Title: Calreticulin promotes migration and invasion of esophageal cancer cells by up-regulating neuropilin-1 expression via STAT5A

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Running Title: CRT promotes cell migration and invasion by up-regulating NRP1

Keywords: esophageal squamous cell carcinoma, CRT, NRP1, STAT5, migration, invasion

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**Translational Relevance**

Esophageal squamous cell carcinoma (ESCC) is one of the most common malignancies worldwide. More than half of the patients have unresectable tumors or metastases at the time of presentation, and metastasis is responsible for most of the deaths. Therefore, identifying the mechanism of ESCC metastasis is critical for effective treatment. Our study revealed a novel signal pathway, CRT-STAT5A-NRP1, contributing to migration and invasion of esophageal cancer cells. We found that inhibition of NRP1 reduced ESCC cell motility *in vitro* and metastasis *in vivo*. And also, NRP1 expression positively correlated with CRT expression in ESCC tissues. These results suggested that they might be potential molecular therapeutic targets for ESCC metastasis.
Abstract

**Purpose:** We previously revealed that calreticulin (CRT) gene is a candidate oncogene promoting cell migration and invasion and that neuropilin-1 (NRP1) is a possible effector downstream of CRT in esophageal squamous carcinoma cells. This study aims to explore the mechanisms underlying the migration and invasion of esophageal cancer cells regulated by CRT through NRP1.

**Experimental Design:** Quantitative reverse-transcription polymerase chain reaction, western blot analysis, Chromatin immunoprecipitation and reporter gene assays were utilized to investigate the relationship between CRT and NRP1. *In vitro* and *in vivo* assays were carried out to evaluate the effects of NRP1 on malignant phenotypes of ESCC cells and tumor metastasis in NOD/SCID mice. Immunohistochemistry was performed to analyze the expression of CRT and NRP1 in esophageal squamous cell carcinomas (ESCC).

**Results:** Knockdown of CRT decreased the expression of NRP1. Inhibition of NRP1 reduced ESCC cell motility *in vitro* and experimental metastasis *in vivo*. Ectopic expression of NRP1 rescued the defects of cell migration and invasion in CRT-shRNA cells. CRT depletion inhibited STAT5A phosphorylation at the Y694 site via a CaMKII-independent pathway. Moreover, STAT5A directly regulated NRP1 transcription. Knockdown of CRT or NRP1 led to a down-regulation of MMP2, MMP9 and FAK. Notably, positive correlation was found between CRT and NRP1 expression in ESCC tissues (*P* = 5.87×10⁻⁵). CRT and NRP1 co-expression was significantly associated with lymph node metastasis (*P* = 0.025).
Conclusions: Our findings suggest that NRP1 is a critical downstream effector of CRT in promoting cell migration and invasion, which might contribute to the metastasis of ESCC.

Keywords: esophageal squamous cell carcinoma, CRT, NRP1, STAT5, migration, invasion

Introduction

Esophageal cancer is one of the most common malignancies worldwide (1). Most Esophageal cancers in China are squamous cell carcinomas (ESCCs). More than half of the patients have unresectable tumors or metastases at the time of presentation, and metastasis is responsible for most of the deaths (2). Cancer cell migration and invasion are parts of the key steps during metastasis. Therefore, identifying the mechanism of ESCC metastasis is critical for effective treatment.

In our previous study, we found that calreticulin (CRT) was up-regulated in ESCC tissues (3). CRT is a calcium binding protein which is crucial for many cellular processes (4). High levels of CRT are significantly associated with poor outcome and metastasis in patients with gastric cancer (5), bladder cancer (6) and breast cancer (7, 8). Several observations show that CRT overexpression contributes to cancer cell survival, migration and invasion (9), but the underlying molecular mechanisms are poorly understood.

We recently found that neuropilin-1 (NRP1) is a potent downstream effector of CRT in esophageal cancer cells (10). NRP1 is a single pass transmembrane glycoprotein
It lacks a defined signaling role, but could mediate functional responses in complex with other receptors, such as VEGFR2 (12) and EGFR (13). NRP1 enhances tumor growth and invasiveness in breast cancer, colorectal cancer, lung cancer, and prostate cancer (14). Here, we demonstrate that CRT promotes the migration, invasion and experimental metastasis of esophageal cancer cells by up-regulating NRP1 expression.

Materials and Methods

Patients and tissue specimens

Fresh ESCC tissues were procured from surgical resection specimens collected by the Department of Pathology at the Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College (CAMS & PUMC), Beijing, China. All patients received no treatment before surgery and signed informed consent forms of the Cancer Hospital, CAMS & PUMC for sample collection. Primary tumor regions and morphologically normal operative margin tissues from the same patients were separated by experienced pathologists, and immediately stored at –70 °C until use. The study has been approved by the Ethics Committee of Cancer Institute (Hospital), CAMS & PUMC (No. 12-097/631).

Immunohistochemistry

Tissue microarrays (TMA) containing 218 primary esophageal tumors and the corresponding normal epithelium were created. Briefly, operative ESCC tissues and morphologically normal operative margins were routinely formalin-fixed and
paraffin-embedded. Then a section of 4 μm was made from the paraffin donor block and stained with hematoxylin-eosin (HE). The HE sections for each case were examined by experienced pathologist, and the areas of best-preserved cancer tissue and normal epithelium (not connective tissue or other) were labeled and chosen for TMA construction. The source areas labeled on the slide were copied onto paraffin block and tissue cores were retrieved and transferred into recipient block. For each case, there were three cancer tissue cores and two morphologically normal operative margin cores. From the TMA blocks, sections were routinely prepared and used for IHC. Immunohistochemical analysis was done as described previously (3). The slides were deparaffinized, rehydrated, and immersed in 3% hydrogen peroxide solution for 10 min. For antigen retrieval, the slides were heated in citrate buffer (pH 6.0) for 25 min at 95°C, and cooled for 60 min at room temperature. Between each incubation step, the slides were washed with PBS (pH 7.4). Then the slides were incubated separately with anti-CRT (Abcam) or anti-NRP1 antibody (Epitomics) overnight at 4°C. Immunostaining was performed using the PV-9000 Polymer Detection System with diaminobenzidine (DAB) according to manufacturer recommendations (GBI, USA) and subsequently counterstained with hematoxylin. The results were evaluated separately by two independent observers. The staining intensity was scored into four grades: 0 (negative), 1 (weak), 2 (moderate), and 3 (strong).

**Cell culture and treatments**

The human ESCC cell lines KYSE150 and KYSE450 were generously provided by Dr. Y. Shimada (Kyoto University, Kyoto, Japan). Cells were cultured in RPMI 1640
medium containing 10% fetal bovine serum (Invitrogen), penicillin (100 U/ml), and streptomycin (100 mg/ml).

Cells were transfected with siRNA or plasmid vectors using Lipofectamin 2000 (Invitrogen) according to the manufacturer’s instruction. The small interfering RNA (siRNA) target sequences, plasmid constructs and stable clone selections are described in the Supplementary Materials and Methods.

ESCC cell lines incubated with CaMKII inhibitor KN-93 (Sigma) at 100 μmol/L for 24 hours.

**Western blot analysis**

Immunoblotting was performed with primary antibodies against CRT (Abcam), NRP1 (Epitomics), STAT5 (Cell signaling Technology), phosphorylated STAT5A (p-STAT5A) (Tyr694) (Cell signaling Technology), MMP2 (Abgent), MMP9 (Abgent), FAK (Cell signaling Technology), E-cadherin (Cell signaling Technology), β-catenin (Cell signaling Technology). GAPDH (Proteintech) was used as a loading control. The signal was visualized with super enhanced chemiluminescence (ECL) detection reagent (Applygen, Beijing, China).

**RNA isolation and real-time PCR**

Total RNA was isolated from KYSE150 and KYSE450 cells transfected with non-specific control or gene-specific siRNAs using the Rneasy Mini kit (Qiagen) according to manufacturer’s instruction. Isolated RNA was used as a template for reverse transcription reaction (Invitrogen).

Quantitative real-time PCR analysis was performed in triplicate using FastStart
Universal SYBR Green Master (Roche) on a CFX96 Real-Time System (Bio-Rad). The relative mRNA expression of the target gene was normalized to an endogenous reference (GAPDH). The primers are listed in the Supplementary Materials and Methods.

**Cell proliferation assay**

Cells (1.5×10³/100 µl) were seeded on 96-well plates with three replicates. A cell Counting Kit 8 (CCK-8; Dojindo Laboratories) was used to evaluate cell proliferation. 10 µl of CCK-8 solution was added to each well and incubated for 1 hour. Absorbance was measured at a wavelength of 450 nm by an Elx 808 Microplate Reader (BioTek).

**Haptotactic migration assay and Matrigel chemoinvasion assay**

The migration and invasion assays were performed on transwell plates. For cell migration assay, 1×10⁵ cells were seeded on a polycarbonate membrane insert in a transwell apparatus (Corning Costar) and cultured in RPMI 1640 without serum. RPMI 1640 containing 20% fetal bovine serum was added to the lower chamber. After incubation for 24 hours at 37 °C in a CO₂ incubator, the insert was washed with PBS, and cells on the top surface of the insert were removed by wiping with a cotton swab. Cells that migrated to the bottom surface of the insert were fixed with methanol, stained with 0.4% crystal violet, and counted in five random fields at 200×.

For the Matrigel chemoinvasion assay, the procedure was similar to the cell migration assay, except that the transwell membrane was coated with 300 ng/mL Matrigel (BD Biosciences), and the cells were incubated for 36 hours at 37 °C.

**NRP1 promoter analysis**
A 2,000 bp sequence upstream of the transcription start site of NRP1 harboring its promoter, was retrieved from the UCSC Genome Bioinformatics site (http://genome.ucsc.edu). Potential transcription factor binding sites were analyzed by Transcription Factor Search (http://mbs.cbrc.jp/research/db/TFSEARCH.html).

**Luciferase assay**

The luciferase reporter assays were performed in triplicate by the Dual-Luciferase Reporter Assay System (Promega). The transfection efficiency was measured by co-transfection with a Renilla luciferase expression plasmid pRL-SV40 (Promega). The data are presented as the ratio of firefly luciferase activity to Renilla luciferase activity. The results are presented as the mean ± SD.

**Chromatin immunoprecipitation**

Chromatin immunoprecipitation (ChIP) assays were performed with the SimpleChIP® Enzymatic Chromatin IP Kit (Cell Signaling Technology). Chromatin samples were immunoprecipitated with either anti-STAT5 antibody (Cell Signaling Technology) or rabbit IgG (Cell Signaling Technology) as a negative control. Precipitated DNA was amplified by PCR using primers provided in the Supplementary Materials and Methods. Non-immunoprecipitated chromatin fragments were used as an input control.

**Xenograft assays in NOD/SCID mice**

The research protocols involving animal studies were approved by Beijing Medical Experimental Animal Care Commission. Stable KYSE150 clones were injected into age-matched female NOD/SCID mice (Beijing HFK Bioscience). For each group, six
mice were injected with $1 \times 10^6$ cells per animal via the tail vein. The mice were sacrificed 6 weeks after injection and examined for lung metastases. The tissues were fixed in Bouin’s solution, embedded in paraffin, sectioned and stained with HE.

**Statistical analysis**

All statistical analyses were performed using SPSS17.0 software. We statistically evaluated experimental results using the Student t test, the Pearson chi-square test, and the ANOVA test. $P < 0.05$ was considered statistically significant.

**Results**

**CRT positively regulates NRP1 expression in ESCC cells**

We previously reported that NRP1 is a potent effector downstream of CRT in esophageal cancer cells (10). Real-time PCR and western blot confirmed that NRP1 mRNA and protein expression were significantly down-regulated in CRT-siRNA cells as compared with those in non-silencing siRNA cells (Fig. 1A-B; Supplementary Fig. S1). In contrast, knockdown of NRP1 did not influence CRT expression (Fig. 2A). The observation indicates that CRT positively regulates NRP1 expression in ESCC cells.

**Reduced NRP1 expression decreases ESCC cell migration and invasion**

Based on that reduced CRT expression decreased ESCC cell migration and invasion (15), we examined the effect of NRP1 on ESCC cell motility. Transwell migration assays with KYSE150 and KYSE450 cells revealed that after treatment with NRP1-siRNA, fewer cells migrated to the bottom of the chamber, compared with
non-silencing siRNA cells (Fig. 2A-C). Whereas the cell proliferation rates were unaltered at 24 hours after seeding (Fig. 2D), suggesting that NRP1 promoted ESCC cell migration. Matrigel chemoinvasion assays showed that, knockdown of NRP1 inhibited ESCC cell invasion (Fig. 2A, B, E and F).

Next, we investigated the functional contribution of NRP1 to metastasis formation in vivo. KYSE150 cells stably expressing a shRNA against NRP1 (sh-NRP1) and control-transfected cells (Ctrl) were injected via tail vein into NOD/SCID mice. Six weeks after injection, the animals were sacrificed. Both groups developed visually observable lung nodules. However, the average number of lung metastasis nodules in sh-NRP1 group was significantly fewer than the control group (Fig. 3B). Tissues were isolated and sectioned, and HE staining showed that lung tissues from control group were heavily infiltrated by metastasized ESCC cells as compared to those from sh-NRP1 group (Fig. 3A). Collectively, inhibition of NRP1 decreased ESCC cell motility in vitro and experimental metastasis in vivo.

**CRT modulates ESCC cell migration and invasion through NRP1**

KYSE150 and KYSE450 cells stably expressing CRT shRNA reduced NRP1 expression, cell migration and invasion potential. Ectopic expression of NRP1 expression restored cell migration and invasion (Fig. 4A-D). The cell proliferation rates were not changed at 24 and 36 hours after seeding (Fig. 4C and D), suggesting that CRT promotes cell migration and invasion via NRP1.

To explore the molecular mechanism by which CRT or NRP1 enhanced cell invasive potential, we examined the effect of CRT or NRP1 on some
metastasis-related proteins: E-cadherin (16), β-catenin (17), FAK (18), MMP2 (19), and MMP9 (20). Western blot analysis indicated that FAK, MMP2 and MMP9 were down-regulated in CRT-siRNA or NRP1-siRNA cells. There was no difference in E-cadherin and β-catenin expression (Fig. 3C). Expression of exogenous NRP1 restored the expression of FAK, MMP2 and MMP9 in CRT-silenced ESCC cells (Fig. 4A).

**CRT promotes NRP1 transcription through STAT5A in ESCC cells**

Knockdown of CRT decreased *NRP1* promoter activity (Fig. 5A). However, there is no information available about CRT as transcriptional factor. Thus we hypothesized that CRT might indirectly modulate *NRP1* transcription through some transcription factors. Online database searching (http://mbs.cbrc.jp/research/db/TFSEARCH.html) revealed several STAT5-binding sites at -1140, -1089 and +139 bp from the transcription initiation site within the *NRP1* promoter region (Fig. 5D). ChIP assays showed that STAT5 directly bound to the -1166/-1020 region of the *NRP1* promoter (Fig. 5C).

Signal transducer and activator of transcription 5 (STAT5) refers to two highly homologous proteins, STAT5A and STAT5B. We tested the effect of STAT5A and STAT5B on NRP1 expression. NRP1 expression was significantly decreased in STAT5A-siRNA group, and no marked change was observed in STAT5B-siRNA group (Fig. 5B; Supplementary Fig. S2). Thus, NRP1 expression was regulated mainly by STAT5A.

We subsequently generated a luciferase reporter vector, pGL3-NRP1, with *NRP1*
promoter fragment (-1185 to -740). Activity of the reporter gene in STAT5A-siRNA group was significantly lower than that in non-silencing group (Fig. 5A). Moreover, deletion of the STAT5A-binding sites (from -1140 to -1132 and from -1089 to -1081), pGL3-NRP1-DM, significantly attenuated \( NRPI \) promoter activity in KYSE150 and KYSE450 cells (Fig. 5E). These results confirmed that \( NRPI \) is a direct transcriptional target of STAT5A.

We further detected possible regulation of CRT to STAT5A activity. Western blot analysis revealed that p-STAT5A (Tyr\(^{694}\)) level was decreased in CRT-siRNA cells (Fig. 5F). It has been reported that CaMKII inhibitor KN-93 could reduce Tyr-694 phosphorylation of STAT5A (21). However, the treatment of ESCC cell lines with KN-93 did not significantly change the p-STAT5A level (Supplementary Fig. S3), suggesting that CRT regulates STAT5A phosphorylation independent of CaMKII.

**NRPI expression positively correlates with CRT in ESCC tissues**

Our previous study has shown that CRT was up-regulated in ESCC tissues (3). To confirm the relationship between CRT and NRPI observed in cell lines, we detected the expression of NRPI in ESCC tissues and analyzed the expression of the two proteins by immunohistochemistry using serial sections from the same tissue microarrays. Among the 218 ESCC specimens, strong CRT and NRPI immunostaining were observed in 34.9% (76 of 218) and 32.6% (71 of 218) of tumors, respectively. High levels of NRPI expression was found in 50% (38 of 76) tumors with CRT overexpression, whereas NRPI presented negative or weak staining in 76.8% (109 of 142) tumors with negative or low CRT expression. Statistical analysis
with Pearson chi-square test revealed a significant positive correlation between the overexpression of CRT and NRP1 ($P = 5.87\times10^{-5}$, Fig. 6 and Supplementary Table S1).

**CRT and NRP1 expression is associated with lymph node metastasis**

We have previously shown that the overexpression of CRT correlated with a poor prognosis (3). We further determined possible correlation between NRP1 expression and clinicopathologic parameters. Statistical analysis displayed that high NRP1 expression was significantly associated with lymph node metastasis ($P = 0.043$) but not with other clinicopathologic parameters. Then we wondered whether there is also a correlation between CRT/NRP1 co-expression and lymph node metastasis. We divided the patients into three groups: both high levels of CRT and NRP1 expression (CRT$_{\text{high}}$ NRP1$_{\text{high}}$), only one high level of CRT or NRP1 expression (CRT$_{\text{high}}$ OR NRP1$_{\text{high}}$) and both low levels of CRT and NRP1 expression (CRT$_{\text{low}}$ NRP1$_{\text{low}}$). Statistical analysis indicated that CRT and NRP1 co-expression was significantly associated with lymph node metastasis ($P = 0.025$, Supplementary Table S2).

**Discussion**

Metastasis is a multistep process in which cancer cell invasion and migration are responsible for penetrating through capillary endothelia and into the secondary organs (22). In this study, we investigated the mechanism of how CRT enhanced cell migration and invasion and identified NRP1 as a downstream effector of CRT.

It has been reported that NRP1 expression is increased in various tumor types (23).
High NRP1 level is significantly associated with metastasis in patients with colon cancer (24), nasopharyngeal carcinoma (25), and esophageal adenocarcinoma (26). Interestingly, NRP1 promotes the invasion of renal carcinoma 786-O cell line (27) and pancreatic cancer COLO-357 cell line (28), but suppresses the migration in another pancreatic cell line, PANC-1 (29). These suggest that the biological role of NRP1 in malignancies probably depends on different types of cancer cells, even in the same kind of cancer. In the present study, we found that inhibition of NRP1 expression abrogated cell aggressiveness, suggesting that NRP1 is an important regulator in the progression of the disease.

Multiple factors and pathways are implicated in cancer metastasis (30). We found that knockdown of CRT or NRP1 decreased MMP2, MMP9, and FAK expression. Although E-cadherin and β-catenin are reported to be regulated by NRP1 (31), we did not find significant changes in expression level of E-cadherin and β-catenin as CRT or NRP1 knockdown. However, our results suggest that CRT promotes cell migration by up-regulating FAK and enhances cell invasion by increasing MMP2 and MMP9 expression via NRP1.

In the present study, we found that STAT5A not STAT5B is the critical transcription factor of NRP1. We previously showed that CRT regulates the transcriptional activity of STAT5A (10). As an important endoplasmic reticulum luminal Ca²⁺-buffering chaperone, CRT regulates both the calmodulin/CaMKII and c-Src pathways by altering intracellular [Ca²⁺] in mouse embryonic fibroblasts (MEFs) (32). It has been reported that the KN-93-induced CaMKII inactivation could reduce Tyr-694
phosphorylation of STAT5A (21). However, we did not observe a reduction of the phosphorylated Tyr-694 in ESCC cells treating with KN-93. Human cellular-Src (c-Src) is a key signaling node for STAT5 (33), we speculate that CRT regulates STAT5A activity probably through c-Src, to which further investigation should be addressed.

Currently, NRP1 is considered a potential therapeutic target. Inhibitor of NRP1 could reduce cell proliferation and tumor growth in non-small cell lung cancer (34), medulloblastoma (35), and breast cancer (36). Based on these reports and our observations that NRP1 promotes cell invasion and migration in vitro, tumor metastasis in animal models, NRP1 could be a candidate molecular target for the therapy of ESCC, to which further study should be addressed.

In summary, our study reveals a novel signal pathway, CRT-STAT5A-NRP1, promoting cancer cell migration and invasion, which probably contributes to ESCC metastasis. NRP1 expression positively correlated with CRT expression in ESCC tissues, suggesting that they may be potential molecular therapeutic targets.

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Reference


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Figure Legends

Figure 1. CRT repression decreases NRP1 mRNA and protein expression in ESCC cells. KYSE150 and KYSE450 cells were transiently transfected with two CRT-specific siRNAs (CRT-siRNA-1, CRT-siRNA-2), a non-specific siRNA (Non-silencing), or nothing (Parental). 48 hours after transfection, cells were harvested for real-time PCR (A) and western blot analysis (B). Columns, mean; Bars, SD (n = 3); **, P < 0.01.

Figure 2. Knockdown of NRP1 reduces ESCC cell migration and invasion. KYSE150 and KYSE450 cells were transiently transfected with two NRP1-specific siRNAs (NRP1-siRNA-1, NRP1-siRNA-2), a non-specific siRNA (Non-silencing), or nothing (Parental). A, Western blot analysis for NRP1 and CRT expression. B, Representative photos of haptotactic migration assay and Matrigel chemoinvasion assay. Original magnification, 200×. C, Statistical plots of migration assays. D, Statistical plots of cell proliferation rates at 24 hours after seeding. E, Statistical plots of invasion assays. F, Statistical plots of cell proliferation rates at 36 hours after seeding. Columns, mean; Bars, SD (n = 3); **, P < 0.01; ***, P < 0.001.

Figure 3. Knockdown of NRP1 reduces lung metastasis in NOD/SCID mice. NOD/SCID mice were injected via tail vein with KYSE150 cells stably expressing scrambled shRNA (Crtl) or NRP1 shRNA (sh-NRP1). All mice were sacrificed 6 weeks after injection. A, Representative photos of mice lungs after Bouin’s fixation (top) and sections of the lungs stained with HE (bottom, original magnification, 100×). B, Statistical plots of visible lung metastasis nodules. C, Levels of MMP2,
MMP9, FAK, E-cadherin and β-catenin expression in parental, non-silencing, CRT-silenced or NRP1-silenced cells. Columns, mean; Bars, SD (n = 6); **, P < 0.01; ***, P < 0.001.

**Figure 4. CRT promotes cell motility through NRP1 in ESCC cells.** KYSE150 and KYSE450 cells stably expressing CRT-shRNA (sh-CRT) were transfected with pcDNA3.1-NRP1 (sh-CRT-NRP1) or empty vector pcDNA3.1 (sh-CRT-Vec), and cells stably expressing scrambled shRNA (Ctrl) infected with empty vector pcDNA3.1 (Ctrl-Vec) were used as control. A, Western blot for CRT, NRP1, MMP2, MMP9 and FAK expression. B, Representative photos of haptotactic migration assay and Matrigel chemoinvasion assay. Original magnification, 200×. C, Statistical plots of migration assays and cell proliferation rates at 24 hours after seeding. D, Statistical plots of invasion assays and cell proliferation rates at 36 hours after seeding. Columns, mean; Bars, SD (n = 3); **, P < 0.01.

**Figure 5. CRT regulates NRP1 transcription through STAT5A.** A, NRP1 promoter activity after transfection with non-silencing, CRT-siRNA or STAT5A-siRNA by luciferase reporter assay. B, NRP1 mRNA expression in non-silencing, STAT5A-siRNA or STAT5B-siRNA cells by quantitative real-time PCR. C, ChIP assay in KYSE150 and KYSE450 cells. D, Schematic diagram of STAT5-binding sites in 2,000 bp of the human NRP1 promoter and primers in the ChIP assay. E, Luciferase reporter assay in cells transfected with pGL3-Basic, pGL3-NRP1 or pGL3-NRP1-DM. F, Western blot analysis for CRT, p-STAT5A, STAT5A and NRP1 expression. Columns, mean; Bars, SD (n = 3); **, P < 0.01.
Figure 6. CRT and NRP1 expression are positively correlated in ESCC tissues.

The expression of CRT and NRP1 in 218 ESCC tissues was evaluated by immunohistochemistry. Representative photos show the consistent expression of CRT and NRP1.
Figure 1

(A) Relative NRP1 mRNA expression in KYSE150 and KYSE450 cells treated with parental or non-silencing CRT-siRNA (CRT-siRNA-1 or CRT-siRNA-2).

(B) Western blot analysis of KYSE150 and KYSE450 cells treated with parental or non-silencing CRT-siRNA (CRT-siRNA-1 or CRT-siRNA-2), showing expression levels of CRT, NRP1, and GAPDH.
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