

Coronin3 Promotes Nasopharyngeal Carcinoma Migration And Invasion By Induction Of Epithelial-To-Mesenchymal Transition

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Purpose: Coronin3 is a cytoskeletal protein that has been implicated in metastasis in many cancer types. Here, we demonstrate its effect on nasopharyngeal carcinoma (NPC) and propose a new probable mechanism of CORO1C-mediated cell migration and invasion by regulation of epithelial-to-mesenchymal transition (EMT) and CDH11.

Patients and methods: First, we measured the differential expression of CORO1C between NPC and non-NPC cells in both cell lines and clinical specimens, using public datasets. Then, we investigated its relationship with clinicopathological factors and its potential as a biomarker to predict the prognosis of NPC patients. We also explored its influence on the cell behaviors of migration and invasion by upregulating and downregulating the expression of CORO1C and attempted to determine the underlying mechanism.

Results: The results verified our original hypothesis. CORO1C was overexpressed in both NPC cell lines and clinical specimens, in both public datasets and our own samples. NPC patients with lower CORO1C expression levels in primary cancer tissues had longer OS (hazard ratio [HR] 1.14, 95% CI 0.831–3.960, $p=0.0341$) and PFS (HR 1.798, 95% CI 0.407–3.56, $p=0.0155$), indicating that it could be used as a prognostic biomarker. It was also confirmed that CORO1C enhanced cells' migration and invasion abilities, by inducing morphological and marker changes typical of EMT. Finally, we found that expression was correlated with and regulated CDH11 expression in NPC cell lines.

Conclusion: Our study provided evidence for the contribution of CORO1C to NPC metastasis, and indicated that it could be used as a new therapeutic target and prognostic biomarker.

Keywords: nasopharyngeal carcinoma, metastasis, GEO, coronin3, CDH11, epithelial to mesenchymal transition

Introduction

Nasopharyngeal carcinoma (NPC) is an uncommon cancer type but is the most common head and neck cancer.^{1,2} It occurs mainly in eastern and southeastern Asia, especially southern China.^{3,4} Males with this disease have higher morbidity and mortality than females.¹ NPC shows an early and high incidence of lymphatic spread, which is a major contributor to the poor outcomes.⁵ Therefore, better understanding the metastatic mechanisms and finding an ideal therapeutic target would be of significant value for developing effective anticancer therapies.

Coronins localize on the dorsal surface of cell. Its knockout mutations displayed a reduction in cell-migration speed, impairment in growth, cytokinesis and

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fluid-phase endocytic uptake.⁶ Coronin3 belongs to the subfamily of the short coronins, which expresses ubiquitously and plays roles in the progression of many cancer types.^{7–9} It was reported to interact with the cofilin and Arp2/3 complex, which play an important role in cell division and migration of normal cells as well as tumor cells.¹⁰ However, the effect of coronin3 in NPC and other mechanisms such as epithelial-to-mesenchymal transition (EMT) progression are still unclear.

EMT is a widely studied process in cancer metastasis. Both EMT and reverse mesenchymal-to-epithelial transition are indicators of tumor cells undergoing metastatic dissemination. EMT is induced and regulated by various signaling molecules and pathways. Previous work showed that coronin3 was upregulated in Madin–Darby canine kidney epithelial cells with epithelial cell repressors directed at Snail, Slug, and E47 compared with control cells.¹¹ This indicated an intimate relationship between coronin3 and EMT progression that deserved further exploration.

One purpose of this work was to prove the effect of coronin3 on cell invasion and migration behaviors. We first examined the differential expression of coronin3 between NPC and normal cells in both cell lines and clinical specimens, following the investigation of the clinical significance of coronin3 as a prognostic biomarker. Then, knockdown and amplification of coronin3 were used to validate its effects on these cell behaviors *in vitro*. Furthermore, we hypothesized that EMT progression is the underlying molecular mechanism by inspecting cell morphology and expression of EMT markers.

Materials And Methods

Cell Culture And Tissues Collection

The NPC cell lines CNE1 and CNE2 were purchased from the Animal Laboratory of Sun Yat-sen University and approved by the institutional ethics committee of Shandong Cancer Hospital (SDTHEC201209041). They were cultured in the RPMI-1640 medium (Gibco, USA) containing 10% fetal bovine serum (FBS; Gibco, Australia), 100 U/mL penicillin and 100 µg/mL streptomycin (Hyclone, USA) at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. When cells were grown to 80% confluence, they were passaged by using 0.25% Trypsin-EDTA (Gibco, USA).

Eighty-seven pairs of paraffin-embedded primary NPC biopsy tissues, the related normal tissues and 27 lymph node metastatic tissues were obtained retrospectively from

Shandong Cancer Hospital affiliated to Shandong University from 2013 to 2014. All patients were selected by at least two senior pathologists according to the histopathological results without any antitumor treatment before biopsy. The data from the original medical records and follow-up information were available. The written informed consent has been provided by the patients. The approval of human tissues studying from the institutional ethics committee of Shandong Cancer Hospital was obtained (SDTHEC201209041).

Total RNA Extraction And Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Total RNA was extracted from NPC cell lines and biopsy tissues by Trizol (Ambion, USA). The cDNA was synthesized according to HiFiScript cDNA synthesis kit (CWBio, People's Republic of China). UltraSYBR Mixture (CWBio, People's Republic of China) and LC450 (Roche, Switzerland) were used for real-time PCR. The PCR reaction condition was based on manufacturer's recommendations. β-actin was used as the internal control. All the samples were reacted three times independently with the three times technical repetitions. The primer sequences were as recorded in [Supplementary Table 1](#).

Western Blotting (WB)

Whole cell protein lysates were extracted by using a mixture of radio-immunoprecipitation (RIPA) lysis buffer (Beyotime, People's Republic of China), Protease and Phosphatase Inhibitor (Servicebio, People's Republic of China) on ice as quickly as possible. Then, the lysates were centrifuged for 10 mins at 12,000 g, 4°C. Protein concentration was determined by Micro BCA Protein Assay Kit (CWBio, People's Republic of China) and protein lysates were denatured at 100°C in 5× loading buffer (CWBio, People's Republic of China) for 10 mins. The same amount of protein was loaded into each well of the 10% sodium dodecyl sulfate polyacrylamide gel (CWBio, People's Republic of China) and separated by electrophoresis. After that, proteins were transferred to a polyvinylidene difluoride (0.45mm PVDF; Millipore, USA) by a wet system (Bio-Rad, USA) at 100V for 1 hr. The PVDF membrane was blocked subsequently by 5% skim milk with 0.1% FBS for more than 1 hr. The primary antibodies including anti-coronin3 (1:500 dilution; Santa Cruz, USA), anti-N-cadherin (1:1000 dilution; CST, USA), anti-β-catenin

(1:1000 dilution; CST, USA), anti-E-cadherin (1:1000 dilution; CST, USA), and anti-CDH11 (1:1000 dilution; Proteintech, USA) were incubated at 4°C overnight, followed by incubation with the HRP-linked anti-rabbit or anti-mouse IgG (1:1000 dilution; CST, USA) at room temperature for 1 hr. An ECL substrate solution (GE, USA) was used for the bolt development.

Cell Proliferation Assay

3000–5000 cells per well were seeded in 96-well plates with 200 μ L medium. Twenty microliter of 5mg/mL solution of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) (Servicebio, Wuhan, People's Republic of China) in 1x phosphate-buffered saline (PBS) was added to each well for 4 hrs incubation and 150 μ L of dimethyl sulfoxide (DMSO) was then added for 10 mins. Then, the OD value was measured. We record the OD value of 24, 48 and 72 hrs of different cell lines for statistical analysis.

Tumorigenicity In Nude Mice

We obtained female Balb/c nu/nu nude mice between 4 and 6 weeks old from HFK Bioscience Cooperation (Beijing, People's Republic of China). All mice experiments were conducted according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the institutional ethics committee of Shandong Cancer Hospital (SDTHC20120904). 5×10^6 cells in 200 μ L phosphate-buffered saline (PBS) were subcutaneously injected into the lower right groin. The tumor sizes were measured every 7 days by a caliper and calculated using the formula $(a \times b^2) \times 0.5$, in which a and b are the long and short dimensions, respectively. Mice were sacrificed one and a half months later. Each group contained six mice.

Wound Healing Assay

For wound-healing assays, 1×10^5 cells were seeded into 12-well plates followed 24-hr incubation. Then, scratches were drawn to create 1-mm-wide or so wound gap using a plastic pipette tip through the center of the well. The cells were washed by PBS 3 times and incubated in medium without FBS for 48 hrs. Cell movement into the wound area was captured by a phase-contrast microscope at 0 hr, 24 hrs and 48 hrs.

Cell Migration And Invasion Assays

Cell migration and invasion assays were performed using transwell plates (8- μ m pore size; Corning Costar, USA)

with or without Matrigel (BD, USA). 8×10^4 cells in 200 μ L medium without FBS were seeded into 24-well transwell plates and 800 μ L medium with FBS was added under the plates. After 24-hr incubation, cells migrated or invaded the Matrigel to the bottom side of the membrane. Microscope was used to count and calculated the relative cell motility activity by the formula below: relative cell motility activity = number of migrated or invaded cells from different groups / number of migrated or invaded cells from the control group.⁸ At least 10 fields were calculated per group in each experiment.

Lentivirus Transduction

Stable increased coronin3 expression cell line was obtained by lentiviral transduction into CNE-1 using pLV-EGFP: T2A-Puro-EGF1A>hcoronin33 (Cyagen, Guangzhou, People's Republic of China) and named as CNE-1-C3. The lentiviral vector pLV[Exp]-EGFP: T2A: Puro-Nucl was used as a control, and the cell line was named as CNE-1-Cnt. Stable decreased coronin3 cell line was generated by lentiviral transduction into CNE-2 using a pCsil-GFP vector (GeneChem, Shanghai, People's Republic of China) and named as CNE-2-ShC3. The control vector expressing green fluorescent protein (LV-GFP) was used as a control, and the cell line was named as CNE-2-ShCntrl.

Immunohistochemistry

The tissues serial sections (4 μ m) were deparaffinized, rehydrated and treated with 3% H₂O₂ in methanol. Then antigen retrieval with citrate buffer (PH 6.0) in a high-pressure steamer followed. The sections were incubated with polyclonal antibody against coronin3 (Santa, USA) after blocked with 5% fat-free dry milk in TBS Tween. The HRP-conjugated secondary antibody was bonded and detected by DAB staining, following by micro imaging under a microscope. Target protein expression was evaluated according to previous description. The histological score (H score) of coronin3 was calculated by the following formula: H score = ratio score \times intensity score. The ratio score respected the proportion of positive cells in each specimen, which was quantitatively evaluated as 0 for 0% staining, 1 for 0.01~25% staining, 2 for 25.01~50% staining, 3 for 50.01~75% staining, 4 for >75% staining. The intensity score was graded as follows: 0 for no signal, 1 for weak, 2 for moderate, 3 for strong. The H score of 0~12 was estimated as negative (-, score: 0), weak (+, score: 1~4), moderate (++, score: 5~8), strong (+++, score: 9~12).

Statistical Analysis

The statistical analyses were performed by using GraphPad Prism7 and IBM SPSS Statistics 22.0 software. All experiments were conducted at least three independent repeats. Data were expressed as the mean \pm standard error of the mean (SE) and analyzed with two-sided unpaired Student's *t*-test for samples with two groups. The Kruskal–Wallis H-test was used to analyze the relationship between coronin3 expression levels and tissue types, while the Mann–Whitney *U*-test was used to analyze its relation to clinic pathological factors. Differences were considered statistically significant for *p* values less than 0.05.

Results

Coronin3 Is Overexpressed In Both NPC Cell Lines And Clinical Specimens

The whole work was presented as a model (Figure 1A). At the beginning of this study, we checked the expression levels of coronin3 in both NPC cells and patients' samples compared with controls. First, we searched the Cancer Genome Atlas (TCGA, <http://gepia.cancer-pku.cn>), Oncomine (<https://www.oncomine.org>), and Gene Expression Omnibus (GEO, <https://www.ncbi.nlm.nih.gov/geo/>) to obtain NPC datasets. The inclusion criteria were: NPC cells or clinical datasets of human samples that contained the expression levels of coronin3 in both tumor and controls. After screening, we focused on the following datasets: GSE15047, GSE5334, GSE53819, GSE13597, and GSE12452 (Supplementary Table 2). Only GSE15047 was from NPC cell set; the others were from clinical datasets. In GSE15047, coronin3 mRNA was highly expressed in NPC cell lines (CNE1, CNE2, and HONE1) compared with a nasopharyngeal epithelial cell line (NP69) as shown in Figure 1B. In the clinical datasets (Figure 1C), two of the four NPC patient sample datasets (GSE53819, GSE12452) and the combined total data showed significantly upregulated levels of coronin3 in NPC versus control tissues.

To confirm this result, we examined the coronin3 mRNA expression in our collected 87 pairs of primary NPC biopsy tissues and related normal tissues (Figure 1D). The results supported the finding that coronin3 was upregulated in NPC cells.

Coronin3 Expression Levels Are Correlated With Clinicopathological Features And Prognosis

Coronin3 protein expression levels were also examined in these 87 primary NPC biopsy tissues, related normal tissues, and lymph node metastatic tissues by IHC analysis. The representative IHC results are presented in Figure 2A. Coronin3 was predominantly located in cytoplasm of cells. As described in Table 1, coronin3 expression levels in both primary NPC tissues and lymph nodes metastatic tissues were significantly higher than those in normal tissues.

The correlation between coronin3 expression levels and clinic pathological factors was analyzed as well. As shown in Table 2, overexpression of coronin3 was statistically related to T and M stage, but not interrelated with gender, age, pathological type, or N stage. Analysis of relationship between coronin3 expression in primary cancer tissues and OS and PFS of NPC patients showed that patients with lower coronin3 expression levels in primary cancer tissues had longer OS [HR (hazard ratio): 1.814, 95% CI (confidence interval): 0.831~3.960, *p*=0.0341] or PFS [HR: 1.728, 95% CI: 0.907~3.564, *p*=0.0155] (Figure 2B). This indicated that coronin3 expression level might be related to clinical stage and could be used as a potential marker to predict prognosis of patients with NPC.

Coronin3 Is Overexpressed In NPC Cell Line With Stronger Migration And Invasive Properties

In GSE15047, coronin3 was overexpressed in CNE-2 compared with CNE-1 (Figure 3A). To verify the microarray results, we used qRT-PCR to measure coronin3 mRNA expression (Figure 3B) and Western blotting (WB) to further explore the protein expression (Figure 3C) in these two cell lines. As described in the previous articles, CNE-2 has stronger migratory ability than CNE-1. To confirm that, we performed wound-healing, migration and invasion assays in vitro. The results showed significant differences between CNE-1 and CNE-2 in migration and invasive behaviors (Figure 3D and E), which proved the poorly differentiated CNE-2 cell line had stronger migration and invasive properties than CNE-1. The results indicated that coronin3 may be associated with the migration and invasive ability of these NPC cell lines.

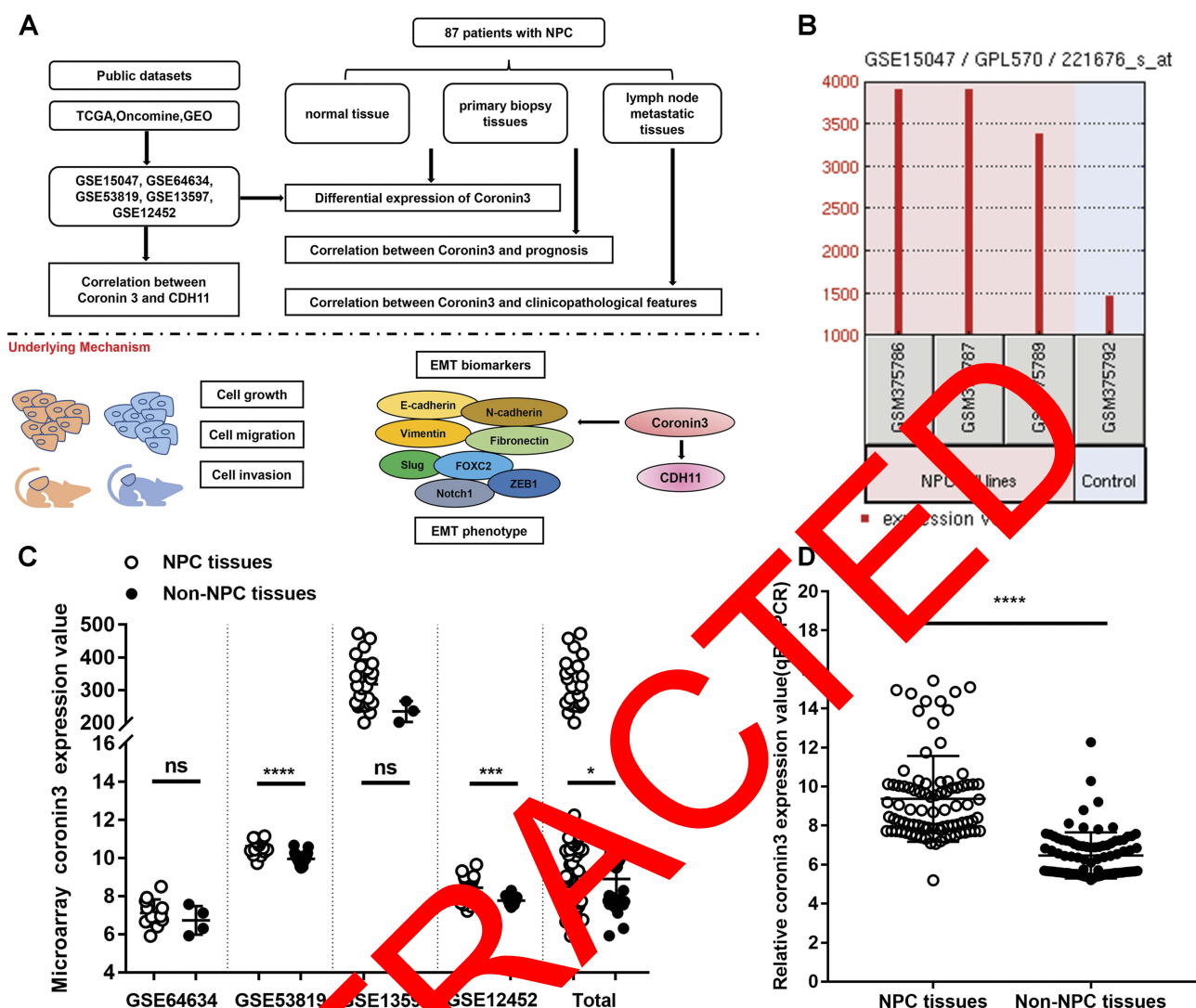


Figure 1 Coronin3 was overexpressed in both NPC cell lines and tissues. **(A)** A model compiled the whole work. **(B)** Coronin3 mRNA was overexpressed in NPC cell lines (CNE-1, CNE-2, and HONE-1) compared with nasopharyngeal epithelial cell line (NP69) according to GSE15047. **(C)** two of four NPC patient sample datasets (GSE53819, GSE12452) and the combined total data showed significantly upregulated levels of coronin3 in NPC versus control tissues. **(D)** Coronin3 was overexpressed in NPC tissues compared with non-NPC tissues in our collected patients ($p < 0.05$; $***p < 0.001$; $****p < 0.0001$).

Abbreviations: NPC, nasopharyngeal carcinoma; NS, not significant.

Coronin3 Promotes NPC Cell Growth In Vitro And In Vivo

For further functional exploration, we ectopically over-expressed coronin3 in CNE-1 or knocked down coronin3 in CNE-2 by lentivirus transduction. The resulting cells were named CNE-1-vector/CNE-1-C3 and CNE-2-ShCntrl/CNE-2-ShC3, respectively. After stable transduction, we used qRT-PCR and WB to test the efficiency. As shown in Figure 4A and B, coronin3 was upregulated in CNE-1-C3 and downregulated in CNE-2-ShC3 in terms of both mRNA and protein levels. Then, we performed an MTT assay to determine the role of coronin3 in NPC cell growth in vitro. Coronin3 overexpression enhanced viability in CNE-1, and decreased coronin3 expression

weakened the viability in CNE-2 (Figure 4C). Furthermore, we injected transfected cells into subcutaneous tissues of nude mice to construct xenograft models. After dynamic detection of tumor volume, we found that cells with high coronin3 expression levels (CNE-1-C3 and CNE-2-ShCntrl) grew faster than those with low coronin3 expression levels (CNE-1-vector and CNE-2-ShC3) (Figure 4D). All the evidence indicated that coronin3 promotes NPC cell growth both in vitro and in vivo.

Coronin3 Promotes NPC Cell Migration And Invasion In Vitro

The effect of coronin3 on cell mobility was also tested by wound healing and transwell assays. Increased expression

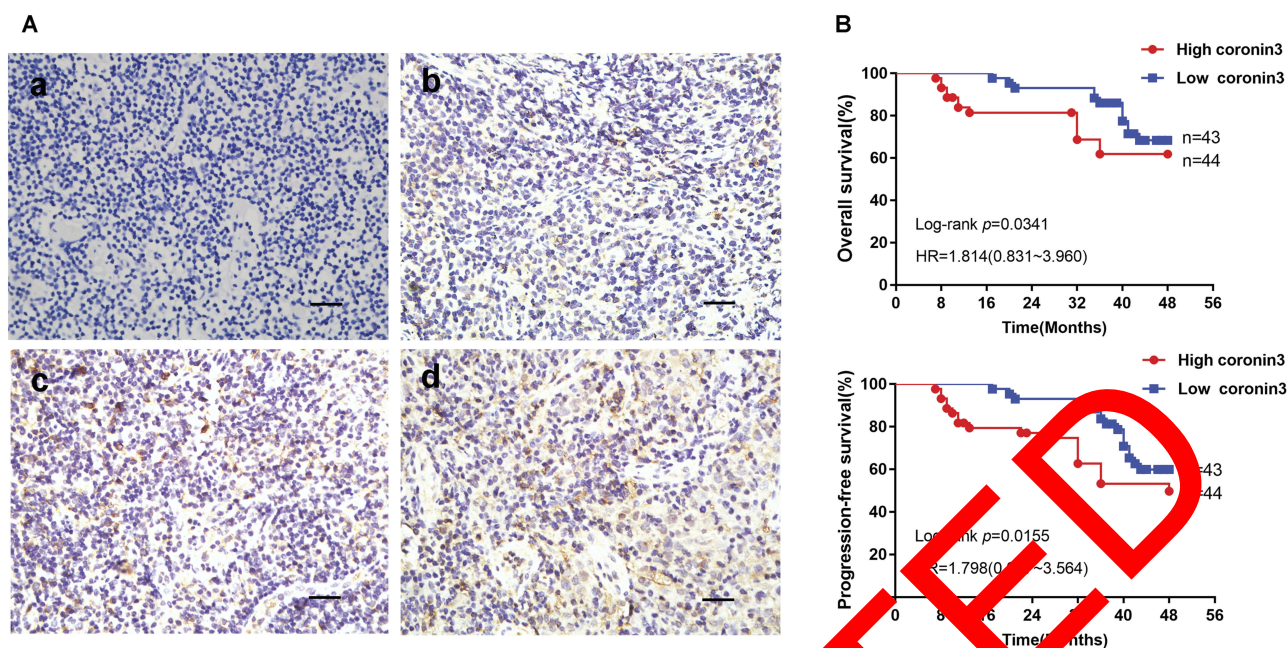


Figure 2 Increased coronin3 protein expression indicated poor prognosis in NPC patients. **(A)** Coronin3 protein expression in NPC patients' biopsy specimens. The typical different expression levels of coronin3 were showed. (a) Negative, -, score: 0; (b) Weak, +, score: 3; (c) Moderate, ++, score: 7; (d) Strong, +++, score: 11. Scale bar=100 μ m. **(B)** Kaplan-Meier curves for the correlation between coronin3 protein expression and overall survival and progression-free survival.

of coronin3 in CNE-1 resulted in a closer wound area in CNE-1-C3 than that in CNE-1-vector (Figure 5A). In accordance with this result, CNE-1-C3 also showed higher mobility than CNE-1-vector in transwell migration and invasion assays (Figure 5B). By contrast, decreased expression of coronin3 in CNE-2 was associated with significant decreases in wound assay, cell migration and invasion in CNE-2-ShC3 compared with CNE-2-ShCntrl (Figure 5C and D). All these results indicated that coronin3 promotes NPC cell mobility in vitro.

Coronin3 Expression is Higher In The Mesenchymal NPC Cell Line And Correlated With The Mesenchymal Phenotype

To clarify the underlying mechanism of coronin3-mediated cell behavior changes in NPC cells, we consulted literature and hypothesized that it influenced cell EMT. Then, we checked the mRNA expression level of EMT-related genes in GSE15047. Notably, there were obvious differences in EMT markers between CNE-1 and CNE-2, with disagreement in conclusion. As shown in Figure 6B–H, epithelial markers E-cadherin had low expression while mesenchymal or mesenchymal-promoting markers Vimentin, FOXC2, Slug, Fibronectin, and ZEB1 had high expression

in CNE-2. This indicated that NPC cells with stronger migratory ability presented stronger EMT. However, the difference of N-cadherin levels (Figure 6A) did not support this conclusion. Given the instability of microarray results, we rechecked the mRNA expression levels of these markers by qRT-PCR (Figure 7A); the results supported the EMT assumption.

Finally, we detected the morphological differences between CNE-1-C3 and CNE-1-vector, and between CNE-2-ShC3 and CNE-2-ShCntrl, to provide further supporting evidence. Typical epithelial to spindle-like phenotype changes occurred in CNE-1-vector to CNE-1-C3, while typical spindle-like to epithelial phenotype changes occurred in CNE-2-ShCntrl to CNE-2-ShC3 (Figure 7B).

Coronin3 Is Correlated With CDH11 Expression Levels In NPC Specimens And Regulates CDH11 Expression In NPC Cell Line

As mentioned above, we analyzed GEO clinical datasets that contained NPC tissues and non-NPC tissues. As well as coronin3, another EMT-related molecule, CDH11, attracted our attention. In GSE12452, CDH11 was overexpressed in NPC tissues (Figure 8A), similar to coronin3. Moreover, in this dataset, coronin3 mRNA expression was related to CDH11

Table 1 Expression Of Coronin3 In Primary NPC Tissues, Normal Tissues And Lymph Nodes Metastatic Tissues

Histological Type	H Score				p
	–	+	++	+++	
Normal tissues (N)	17	43	22	5	0.000 ^a
Primary NPC tissues (P)	12	32	31	12	0.015 ^b
Lymph nodes metastatic tissues (L)	8	18	30	31	0.001 ^c

Notes: ^aThis p-value refers to the difference of three groups (N, P, and L) by Kruskal–Wallis H-test. ^bThis p value refers to the difference of two groups (N and P) by Mann–Whitney U-test. ^cThis p value refers to the difference of two groups (P and L) by Mann–Whitney U-test.

Table 2 Correlation Between Primary Tumor Tissue Coronin-1C Expression And Clinicopathological Factors Of NPC Patients

Clinicopathological Factors	n	H Score				p ^c
		–	+	++	+++	
Gender						
Male	43	5	15	13	7	0.619
Female	44	7	17	13	7	
Age						
<50	42	10	12	14	6	0.316
≥50	45	2	20	17	6	
Pathological type						
KSCC ^a	65	8	23	26	8	0.540
NKC ^b	22	4	9	5	4	
T stage						
T1-2	41	1	1	11	3	0.019*
T3-4	46	1	11	20	9	
N stage						
N0-1	40	5	16	15	4	0.683
N2-3	7	7	16	16	8	
M stages						
M0	50	8	22	18	2	0.012*
M1	37	4	10	13	10	

Notes: ^aKSCC, keratinizing squamous cell carcinoma; ^bNKC, non-keratinizing carcinoma; ^cthe Mann–Whitney U-test was used to analyze the relationship between coronin3 expression and clinicopathological factors. *p<0.05.

mRNA expression (Figure 8B). These observations promoted further exploration of the relationship between coronin3 and CDH11 as well as other EMT markers in NPC cell lines. Regarding mRNA expression level, the qRT-PCR results showed that epithelial marker E-cadherin was downregulated, while mesenchymal marker N-cadherin and CDH11 were upregulated in CNE-1-C3 compared with CNE-1. By contrast, the expression of epithelial marker E-cadherin was upregulated while mesenchymal marker N-cadherin and CDH11 were downregulated in CNE-2-ShC3 compared with CNE-2 (Figure 8C). The same tendency was observed for protein expression levels (Figure 8D). These suggested a role for coronin3 in EMT, which may be related to regulation of CDH11 expression.

Discussion

Coronins are highly conserved but poorly understood proteins. The immunofluorescence images firstly indicated this protein, which localized on the dorsal surface of the cell.¹² It was named coronin because of its “crown-like” structures and derived from the Latin root corona, which means “crown”. Mammalian coronins were constitutive of a number of family members and grouped into three types (I, II and III).¹³ The nomenclature of coronin genes and their protein products is of fundamental importance for the annotation and information exchange but it is various (CORO1C, Coronin3, Coronin2).¹⁴ We used de Hostos nomenclature (Coronin3) for this article. Coronin3 protein belonged to the subfamily of the short coronins (type I)

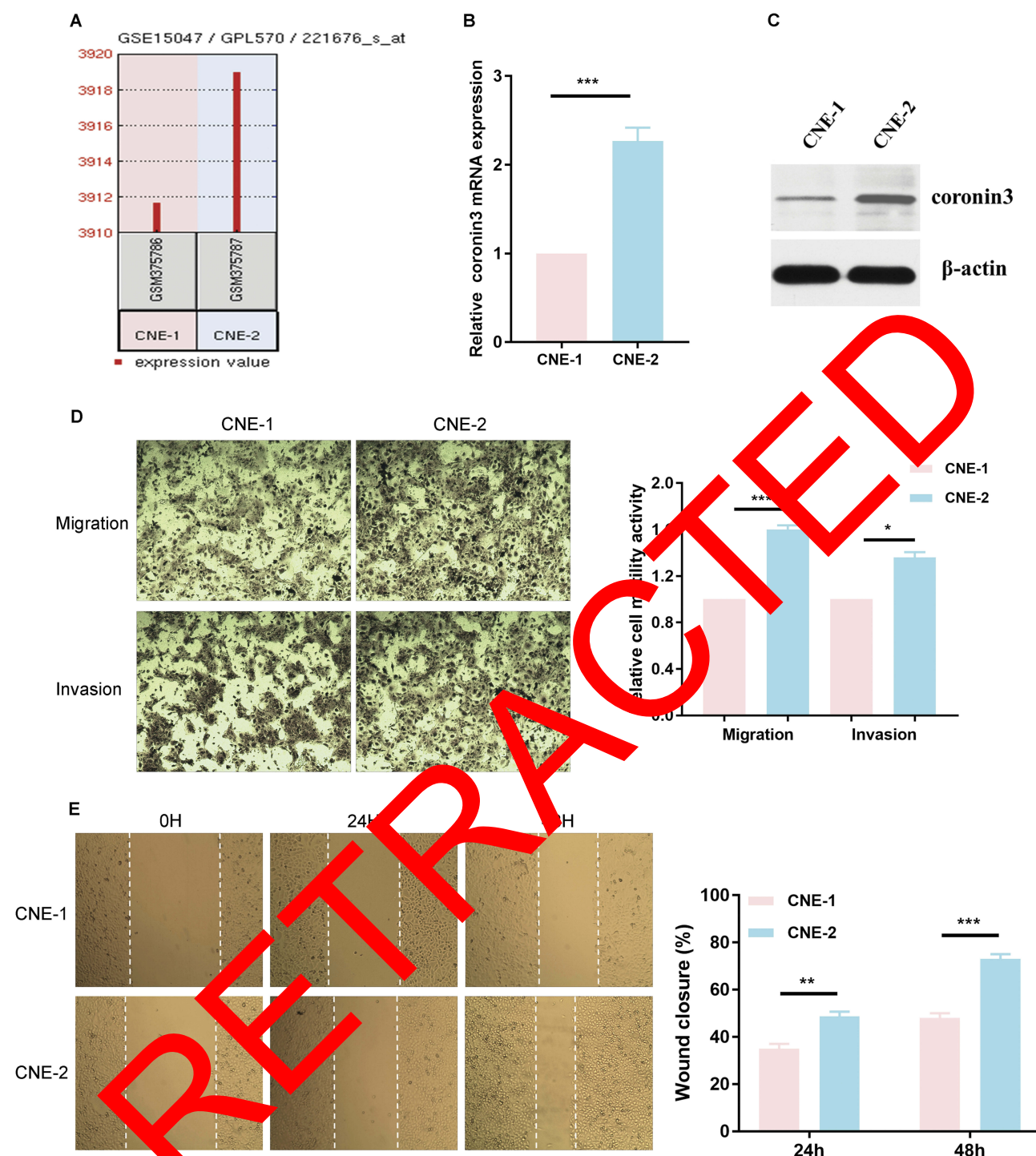


Figure 3 High coronin3 expression was correlated with stronger migration and invasive properties in NPC cell lines. **(A)** Coronin3 mRNA overexpressed in CNE-2 compared with CNE-1 according to GSE15047. **(B)** The mRNA expression of coronin3 was estimated by qPCR. β -actin was used as the internal control. $2^{-\Delta\Delta CT}$ method was used for results analysis. *** $p < 0.001$. $n = 3$ per group, three repeats per time. **(C)** The protein expression of coronin3 was estimated by Western Blotting. **(D)** The migratory and invasive ability was evaluated by counting the number of cells that transferred the 8- μ m-pore transwell membrane with or without the Matrigel. * $p < 0.05$; *** $p < 0.001$. Magnification $\times 200$, $n = 3$ per group. **(E)** Wound-healing assay was performed by assessing the closure at 0 hr, 24 hrs and 48 hrs. ** $p < 0.01$; *** $p < 0.001$. Magnification $\times 200$, $n = 3$ per group.

and consisted of 474 amino acids, whose molecular weight by SDS-PAGE analysis was 57 kDa.¹² It expresses ubiquitously and plays roles in wound healing, protrusion

formation including lamellipodia, filopodia and axonal growth, migration, invasion, cell proliferation, cytokinesis, endocytosis, secretion of norepinephrine and matrix

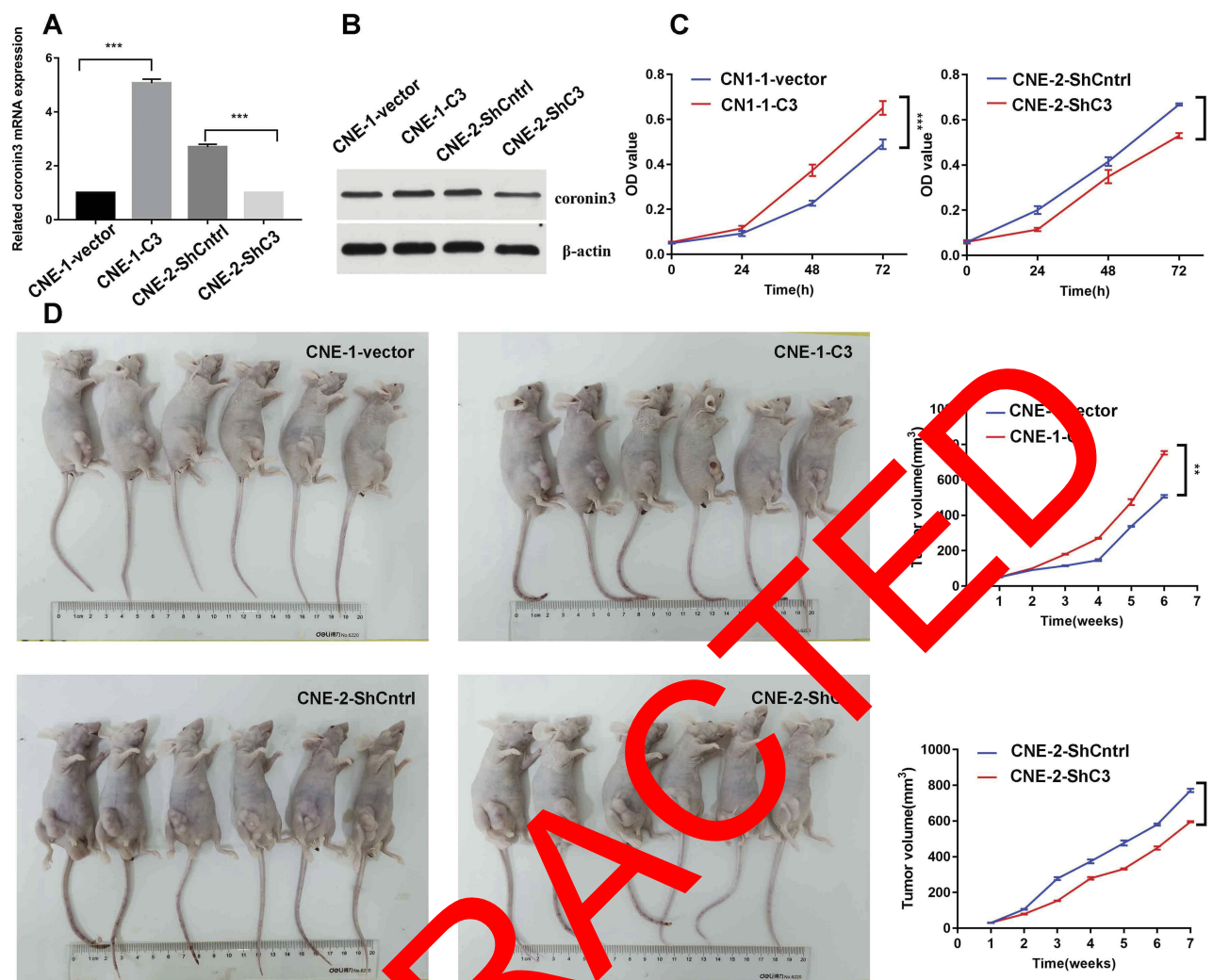


Figure 4 Coronin3 promoted NPC cell growth both in vitro and in vivo. **(A)** The mRNA expression of coronin3 was estimated by qPCR after lentivirus transduction. Lentivirus with pLV-EGFP or pGCSil-GFP were used for overexpressing or knockdown. Null vectors were used as controls. *** $p < 0.001$. $n = 3$ per group, three repeats per time. **(B)** The protein expression of coronin3 was estimated by Western Blotting. **(C)** NPC cell viability was measured by MTT assay after stable lentivirus transduction. ** $p < 0.01$; *** $p < 0.001$. $n = 6$ per group. **(D)** Subcutaneous tumorigenicity to assess the effect of coronin3 on NPC cell growth in vivo. * $p < 0.05$; ** $p < 0.01$. $n = 6$ per group.

metalloproteinases. As we got increasing number of information about this protein, people paid more attention to its function on cancer metastasis, such as hepatocellular carcinoma,¹⁶ gastric cancer⁹ and diffuse gliomas.¹⁷ However, the effect of coronin3 on NPC metastasis has not been explored.

In this work, we studied highly and poorly differentiated human CNE-1 and CNE-2 cell lines. Their different cell migration and invasion behaviors were verified, as well as the differential expression of coronin3. This led to the hypothesis that the differential expression of coronin3 was associated with these cell behaviors, as well as the migration and invasive properties of NPC cells. A lentivirus was used to increase or decrease the expression

of coronin3 and the cell behaviors were detected again. The consistent results confirmed our previous hypothesis. The underlying mechanism was then further investigated.

Cancer metastasis refers to the spread of tumor cells from primary sites to new secondary sites in the body, during which EMT is a well-known fundamental process involving in several defined stages.⁸ Cell-cell cohesion and tissue integrity were affected by epithelial-like cells mesenchymal-like cells transition, which results in the enhancement of cell invasion, migration, and subsequent metastasis in vivo. Then, reverse MET is activated revert cells back to an epithelial state¹⁸ so that the tumor cells settle down at new sites and cancer metastasis occurs. In this progress, the phenotype and molecular expression

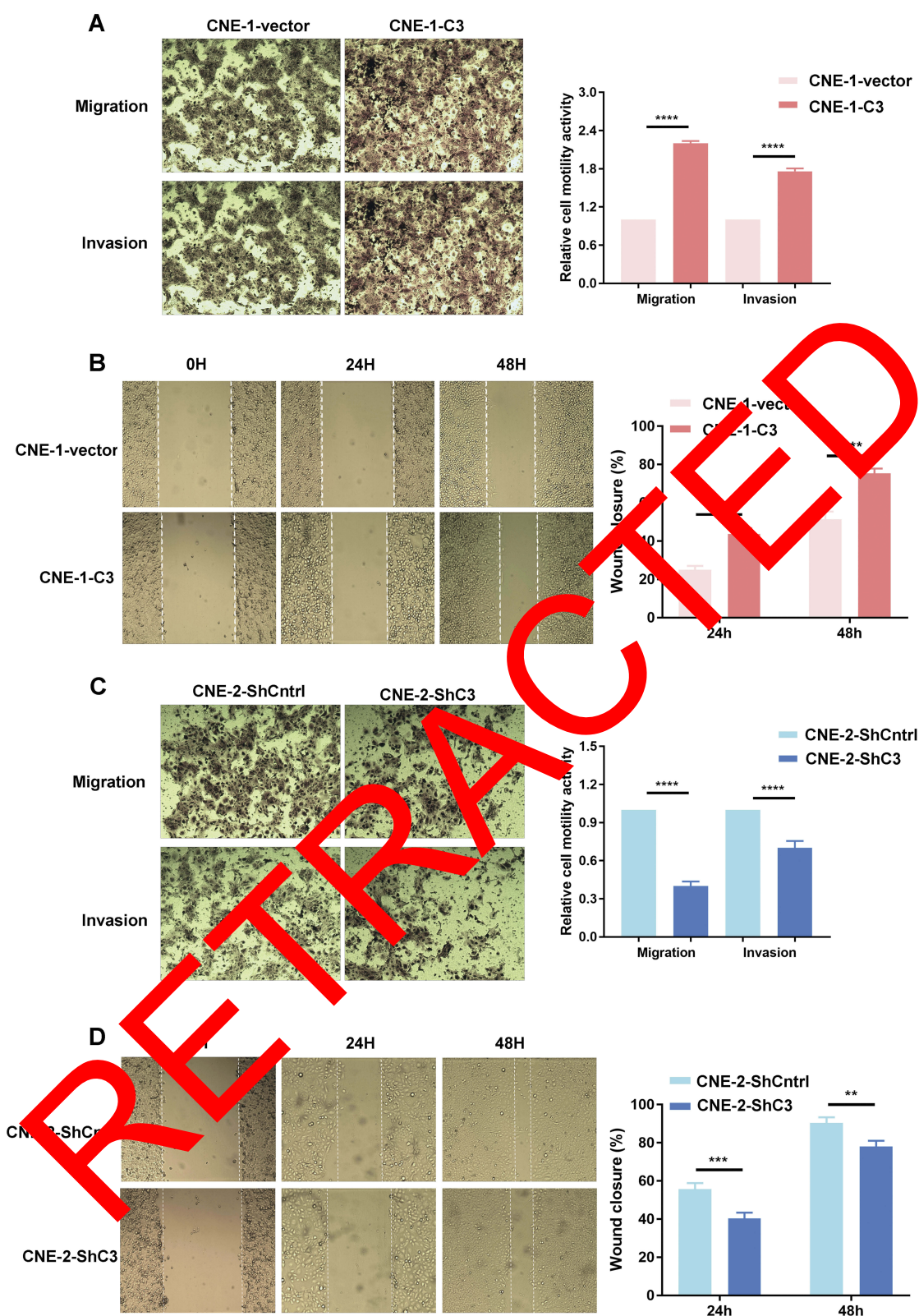


Figure 5 The effects of coronin3 on the migration and invasion of NPC cells. **(A and C)** The migratory and invasive ability was evaluated by counting the number of cells that transferred the 8- μ m-pore transwell membrane with or without the Matrigel. **(B and D)** The wound-healing assay was also performed. The closure was recorded at 0 hr, 24 hrs and 48 hrs. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Magnification $\times 200$, $n = 3$ per group.

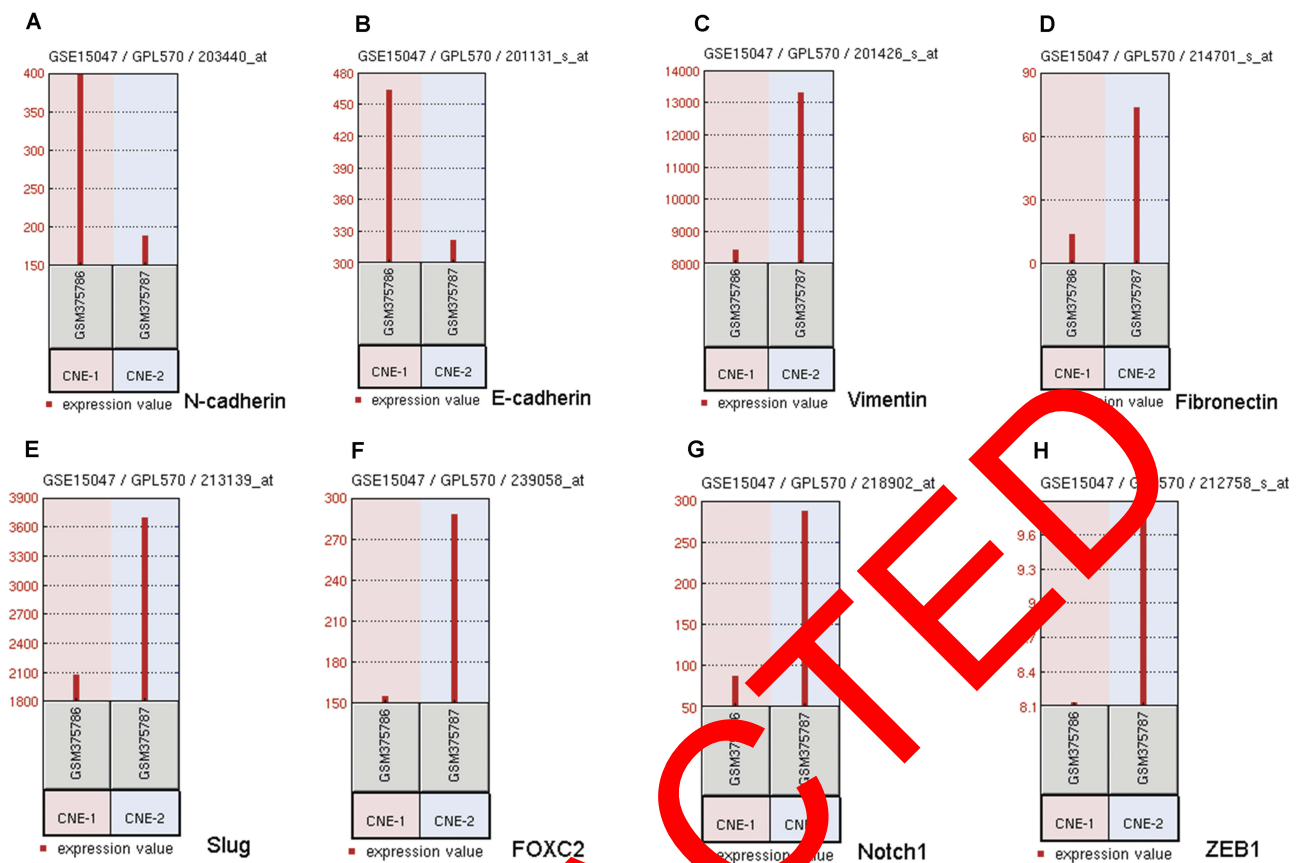


Figure 6 The differential mRNA expression levels of EMT markers between CNE-1 and CNE-2. (A) N-cadherin expression levels differences. (B) E-cadherin expression levels differences. (C) Vimentin expression levels differences. (D) Fibronectin expression levels differences. (E) Slug expression levels differences. (F) FOXC2 expression levels differences. (G) Notch1 expression levels differences. (H) ZEB1 expression levels differences.

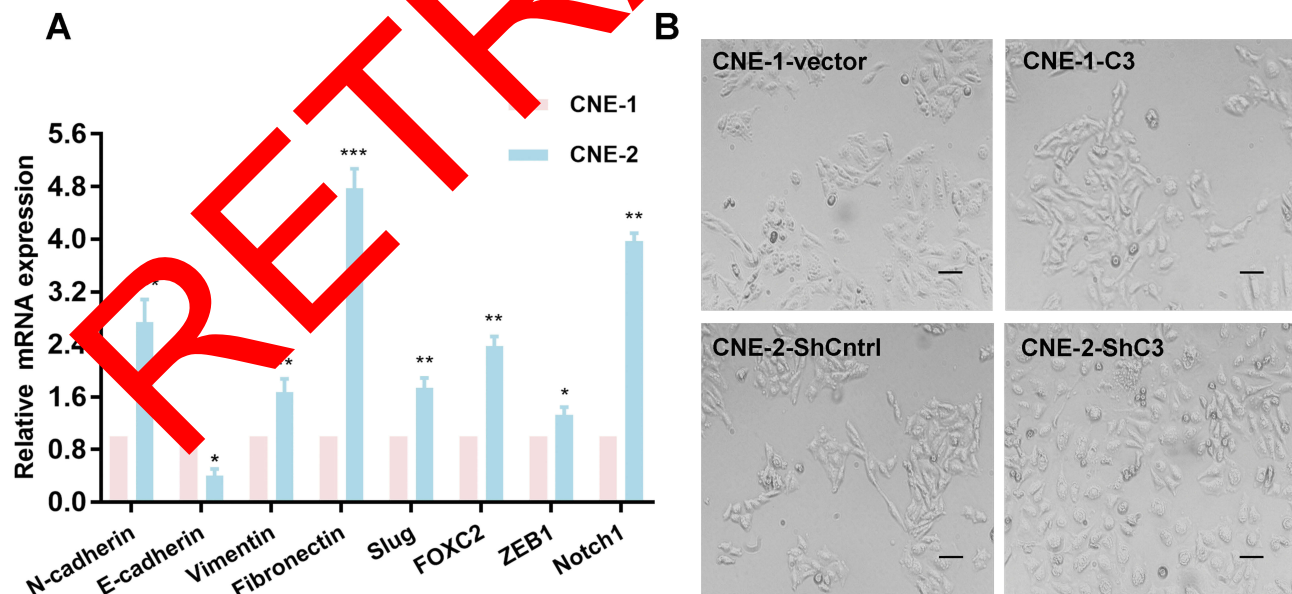


Figure 7 Increased coronin3 induced EMT in NPC cells. (A) The verification of EMT markers in CNE-1 and CNE-2 by qRT-PCR. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (B) The morphology changed in NPC cells upregulation or downregulation of coronin3.

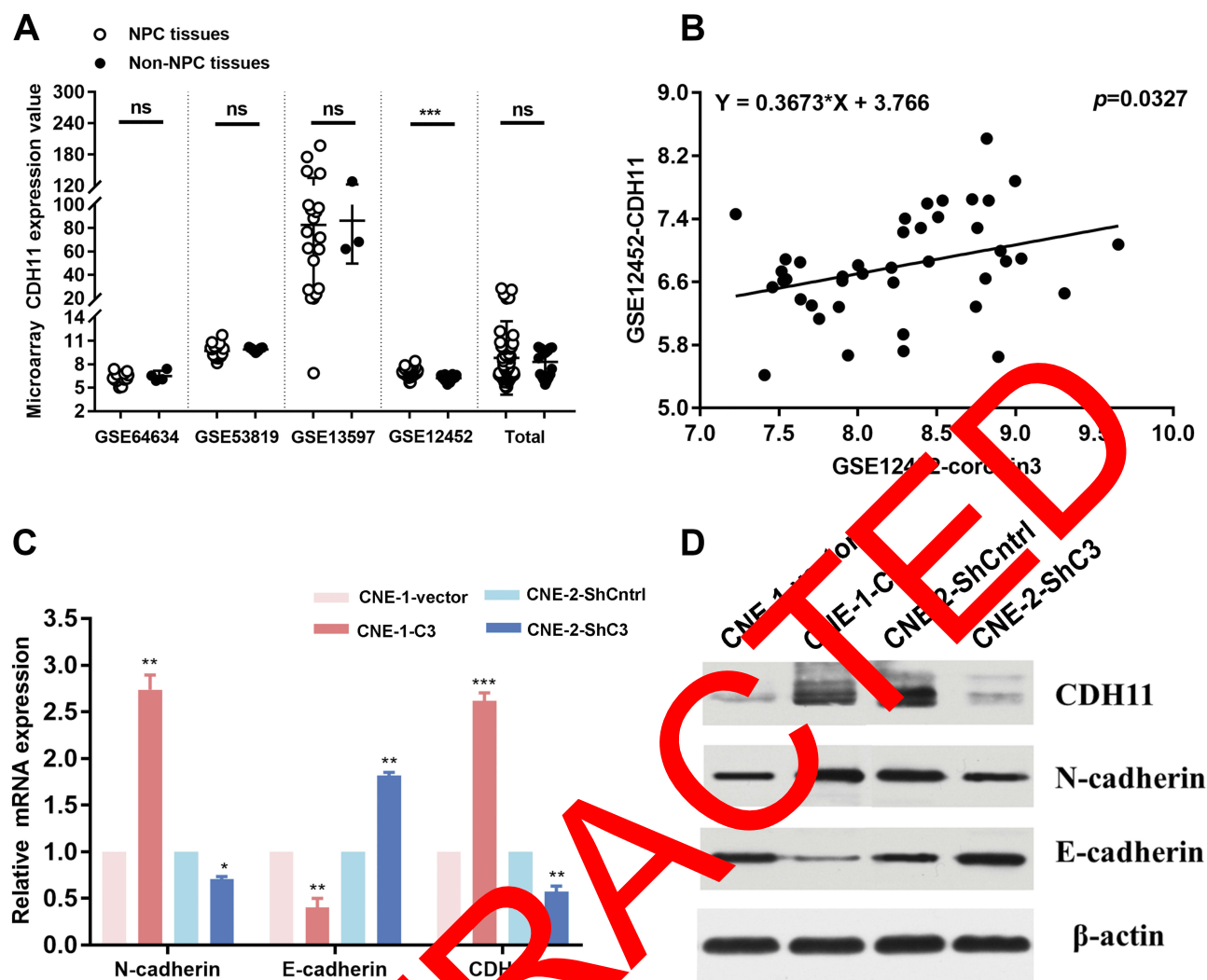


Figure 8 Coronin3 was correlated with CDH11 expression and regulated EMT markers expression. **(A)** CDH11 was high expressed according to GSE12452, which consisted with coronin3. **(B)** In GSE12452, coronin3 mRNA expression was related to CDH11 mRNA expression. **(C)** mRNA expression levels and **(D)** protein expression levels of EMT typical markers (E-cadherin and N-cadherin) and CDH11 were regulated after increase of coronin3 in CNE-1 and decrease of coronin3 in CNE-2. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. $n = 3$ per group.

Abbreviations: NS, not significant.

levels changes are the main markers. The characteristics of epithelial cells include general flat and polygonal shape with cell-cell interactions like tight junctions, adherent junctions, desmosomes, gap junctions and basement-membrane. Their shapes and attachments restrict the movement.¹⁹ However, the mesenchymal cells possess neither uniform composition nor cellular adhesion. They are generally morphologically irregular with elongated and spin-like shapes that make sense of less rigid topological functionality.¹⁸ As for molecular markers, EMT is regulated by several pathways and involves in numerous signaling mechanisms. E-cadherin and N-cadherin were taken as our main tested markers. E-cadherin is an extractive epithelial gene, which modulates cytoskeletal organization

and secures connections between adjacent cells. N-cadherin is a cadherin of neural specificity, which facilitates cell migration and invasion by promoting the cells to other mesenchymal types. In the EMT, the expression of E-cadherin is upregulated while the N-cadherin is down-regulated. As a cytoskeleton-associated protein, coronin3 protein was suspected to facilitate cancer metastasis by accelerating EMT. In our study, after coronin3 overexpression, the cell phenotype was transited from epithelial-like to mesenchymal-like while the E-cadherin was downregulated, and the N-cadherin was upregulated. After coronin3 decreasing, the results were to the contrary.

The different expression levels of coronin3 appear to be characteristic features in many solid tumors. Thal et al

found that coronin3 expression was related to malignancy of diffuse gliomas but was uncorrelated to that of pilocytic astrocytoma while no immunoreactive cells were showed in medulloblastomas and neuroblastomas.¹⁷ An IHC study by Wu et al in 115 human hepatocellular carcinoma specimens demonstrated that the higher coronin3 expression in patients indicated more advanced stage.¹⁶ Ren et al reported that coronin3 was highly expressed in gastric carcinoma tissues and metastatic lymph nodes⁹ and Sun et al determined that higher coronin3 expression levels indicated a relatively shorter overall survival of patients with gastric sarcoma.⁸ In our study, coronin3 expression in NPC patients was tested and the correlations between coronin3 expression and clinical features or prognosis were explored. The trend is like that in other malignant tumors. Taken together, these findings validated the clinical significance of coronin3 as a potential biomarker in many tumors.

This work had some limitations. First, there were no experiments in vivo, which weakens the evidence for coronin3 having a regulatory role in the migration and invasion of NPC. Second, the underlying mechanism by which coronin3 induces EMT via CDH11 regulation was not explored in depth. Third, a larger sample size is required to provide convincing evidence of this protein's clinical potential as a biomarker. In brief, further study on the role of coronin3 in NPC is required.

Conclusion

Thus far, our study provided evidence that coronin3 contributes NPC metastasis partly by EMT induction, which could be used as a new therapeutic target. Furthermore, our findings also confirmed the potential of coronin3 as a biomarker for prognosis in patients with NPC.

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Disclosure

The authors report no conflicts of interest in this work.

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