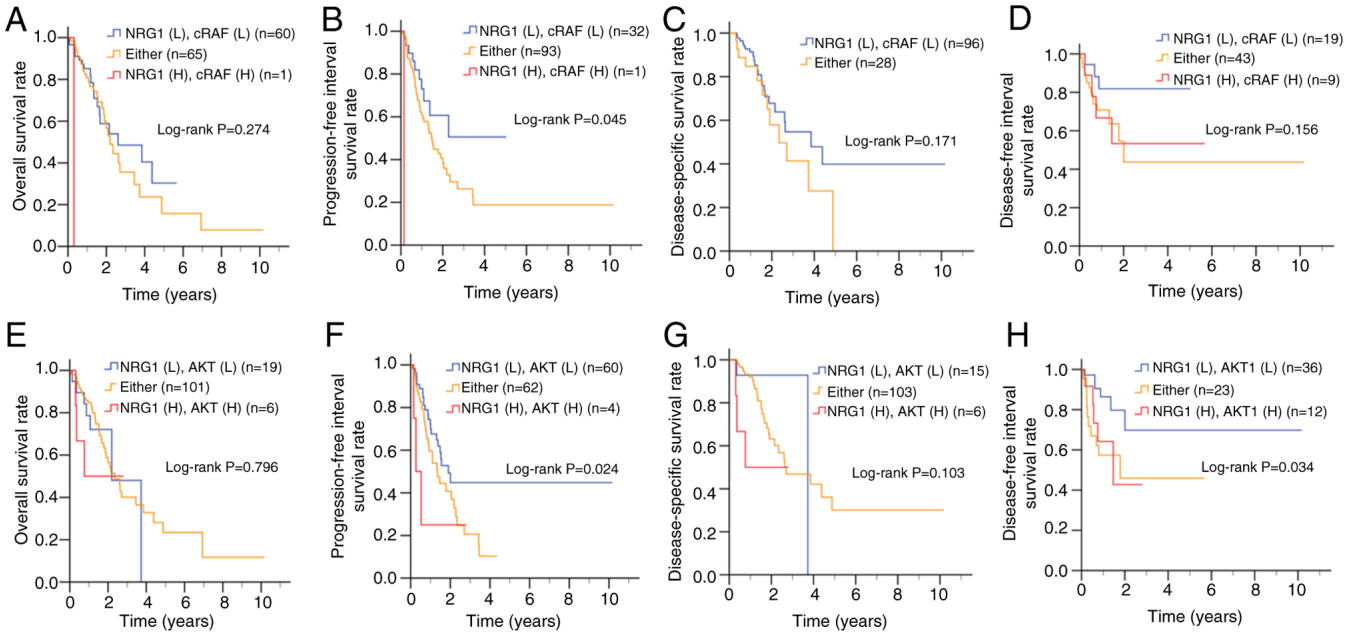

Figure 4. Clinical association between gene expression of NRG1, cRAF and AKT in patients with ESCC. Based on an ESCC dataset from The Cancer Genome Atlas, Kaplan-Meier plots were used to analyze the association between NRG1 and cRAF expression of patients with ESCC and (A) overall, (B) progression-free interval, (C) disease-specific and (D) disease-free interval survival. Association between NRG1 and AKT gene expression and (E) overall (F) progression-free interval, (G) disease-specific and (H) disease-free interval survival. NRG, neuregulin-1; ESCC, esophageal squamous cell carcinoma; cRAF, cellular rapidly accelerated fibrosarcoma; L, low; H, high expression; Either, NRG1(H) cRAF(L) or NRG1(L)/cRAF(H); yrs, years.

live and an increase in that of dead cells following transfection with siNRG1 (Fig. 3E). Therefore, siNRG1 inhibited sphere formation ability of ESCC cells and increased the proportion of dead cells.

Co-expression of NRG1 and its signaling molecules are associated with poor prognosis of ESCC. The expression of NRG1 and its downstream signaling molecules, cRAF and AKT, was associated with enhanced migratory and invasive capabilities in ESCC cells, which are associated with metastasis and cancer aggressiveness (54). The present study examined the prognostic value of NRG1, cRAF and AKT expression in patients with ESCC (Fig. 4). Kaplan-Meier survival analysis demonstrated that patients with high co-expression of NRG1 and cRAF had significantly shorter progression-free interval survival (Fig. 4B), although no significant differences were observed in overall (Fig. 4A), disease-specific or disease-free survival (Fig. 4C and D) compared with those with low NRG1 and cRAF co-expression. Additionally, high co-expression of NRG1 and AKT was significantly associated with worse overall survival (Fig. 4F) and shorter disease-free interval survival (Fig. 4H) but did not show a significant association with progression-free interval (Fig. 4E) or disease-specific survival (Fig. 4G).

To account for variations in cell differentiation and AJCC pathological stage, multivariate Cox proportional hazard model was used to evaluate association between survival outcomes and NRG1 expression alone or in combination with cRAF expression (Table III). ESCC patients with high co-expression of NRG1 and cRAF exhibited a markedly increased risk of mortality compared with those with low co-expression. These patients had significantly higher risks for both overall (AHR, 44.72, CI, 4.54-440.89, Table III) and progression-free interval survival (AHR, 93.44, CI,7.93-1101.57). Although patients with high expression levels of NRG1 and AKT showed poorer outcomes in progression-free interval, disease-specific survival, and disease-free survival, these associations were not significant (Table IV). These results highlight the complex nature of the effects of NRG1on the ESCC prognosis.

Silencing NRG1 induces cytoprotective autophagy in ESCC cells. Downstream signals of NRG1, namely p-AKT and p-cRAF, were inhibited in ESCC cells in which NRG1 was silenced. The inactivation of AKT decreases activity of its substrate protein, mTOR, which is a negative regulator of autophagy (53). RAF inhibitors activate cytoprotective autophagy to facilitate resistance to stressed conditions (54). Therefore, it was hypothesized that autophagy is activated in ESCC cells in response to downregulation of NRG1. To confirm whether siNRG1 promotes autophagy in ESCC cells, a fluorescence assay was used to observe the changes in autophagosomes. ESCC cells were transfected with siNRG1 and treated with EBSS and CQ as controls for autophagy inducer and inhibitor, respectively (Fig. 5A-C). More autophagosome (DAP) and autolysosomes (DAL) puncta were observed in the siNRG1 and EBSS groups compared with the control group, indicating activation of autophagy. Conversely, fluorescence of DAL decreased in the CQ group, indicating inhabitation of autophagy (Fig. 5A-C). To investigate the effects of silencing NRG1 on autophagic activity, expression of autophagy marker LC3B-II and p62 was determined using western blot analysis (Fig. 5D and E). The results demonstrated decreased expression levels of p62 and LC3B-II in ESCC cells following transfection with siNRG1 (Fig. 5D and E). CQ was added to examine the effect of siNRG1 on LC3B-II turnover, indicative of autophagic flux. The silencing NRG1 increased net LC3B-II protein levels in ESCC cells treated with or without autophagy inhibitor CQ

Table III. Effect of NRG1/cRAF co-expression on survival of patients with esophageal squamous cell carcinoma.

A, Overall survival

Variable	Expression	n (%)	CHR (95% CI)	P-value	AHR (95% CI)	P-value
NRG1	Low	120 (95.2)	1.00		1.00	
	High	6 (4.8)	2.01 (0.62-6.52)	0.244[a]	3.40 (0.99-11.71)	0.052[b]
cRAF	Low	65 (52.4)	1.00		1.00	
	High	61 (47.6)	1.22 (0.69-2.15)	0.501[a]	1.07 (0.60-1.91)	0.809[b]
NRG1 (L), cRAF (L)	-	60 (47.6)	1.00		1.00	
Either	-	65 (51.6)	1.17 (0.66-2.07)	0.591[a]	1.23 (0.69-2.20)	0.489[c]
NRG1 (H), cRAF (H)	-	1 (0.8)	39.89 (4.15-383.56)	0.001[a]	44.72 (4.54-440.89)	0.001[c]

B, Progression-free interval survival

Variable	ROC	n (%)	CHR (95% CI)	P-value	AHR (95% CI)	P-value
NRG1	Low	120 (95.2)	1.00		1.00	
	High	6 (4.8)	2.93 (1.16-7.38)	0.023[a]	5.11 (1.90-13.72)	0.001[b]
cRAF	Low	37 (29.4)	1.00		1.00	
	High	89 (70.6)	1.38 (0.76-2.50)	0.297[a]	1.32 (0.73-2.41)	0.360[b]
NRG1 (L), cRAF (L)	-	32 (25.4)	1.00		1.00	
Either	-	93 (73.8)	1.61 (0.84-3.09)	0.155[a]	1.77 (0.90-3.49)	0.101[c]
NRG1 (H), cRAF (H)	-	1 (0.8)	59.49 (5.39-656.09)	0.001[a]	93.44 (7.93-1101.57)	<0.001[c]

C, Disease-specific survival

Variable	ROC	n (%)	CHR (95% CI)	P-value	AHR (95% CI)	P-value
NRG1	Low	118 (95.2)	1.00		1.00	
	High	6 (4.8)	3.17 (0.96-10.53)	0.059[a]	7.76 (2.04-29.53)	0.003[b]
cRAF	Low	102 (82.3)	1.00		1.00	
	High	22 (17.7)	1.27 (0.59-2.73)	0.542[a]	1.31 (0.60-2.85)	0.497[b]
NRG1 (L), cRAF (L)	-	96 (77.4)	1.00		1.00	
Either	-	28 (22.6)	1.63 (0.80-3.31)	0.175[a]	1.63 (0.80-3.31)	0.175[c]
NRG1 (H), cRAF (H)	-	0 (0.0)	-	-	-	-

D, Disease-free interval survival

Variable	ROC	n (%)	CHR (95% CI)	P-value	AHR (95% CI)	P-value
NRG1	Low	52 (73.2)	1.00		1.00	
	High	19 (26.8)	1.29 (0.52-3.20)	0.580[a]	1.97 (0.75-5.16)	0.169[b]
cRAF	Low	29 (40.8)	1.00		1.00	
	High	42 (59.2)	1.94 (0.75-4.99)	0.172[a]	2.03 (0.79-5.26)	0.144[b]
NRG1 (L), cRAF (L)	-	19 (26.8)	1.00		1.00	
Either	-	43 (60.6)	1.74 (0.70-4.34)	0.234[a]	2.72 (0.78-9.49)	0.117[c]
NRG1 (H), cRAF (H)	-	9 (12.7)	1.30 (0.44-3.89)	0.636[a]	2.70 (0.60-12.10)	0.194[c]

L, low; H, high expression; Either, NRG1(H)cRAF(L) or NRG1(L)/cRAF(H); NRG, neuregulin-1; cRAF, cellular rapidly accelerated fibrosarcoma; CHR, crude hazard ratio; CI, confidence interval; AHR, adjusted hazard ratio. [a]Cox's regression; [b]adjusted for differentiation and American Joint Committee on Cancer pathological stage; [c]multivariate Cox's regression; -, not applicable.

(Fig. 5F and G). Autophagic flux was higher in ESCC cells in which NRG1 was silenced compared with control. Therefore, silencing NRG1 increased autophagosome and autolysosome formation and promoted autophagic activity in ESCC cells.

Table IV. Effect of NRG1 and AKT co-expression on patients with esophageal squamous cell carcinoma.

A, Overall survival

Variable	Expression	n (%)	CHR (95% CI)	P-value	AHR (95% CI)	P-value
NRG1	Low	120 (95.2)	1.00		1.00	
	High	6 (4.8)	2.01 (0.62-6.52)	0.244[a]	3.40 (0.99-11.71)	0.052[b]
AKT	Low	19 (15.1)	1.00		1.00	
	High	107 (84.9)	0.88 (0.39-1.96)	0.746[a]	0.75 (0.33-1.71)	0.500[b]
NRG1 (L), AKT (L)	-	19 (15.1)	1.00		1.00	
Either	-	101 (80.2)	0.73 (0.36-1.48)	0.387[a]	0.84 (0.37-1.89)	0.671[c]
NRG1 (H), AKT (H)	-	6 (4.8)	2.01 (0.62-6.52)	0.244[a]	1.74 (0.45-6.73)	0.426[c]

B, Progression-free interval survival

Variable	ROC	n (%)	CHR (95% CI)	P-value	AHR (95% CI)	P-value
NRG1	Low	120 (95.2)	1.00		1.00	
	High	6 (4.8)	2.93 (1.16-7.38)	0.023[a]	5.11 (1.90-13.72)	0.001[b]
AKT	Low	62 (49.2)	1.00		1.00	
	High	64 (50.8)	1.53 (0.92-2.55)	0.103[a]	1.62 (0.97-2.71)	0.066[b]
NRG1 (L), AKT (L)	-	60 (47.6)	1.00		1.00	
Either	-	62 (49.2)	1.53 (0.92-2.55)	0.101[a]	1.66 (0.98-2.81)	0.061[c]
NRG1 (H), AKT (H)	-	4 (3.2)	2.18 (0.68-7.03)	0.192[a]	2.87 (0.85-9.66)	0.088[c]

C, Disease-specific survival

Variable	ROC	n (%)	CHR (95% CI)	P-value	AHR (95% CI)	P-value
NRG1	Low	118 (95.2)	1.00		1.00	
	High	6 (4.8)	3.17 (0.96-10.53)	0.059[a]	7.76 (2.04-29.53)	0.003[b]
AKT	Low	15 (12.1)	1.00		1.00	
	High	109 (87.9)	1.77 (0.42-7.42)	0.436[a]	1.44 (0.34-6.16)	0.621[b]
NRG1 (L), AKT (L)	-	15 (12.1)	1.00		1.00	
Either	-	103 (83.1)	0.86 (0.33-2.24)	0.753[a]	1.64 (0.39-6.93)	0.499[c]
NRG1 (H), AKT (H)	-	6 (4.8)	3.17 (0.96-10.53)	0.059[a]	4.97 (0.83-29.88)	0.080[c]

D, Disease-free interval survival

Variable	ROC	n (%)	CHR (95% CI)	P-value	AHR (95% CI)	P-value
NRG1	Low	52 (73.2)	1.00		1.00	
	High	19 (26.8)	1.29 (0.52-3.20)	0.580[a]	1.97 (0.75-5.16)	0.169[b]
AKT	Low	43 (60.6)	1.00		1.00	
	High	28 (39.4)	3.42 (1.40-8.35)	0.007[a]	4.24 (1.69-10.66)	0.002[b]
NRG1 (L), AKT (L)	-	36 (50.7)	1.00		1.00	
Either	-	23 (32.4)	2.25 (0.95-5.30)	0.064[a]	3.21 (1.16-8.84)	0.024[c]
NRG1 (H), AKT (H)	-	12 (16.9)	1.65 (0.60-4.54)	0.329[a]	2.91 (0.88-9.60)	0.079[c]

L, low; H, high expression; Either, either NRG1(H)AKT(L) or NRG1(L)/AKT(H); NRG, neuregulin-1; cRAF, cellular rapidly accelerated fibrosarcoma; CHR, crude hazard ratio; CI, confidence interval; AHR, adjusted hazard ratio. [a]Cox's regression; [b]adjusted for differentiation and American Joint Committee on Cancer pathological stage; [c]multivariate Cox's regression; -, not applicable.

Autophagy functions as a detrimental or survival pathway in cells in response to stress (31,55). Cells treated with siNRG1 and CQ displayed a significant decrease in cell viability compared with siNRG1-alone (Fig. 6A). In addition,

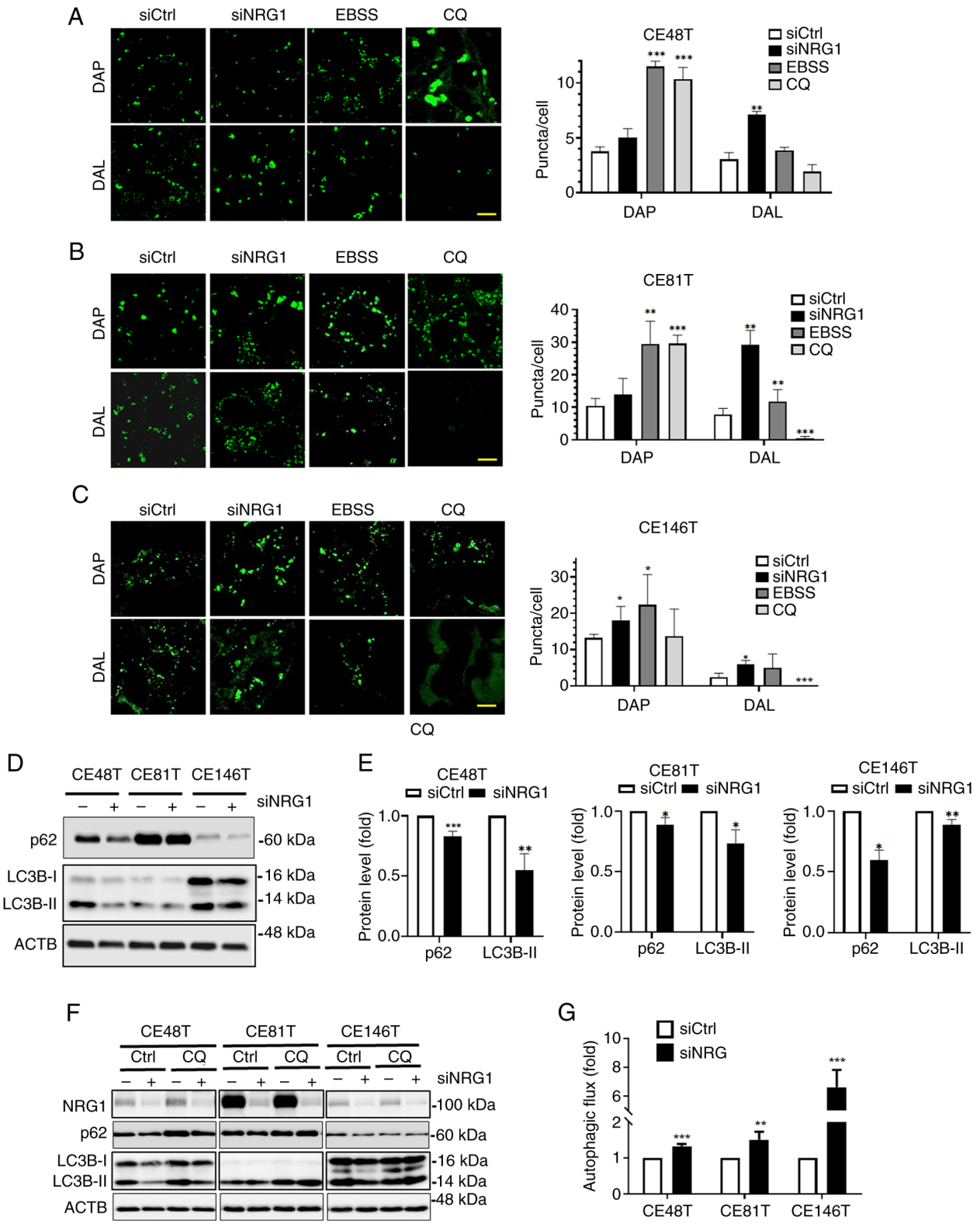

Figure 5. Effect of NRG1 on autophagy activity of ESCC cells. (A) Transfected CE48T, (B) CE81T and (C) CE146T ESCC cells were stained with autophagosome (DAP, 0.1 μM) or autolysosome dye (DAL, 0.5 μM). ESCC cells were treated with CQ for autophagy induction and inhibition, respectively. The autophagosome and autolysosome puncta were observed under a confocal microscope. Scale bar, 10 μm. (D) Protein levels of NRG1, p62 and LC3B following silencing were (E) quantified. (F) Transfected ESCC cells were treated with CQ (20 μM) to examine LC3B-II turnover using immunoblotting. (G) LC3B-II was used to determine autophagic flux. *P<0.05, **P<0.01, ***P<0.001 vs. siCtrl. NRG, neuregulin-1; ESCC, esophageal squamous cell carcinoma; DAP, dye of autophagosome; DAL, dye of autolysosome; EBSS, Earle's Balanced Salt Solution; CQ, chloroquine; si, small interfering; Ctrl, control; ACTB, β-actin.

sphere formation assay and live (green)/dead (red) staining were employed to assess whether combined siNRG1 and CQ affected the ratio of live and dead cells within an ESCC sphere (Fig. 6B and C). ESCC cells transfected with siNRG1 and treated with CQ displayed a significant decrease in live and a significant increase in dead cells within spheres

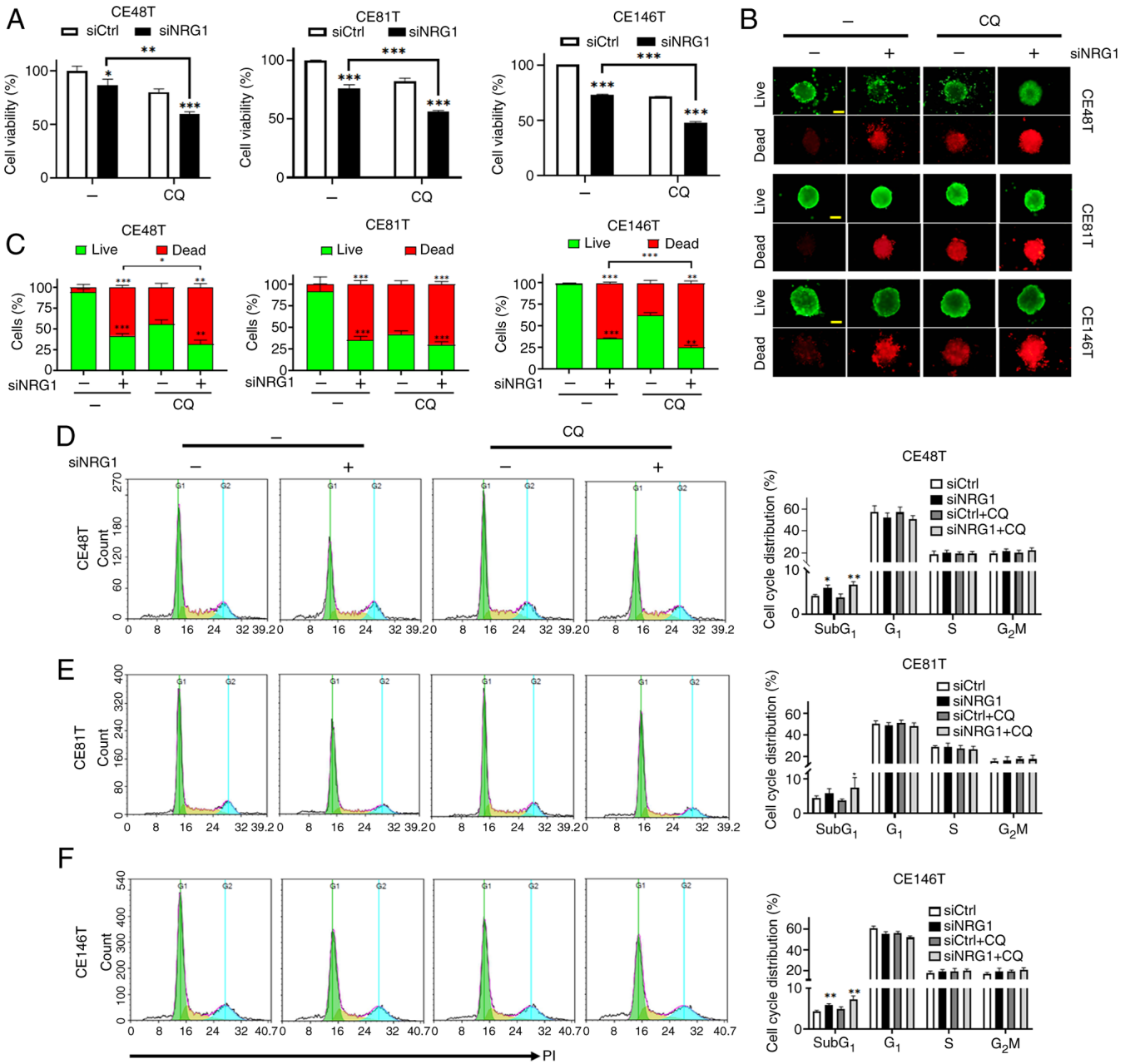

Figure 6. Effects of siNRG1-induced autophagy on cell death of ESCC cells. (A) NRG1 was silenced in ESCC cells. Viability was assessed with CellTiter Glo. (B) CE48T, (C) CE81T and (D) CE146T ESCC cells were stained with propidium iodide to analyze cell cycle progression. (E) Tumor spheres were stained to determine live (green) and dead (red) cells. Scale bar, 100 μm. (F) Viable and dead ESCC cells were quantified. *P<0.05, **P<0.01, ***P<0.001 vs. siCtrl). NRG1, neuregulin-1; ESCC, esophageal squamous cell carcinoma; si, small interfering; Ctrl, control; CQ, chloroquine.

(Fig. 6B and C). Compared with siNRG1, siNRG1 + CQ increased the proportion of dead cells. Therefore, the effect on proliferation or death of ESCC cells was assessed. ESCC cells transfected with siNRG1 with or without CQ exhibited an increase the numbers of cells in subG$_1$ phase (Fig. 6D-F), indicating cell cycle arrest was not induced in a specific phase. Therefore, the combined use of siNRG1 and CQ may not primarily affect proliferation of ESCC cells by regulating the cell cycle progression.

Co-expression of NRG1 and LC3B is associated with unfavorable prognosis of patients with ESCC. Based on the aforementioned results that NRG1 and autophagy may contribute to survival pathways and silencing NRG1 decreases LC3B levels in ESCC cell lines, the present study analyzed data from TCGA to explore the association between NRG1 and LC3B in patients with ESCC (Fig. 7; Table V). LC3B has two isoforms, differing by a single amino acid (C113 vs. Y113) (56); therefore, LC3B1 and LC3B2 were included in the analysis. Kaplan-Meier survival curves revealed that patients with high co-expression of NRG1 and LC3B1 had significantly shorter overall, progression-free interval and disease-specific survival (Fig. 7A-C) compared with those with low co-expression of NRG1 and LC3B1. However, no significant difference was observed in disease-free interval survival (Fig. 7D). Similarly, high co-expression of NRG1 and LC3B2 was associated with

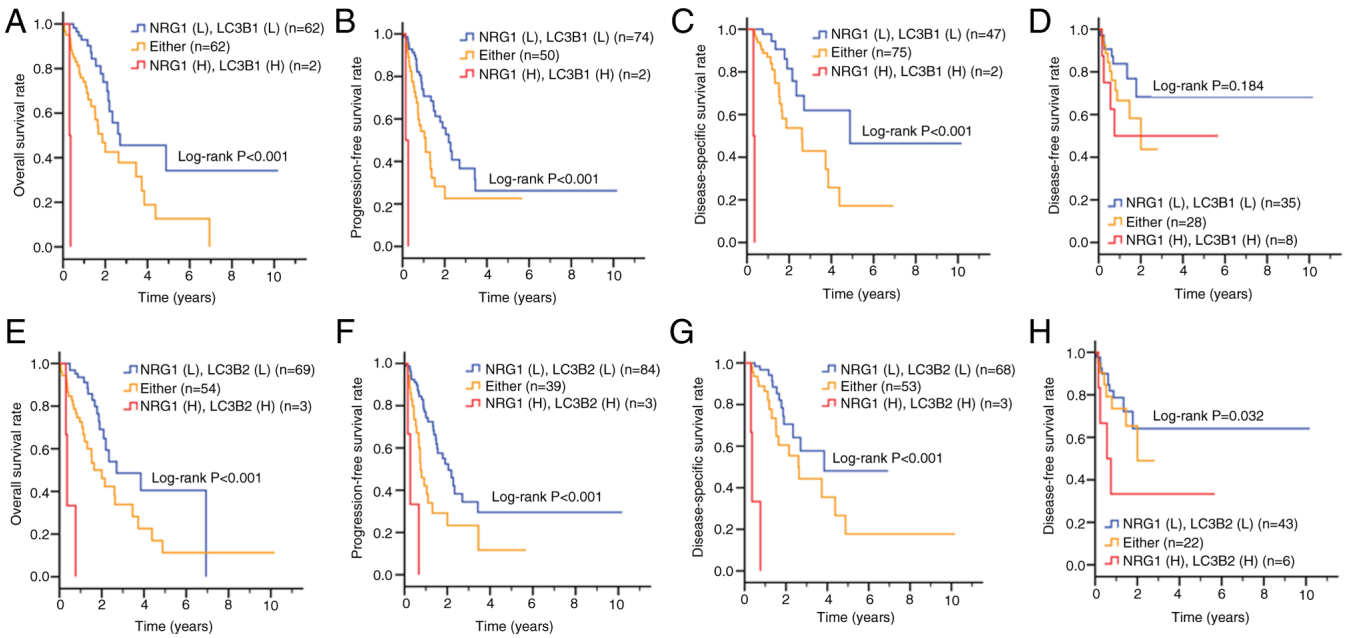

Figure 7. Clinical association between gene expression of NRG1 and LC3B in patients with ESCC. Based on an ESCC dataset from The Cancer Genome Atlas, Kaplan-Meier plots were used to analyze the association between NRG1 and LC3B1 expression of patients with ESCC and (A) overall, (B) progression-free interval, (C) disease-specific and (D) disease-free interval survival. Association between NRG1 and LC3B2 gene expression and (E) overall, (F) progression-free interval, (G) disease-specific and (H) disease-free interval survival. NRG1, neuregulin-1; ESCC, esophageal squamous cell carcinoma; L, low expression; H, high expression; Either, NRG1(H)cRAF(L) or NRG1(L)/cRAF(H); yrs, years.

poorer overall, progression-free interval, disease-specific (Fig. 7E-G) and disease-free interval survival (Fig. 7H).

To account for variations in cell differentiation and AJCC pathological stage, multivariate Cox proportional hazard model was used to evaluate the association between survival outcomes and NRG1 expression alone or in combination with LC3B1 (Table V). Patients with high co-expression of NRG1 and LC3B1 showed significantly increased risk of mortality compared with those with NRG1(low)/LC3B1(low). Patients with high co-expression of NRG1 and LC3B1 had higher risk for overall (AHR, 50.98; CI, 9.25-280.88), progression-free interval (AHR, 34.31, CI, 6.86-171.71) and disease-specific survival (AHR, 360.05, CI, 29.44-4403.07; Table V). Likewise, patients with high co-expression of NRG1 and LC3B2 had significantly worse overall (AHR, 23.11, CI, 6.19-86.25), progression-free interval (AHR, 12.65, CI, 3.68-43.47), disease-specific (AHR, 48.50, CI, 11.53-204.10) and disease-free interval survival (AHR, 3.71, CI, 1.16-11.87; Table VI). These findings suggest that high co-expression of NRG1 and LC3B may contribute to tumor progression and relapse in ESCC.

Discussion

NRG1 serves a dual role in cancer development; however, its specific role in ESCC remains unclear. Here, NRG1 gene and protein levels were elevated in tumor tissue and associated with poor outcomes in patients with ESCC (Fig. 8). Silencing NRG1 led to cancer cell death and decreased tumor sphere formation, accompanied by decreased phosphorylation of AKT and cRAF. Co-expression of NRG1 and cRAF increased mortality risk of overall and progression-free survival. Silencing NRG1 triggered cytoprotective autophagy, evidenced by increased autophagosome/autolysosome formation and autophagic flux. CQ enhanced cancer cell death in NRG1-deficient ESCC cells. Patients with high co-expression of NRG1 and LC3B1 or LC3B2 had worse prognosis compared with those with low co-expression. Given the poor prognosis and treatment outcomes for ESCC, the present findings suggested that NRG1 may serve as a promising biomarker and therapeutic target. Combination of siNRG1 and CQ, which showed an enhanced inhibitory effect, highlights its potential for use as a viable treatment strategy for ESCC.

NRG1, a member of the NRG family, is a ligand for the HER3 receptor associated with aspects of tumor progression in numerous types of human cancer, such as lung cancer, breast cancer and prostate cancer (57-59). These aspects include cell proliferation, differentiation, angiogenesis and metastasis. NRG1 is overexpressed in various types of cancer (57-59) and activates downstream signaling pathways such as MAPK and PI3K by binding members of the HER family (60). In non-small cell lung cancer, blocking the NRG1 signaling pathway may inhibit tumor growth and enhance response to chemotherapy (61). These findings indicate that NRG1 serves as an oncogene in cancer development. Conversely, other studies have reported decreased NRG1 expression in breast cancer cell lines due to gene methylation; loss of NRG1 gene can lead to chromosomal abnormalities in breast and colon cancer (62,63). NRG1 may serve a suppressor role in the development of lung adenocarcinoma and may be associated with AKT and ERK1/2 pathways (64). Hence, NRG1 may serve a dual role in tumors, functioning as both an oncogene and tumor suppressor gene depending on the type of cancer. Despite elevated expression of NRG1 in numerous types of cancer (57-59), its role in ESCC remains unclear. Here, the upregulation of mRNA and protein levels of NRG1 was observed in ESCC specimens. The

Table V. Effect of NRG1 and LC3B1 co-expression on survival of patients with esophageal squamous cell carcinoma.

A, Overall survival

Variable	Expression	n (%)	CHR (95% CI)	P-value	AHR (95% CI)	P-value
NRG1	Low	120 (95.2)	1.00		1.00	
	High	6 (4.8)	2.01 (0.62-6.52)	0.244[a]	3.40 (0.99-11.71)	0.052[b]
LC3B1	Low	66 (52.4)	1.00		1.00	
	High	60 (47.6)	2.56 (1.43-4.57)	0.001[a]	2.17 (1.21-3.91)	0.010[b]
NRG1 (L), LC3B1 (L)	-	62 (49.2)	1.00		1.00	
Either	-	62 (49.2)	2.10 (1.18-3.72)	0.011[a]	2.36 (1.30-4.27)	0.005[c]
NRG1 (H), LC3B1 (H)	-	2 (1.6)	30.50 (5.82-159.70)	<0.001[a]	50.98 (9.25-280.88)	<0.001[c]

B, Progression-free interval survival

Variable	ROC	n (%)	CHR (95% CI)	P-value	AHR (95% CI)	P-value
NRG1	Low	120 (95.2)	1.00		1.00	
	High	6 (4.8)	2.93 (1.16-7.38)	0.023[a]	5.11 (1.90-13.72)	0.001[b]
LC3B1	Low	78 (61.9)	1.00		1.00	
	High	48 (38.1)	1.94 (1.16-3.24)	0.012[a]	1.90 (1.09-3.31)	0.024[b]
NRG1 (L), LC3B1 (L)	-	74 (58.7)	1.00		1.00	
Either	-	50 (39.7)	1.81 (1.09-3.02)	0.022[a]	1.95 (1.16-3.27)	0.012[c]
NRG1 (H), LC3B1 (H)	-	2 (1.6)	25.06 (5.14-122.25)	<0.001[a]	34.31 (6.86-171.71)	<0.001[c]

C, Disease-specific survival

Variable	ROC	n (%)	CHR (95% CI)	P-value	AHR (95% CI)	P-value
NRG1	Low	118 (95.2)	1.00		1.00	
	High	6 (4.8)	3.17 (0.96-10.53)	0.059[a]	7.76 (2.04-29.53)	0.003[b]
LC3B1	Low	51 (41.1)	1.00		1.00	
	High	73 (58.9)	2.78 (1.32-5.84)	0.007[a]	2.42 (1.14-5.16)	0.022[b]
NRG1 (L), LC3B1 (L)	-	47 (37.9)	1.00		1.00	
Either	-	75 (60.5)	2.16 (1.05-4.46)	0.037[a]	2.71 (1.25-5.89)	0.012[c]
NRG1 (H), LC3B1 (H)	-	2 (1.6)	177.84 (15.73-2010.96)	<0.001[a]	360.05 (29.44-4403.07)	<0.001[c]

D, Disease-free interval survival

Variable	ROC	n (%)	CHR (95% CI)	P-value	AHR (95% CI)	P-value
NRG1	Low	52 (73.2)	1.00		1.00	
	High	19 (26.8)	1.29 (0.52-3.20)	0.580[a]	1.97 (0.75-5.16)	0.169[b]
LC3B1	Low	46 (64.8)	1.00		1.00	
	High	25 (35.2)	2.53 (1.07-5.98)	0.035[a]	3.24 (1.34-7.84)	0.009[b]
NRG1 (L), LC3B1 (L)	-	35 (49.3)	1.00		1.00	
Either	-	28 (39.4)	1.47 (0.63-3.47)	0.375[a]	1.94 (0.74-5.10)	0.179[c]
NRG1 (H), LC3B1 (H)	-	8 (11.3)	2.10 (0.70-6.24)	0.184[a]	2.94 (0.86-10.05)	0.087[c]

L, low; H, high expression; Either, either NRG1(H)LC3B1(L) or NRG1(L)/LC3B1(H); NRG, neuregulin-1; CHR, crude hazard ratio; CI, confidence interval; AHR, adjusted hazard ratio. [a]Cox's regression; [b]adjusted for differentiation and American Joint Committee on Cancer pathological stage; [c]multivariate Cox's regression; -, not applicable.

high expression of NRG1 was associated with worse survival in patients with ESCC with poorly differentiated tumors and advanced AJCC stage and lymph node invasion. The present results demonstrated that silencing NRG1 leads to a significant

Table VI. Effect of NRG1 and LC3B2 co-expression on survival of patients with esophageal squamous cell carcinoma.

A, Overall survival

Variable	Expression	n (%)	CHR (95% CI)	P-value	AHR (95% CI)	P-value
NRG1	Low	120 (95.2)	1.00		1.00	
	High	6 (4.8)	2.01 (0.62-6.52)	0.244[a]	3.40 (0.99-11.71)	0.052[b]
LC3B2	Low	72 (57.1)	1.00		1.00	
	High	54 (42.9)	2.79 (1.56-4.98)	0.001[a]	2.39 (1.32-4.34)	0.004[b]
NRG1 (L), LC3B2 (L)	-	69 (54.8)	1.00		1.00	
Either	-	54 (42.9)	1.98 (1.12-3.47)	0.018[a]	2.29 (1.27-4.14)	0.006[c]
NRG1 (H), LC3B2 (H)	-	3 (2.4)	14.94 (4.22-52.93)	<0.001[a]	23.11 (6.19-86.25)	<0.001[c]

B, Progression-free interval survival

Variable	ROC	n (%)	CHR (95% CI)	P-value	AHR (95% CI)	P-value
NRG1	Low	120 (95.2)	1.00		1.00	
	High	6 (4.8)	2.93 (1.16-7.38)	0.023[a]	5.11 (1.90-13.72)	0.001[b]
LC3B2	Low	87 (69.0)	1.00		1.00	
	High	39 (31.0)	2.53 (1.51-4.26)	<0.001[a]	2.33 (1.37-3.98)	0.002[b]
NRG1 (L), LC3B2 (L)	-	84 (66.7)	1.00		1.00	
Either	-	39 (31.0)	2.11 (1.25-3.55)	0.005[a]	2.30 (1.36-3.92)	0.002[c]
NRG1 (H), LC3B2 (H)	-	3 (2.4)	9.34 (2.78-31.35)	<0.001[a]	12.65 (3.68-43.47)	<0.001[c]

C, Disease-specific survival

Variable	ROC	n (%)	CHR (95% CI)	P-value	AHR (95% CI)	P-value
NRG1	Low	118 (95.2)	1.00		1.00	
	High	6 (4.8)	3.17 (0.96-10.53)	0.059[a]	7.76 (2.04-29.53)	0.003[b]
LC3B2	Low	71 (57.3)	1.00		1.00	
	High	53 (42.7)	2.66 (1.33-5.34)	0.006[a]	2.15 (1.06-4.38)	0.034[b]
NRG1 (L), LC3B2 (L)	-	68 (54.8)	1.00		1.00	
Either	-	53 (42.7)	1.68 (0.85-3.30)	0.136[a]	2.06 (1.01-4.24)	0.048[c]
NRG1 (H), LC3B2 (H)	-	3 (2.4)	33.66 (8.53-132.90)	<0.001[a]	48.50 (11.53-204.10)	<0.001[c]

D, Disease-free interval survival

Variable	ROC	n (%)	CHR (95% CI)	P-value	AHR (95% CI)	P-value
NRG1	Low	52 (73.2)	1.00		1.00	
	High	19 (26.8)	1.29 (0.52-3.20)	0.580[a]	1.97 (0.75-5.16)	0.169[b]
LC3B2	Low	56 (78.9)	1.00		1.00	
	High	15 (21.1)	3.00 (1.23-7.27)	0.015[a]	3.87 (1.55-9.69)	0.004[b]
NRG1 (L), LC3B2 (L)	-	43 (60.6)	1.00		1.00	
Either	-	22 (31.0)	1.08 (0.43-2.67)	0.876[a]	1.36 (0.52-3.58)	0.537[c]
NRG1 (H), LC3B2 (H)	-	6 (8.5)	3.31 (1.11-9.89)	0.032[a]	3.71 (1.16-11.87)	0.027[c]

L, low; H, high expression; Either, either NRG1(H)LC3B2(L) or NRG1(L)/LC3B2(H); NRG, neuregulin-1; CHR, crude hazard ratio; CI, confidence interval; AHR, adjusted hazard ratio. [a]Cox's regression; [b]adjusted for differentiation and American Joint Committee on Cancer pathological stage; [c]multivariate Cox's regression; -, not applicable.

decrease in viability, colony formation, migration and invasion of ESCC cell lines.

NRG1 isoforms are predominantly expressed in different organs, serving a key role in proliferation, survival, migration

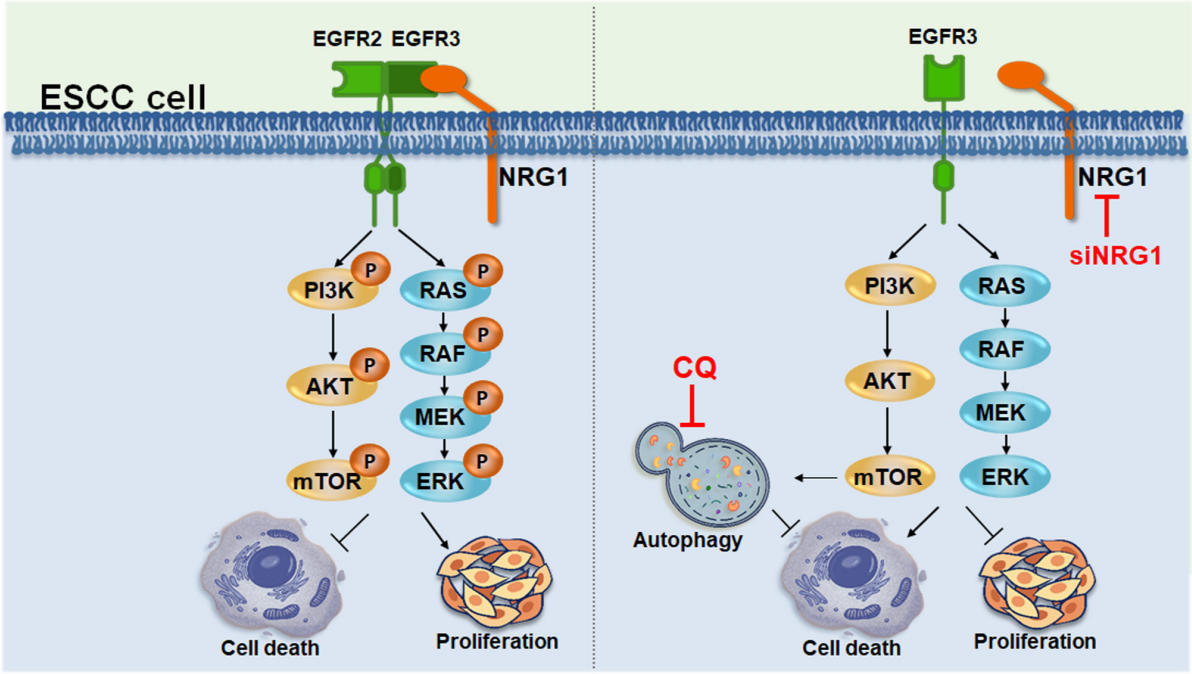

Figure 8. Effects of NRG1 on autophagy and viability of ESCC cells. NRG1 modulates signaling for cell proliferation and survival in ESCC. Downregulation of NRG1 reduces cell proliferation induces death and activates autophagy as a survival pathway in response to NRG1 silencing-induced cell death. ESCC, esophageal squamous cell carcinoma; NRG, neuregulin-1; si, small interfering; CQ, chloroquine.

and differentiation of various types of cell, including epithelial, nerve, cardiac and skeletal muscle cells (65). NRG1 mediates activation of downstream signaling pathways associated with malignancy. Several gene fusions associated with NRG1 have been identified in lung cancer, including CD47-NRG1, Syndecan-4-NRG1, RNA binding protein with multiple splicing -NRG1, Werner syndrome protein (WRN)-NRG1 and Solute carrier family 3 member 2 (SLC3A2)-NRG1 (20,66,67). NRG1 is abnormally expressed in various types of tumor and is associated with aspects of tumor progression, such as cell proliferation, differentiation, invasion and metastasis (58,59). The molecular weight of NRG1 observed in SDS-PAGE is 25% higher than expected, which is due to protein modification glycosylation (68). The present study indicated that NRG1 was highly expressed in patients with ESCC and associated with poor prognosis. However, the role of specific isoforms, gene translocation or post-translation modification of NRG1 in ESCC remains unclear; thus, further investigations are required to determine the association between NRG1 isoforms/localization and post-translation modification with prognosis in patients with ESCC. The investigation of these isoforms and modifications may lead to identification of therapeutic biomarkers for ESCC and facilitate development of treatment strategies.

siRNA-mediated NRG1 silencing experiments in CE48T, CE81T and CE146T cell lines revealed a decrease in downstream signaling molecules, including p-AKT and p-cRAF, following NRG1 silencing, thereby influencing the associated MAPK and PI3K pathway. Both MAPK and PI3K pathways are required for cell proliferation and mobility (40). ESCC cell lines silenced with siNRG1 exhibited decreased cell proliferation, migration, viability and spheroid formation, confirming the key role of NRG1 as an oncogene in ESCC. Moreover, AKT and cRAF negatively regulate autophagy (53,54), which allows cancer cell survive in stressed conditions, such as hypoxia, suspension growth and chemotherapeutic stress. Induction of autophagy was evident following NRG1 silencing. Using DAP and DAL, the present study observed a significant increase in numbers of autophagosomes and autolysosomes following NRG1 silencing. p62 and LC3-II protein levels decreased following NRG1 silencing. Silencing NRG1 increased LC3-II flux when co-treated with autophagy inhibitor. These findings suggested that NRG1 silencing may inactivate AKT and cRAF to enhance autophagy in ESCC cells. Notably, combination of NRG1 silencing and autophagy inhibition, as demonstrated by live/dead staining following treatment with CQ, resulted in increased cytotoxicity against ESCC cells. Following NRG1 silencing, autophagy was activated to allow cancer cells to survive, suggesting that a potential treatment strategy for ESCC may involve autophagy inhibitors. Moreover, NRG1 and autophagy serve key roles on survival of ECSS cells. Patients with ESCC with high co-expression of NRG1 and LC3B had higher mortality risk compared with those with low co-expression of NRG1 and LC3B. Although further studies with a greater number of cases and different cohorts are required to determine the association between NRG1 and autophagy markers in ESCC, combining siNRG1 and autophagy inhibitors may be an alternative treatment strategy to improve outcomes for patients with ESCC.

Taken together, the present study demonstrated that elevated levels of NRG1 were associated with tumor progression of ESCC. Silencing NRG1 inhibited proliferation and migration of ESCC cells. Co-expression of NRG1 and cRAF may be key for malignancy and prognosis of patients with ESCC. Furthermore, autophagy may serve as a survival mechanism in ESCC cells in which NRG1 is silenced. While siRNA-based

results of the present study support the oncogenic role of NRG1 in ESCC cells, further investigations involving overexpression of NRG1 in ESCC cells with low NRG1 expression are necessary to confirm whether NRG1-mediated downstream factors contribute to cell proliferation and mobility.

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Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Authors' contributions

CWS and YGG conceived the study, confirm the authenticity of all the raw data and reviewed the manuscript. HWC, CHL and CCC analyzed data. YRC, WHY, CWS, HWC, CCC, YCT and PFL performed experiments and interpreted data. PFL, YCT and CWS designed the experiments. CWS and YCT wrote the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

This project was approved by the Ethics Committee of the Kaohsiung Veterans General Hospital (approval nos. VGHKS 95-CT3-21 and VGHKS 15-CT12-10). Written informed consent was obtained from all subjects.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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