Calreticulin Promotes Migration and Invasion of Esophageal Cancer Cells by Upregulating Neuropilin-1 Expression via STAT5A

Feng Shi, Li Shang, Bei-Qing Pan, Xiao-Min Wang, Yan-Yi Jiang, Jia-Jie Hao, Yu Zhang, Yan Cai, Xin Xu, Qi-Min Zhan, and Ming-Rong Wang

Abstract

**Purpose:** We previously revealed that the 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 used 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 downregulation of MMP2, MMP9, and FAK. Notably, positive correlation was found between CRT and NRP1 expression in ESCC tissues \( (P = 5.87 \times 10^{-5}) \). CRT and NRP1 coexpression 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. Clin Cancer Res; 20(23); 6153–62. ©2014 AACR.

Introduction

Esophageal cancer is one of the most common malignancies worldwide (1). Most esophageal cancers in China are squamous cell carcinomas (ESCC). 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 upregulated in ESCC tissues (3). CRT is a calcium-binding protein that 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 (11). 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 upregulating NRP1 expression.
Translational Relevance

Esophageal squamous cell carcinoma (ESCC) is one of the most common malignancies worldwide. More than half of the patients have unsectable 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.

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 were created. Briefly, operative ESCC tissues and morphologically normal operative margin cores. From the primary areas labeled on the slide were copied onto the paraffin block and tissue cores were retrieved and transferred into the recipient block. For TMA construction. The source areas labeled on the slide were copied onto the paraffin block and tissue cores were retrieved and transferred into the recipient block. For TMA construction. The source areas labeled on the slide were copied onto the paraffin block and tissue cores were retrieved and transferred into the recipient block. For TMA construction.

For immunohistochemistry. Immunohistochemical (IHC) analysis was done as described previously (3). The slides were deparaffinized, rehydrated, and immersed in 3% hydrogen peroxide solution for 10 minutes. For antigen retrieval, the slides were heated in citrate buffer (pH 6.0) for 25 minutes at 95°C, and cooled for 60 minutes 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 the manufacturer’s recommendations (GBI Labs) 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 Lipofectamine 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 the 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), and 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).

RNA isolation and real-time PCR

Total RNA was isolated from KYSE150 and KYSE450 cells transfected with nonspecific control or gene-specific siRNAs using the RNeasy Mini kit (Qiagen) according to the manufacturer’s instruction. Isolated RNA was used as a template for the 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 Supplementary Materials and Methods.

Cell proliferation assay

Cells (1.5 × 10^4/100 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. Ten microliters 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 × 10^5 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.crbj.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 cotransfection 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 Supplementary Materials and Methods. Nonimmunoprecipitated 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, 6 mice were injected with 1 × 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 H&E.

**Statistical analysis**

All statistical analyses were performed using the SPSS17.0 software. We statistically evaluated the experimental results using the Student t test, the Pearson χ² 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 analysis confirmed that NRP1 mRNA and protein expression were significantly downregulated in CRT-siRNA cells as compared with those in nonsilencing 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.

![Figure 1](https://example.com/figure1.png)

**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 nonspecific siRNA (nonsilencing), or nothing (Parental). Forty-eight 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.
Reduced NRP1 expression decreases ESCC cell migration and invasion

On the basis 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 nonsilencing siRNA cells (Fig. 2A–C). However, 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...
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 the sh-NRP1 group was significantly fewer than the control group (Fig. 3B). Tissues were isolated and sectioned, and H&E staining showed that lung tissues from the control group were heavily infiltrated by metastasized ESCC cells as compared with those from the 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 downregulated 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 the NRPI promoter activity (Fig. 3A). However, there is no information available about CRT as a transcriptional factor. Thus, we
hypothesized that CRT might indirectly modulate NRPI transcription through some transcription factors. Online database searching (http://mbs.cbrc.jp/research/db/TFSEARCH.html) revealed several signal transducer and activator of transcription 5 (STAT5)–binding sites at −1140, −1089, and +139 bp from the transcription initiation site within the NRPI promoter region (Fig. 5D). ChIP assays showed that STAT5 directly bound to the −1166/−1020 region of the NRPI promoter (Fig. 5C).

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

We subsequently generated a luciferase reporter vector, pGL3-NRPI, with NRPI promoter fragment (−1185 to −740). Activity of the reporter gene in the STAT5A-siRNA group was significantly lower than that in the nonsilencing group (Fig. 5A). Moreover, deletion of the STAT5A-binding sites (from −1140 to −1132 and from −1089 to −1081), pGL3-NRPI-DM, significantly attenuated the 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 (Tyr694) level was decreased in CRT-siRNA cells (Fig. 5F). It has been reported that the 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.

**NRP1 expression positively correlates with CRT in ESCC tissues**

Our previous study has shown that CRT was upregulated in ESCC tissues (3). To confirm the relationship between CRT and NRP1 observed in cell lines, we detected the expression of NRP1 in ESCC tissues and analyzed the expression of the two proteins by IHC using serial sections from the same TMAs. Among the 218 ESCC specimens, strong CRT and NRP1 immunostaining were observed in 34.9% (76 of 218) and 32.6% (71 of 218) of tumors, respectively. High levels of NRP1 expression was found in 50% (38 of 76) of the tumors with CRT overexpression, whereas NRP1 presented negative or weak staining in 76.8% (109 of 142) of the tumors with negative or low CRT expression. Statistical analysis with the Pearson $\chi^2$ test revealed a significant positive correlation between the overexpression of CRT and NRP1 ($P = 5.87 \times 10^{-5}$, 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 coexpression 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 coexpression 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 the 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 biologic 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 the 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 upregulating FAK and enhances cell invasion by increasing MMP2 and MMP9 expression via NRP1.

In the present study, we found that STAT5A, and 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 Ca$^{2+}$-buffering chaperone, CRT regulates both the calmodulin/CaMKII and c-Src pathways by altering intracellular [Ca$^{2+}$] in mouse embryonic fibroblasts (MEF; ref. 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 treated 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). On the basis of 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.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Development of methodology: F. Shi

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Shi, L. Shang, X.-M. Wang, Y.-Y. Jiang, M.-R. Wang

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X.-M. Wang, Y.-Y. Jiang, J.-J. Hao, Y. Cai, X. Xu

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