

Expression of cytoskeleton-associated protein 4 is associated with poor prognosis and metastasis in nasopharyngeal carcinoma

Manbo Cai¹ , Weijun Wu¹, Shengling Deng², Qiao Yang¹, Haibiao Wu¹, Haiyun Wang³, Jiaying Zhang³, Qisheng Feng³, Jianyong Shao³, Yixin Zeng³ and Jianjun Li⁴

¹Department of Radiotherapy, The First Affiliated Hospital, Hengyang Medical School, University of South China, Hengyang 421001, China; ²Department of Anesthesia, The Second Affiliated Hospital, Hengyang Medical School, University of South China, Hengyang 421001, China; ³State Key Laboratory of Oncology in South China, Cancer Center, Sun Yat-Sen University, Guangzhou 510060, China; ⁴Department of Urological Surgical, The Second Affiliated Hospital, Hengyang Medical School, University of South China, Hengyang 421001, China

Corresponding author: Jianjun Li. Email: 1995020005@usc.edu.cn

Impact statement

Distant organ metastasis is the major factor resulting in treatment failure in nasopharyngeal carcinoma (NPC) patients. However, the molecular mechanism underlying metastasis is still not clearly understood. In this study, immunohistochemistry assisted in detecting the high expression of cytoskeleton-associated protein 4 (CKAP4) protein in a large sample of NPC tissues. According to western assays, NPC cell lines exhibited high CKAP4 expression. Moreover, a high level of CKAP4 related to NPC patients' poor survival and exhibited a positive relevance to metastasis. As revealed by the *in vivo* and *in vitro* experiments, stable knockdown of CKAP4 expression suppressed cell migration, invasion, and metastasis and was correlated with vimentin and E-cadherin levels. Accordingly, CKAP4 may promote tumor development in progression and metastasis in NPC patients by influencing epithelial mesenchymal transformation of NPC, which indicated that CKAP4 could serve as a predictor or promising new therapeutic targeted for metastatic NPC.

Abstract

Cytoskeleton-associated protein 4 (CKAP4) acts as a key transmembrane protein that connects the endoplasmic reticulum (ER) to microtubule dynamics. Researchers have not examined the roles of CKAP4 in nasopharyngeal carcinoma (NPC). The study aimed at evaluating the prognostic value and metastasis-regulating effect of CKAP4 in NPC. CKAP4 protein could be observed in 86.36% of 557 NPC specimens but not in normal nasopharyngeal epithelial tissue. According to immunoblot assays, NPC cell lines presented high CKAP4 expression relative to NP69 immortalized nasopharyngeal epithelial cells. Moreover, CKAP4 was highly expressed at the NPC tumor front and in matched liver, lung, and lymph node metastasis samples. Furthermore, high CKAP4 expression reported poor overall survival (OS) and presented a positive relevance to tumor (T) classification, recurrence, and metastasis. According to multivariate analysis, CKAP4 could independently and negatively predict patients' prognosis. Stable knockdown of CKAP4 expression in NPC cells inhibited cell migration, invasion and metastasis *in vitro* and *in vivo*. Moreover, CKAP4 promoted epithelial-mesenchymal transition (EMT) in NPC cells. CKAP4 knockdown was followed by the downregulation of the interstitial marker vimentin, and upregulation of the epithelial marker E-cadherin. In NPC tissues, high CKAP4 expression exhibited a positive relevance to vimentin expression and a negative relevance to E-cadherin expression. In conclusion, CKAP4 is an independent predictor of NPC, and CKAP4 might contribute NPC progression and metastasis, which may be involved in EMT with vimentin and E-cadherin.

Keywords: Nasopharyngeal carcinoma, CKAP4, prognosis, metastasis, EMT

Experimental Biology and Medicine 2023; 248: 1085–1094. DOI: 10.1177/15353702231167940

Introduction

Nasopharyngeal carcinoma (NPC) refers to an epithelial carcinoma originated from the nasopharyngeal mucosal lining and patients with NPC are geographically concentrated in South China, Southeast Asia and North Africa.¹ Radiotherapy (RT) is the backbone of NPC treatment. Due

to the widespread application of RT modulated by intensity, induction, and concurrent chemotherapy and the advancement of an accurate cancer staging system, the management of NPC has been greatly improved in recent decades.² The reported 5-year local control rates are outstanding, ranging from 70% to 90%. However, approximately about 20–30% of patients eventually develop distant metastasis or local

recurrence resulting in treatment failure after the administration of radical chemoradiotherapy for locoregionally advanced NPC (stages III–IVB).³ Local recurrence and distant metastasis are the main factors that lead to poor overall survival (OS) of NPC patients with advanced-stage disease.⁴ Furthermore, the molecular mechanism of metastasis is still not clearly understood. Therefore, studying the molecular mechanism of NPC metastasis and developing new molecular targets are important for the optimization of treatment strategies.

Cytoskeleton-associated protein 4 (CKAP4), a 63-kDa palmitoylated type II transmembrane protein, was initially identified to anchor the endoplasmic reticulum (ER) by binding to microtubules in epithelial cells.⁵ It remarkably impacts the formation and maintenance of microtubule network structure and the dynamic balance of the ER. It is also a plasma cell markers. However, in addition to being expressed in normal plasma cells and plasmacytoma cells, CKAP4 can also be observed in several cell types' cell surface membrane, such as type II pneumocytes and vascular smooth muscle cells (VSMCs).⁶ CKAP4 has been identified as a receptor for tissue plasminogen activator (TPA),⁷ surfactant protein A (SP-A),⁸ antiproliferative factor (APF),⁹ alginate exopolysaccharides,¹⁰ and Dickkopf (DKK) proteins.¹¹ Due to the binding to various ligands, CKAP4 protein exerts various effects. Moreover, CKAP4 palmitoylation and phosphorylation can critically help to regulate its translocation between the ER and plasma membrane or nucleus where it can exert different biological effects through its translocation between organelles.¹² Further research has demonstrated that phosphorylated CKAP4 is capable of translocating to the nucleus in HeLa cells, and binds DNA through its outer domain, and subsequently alters the transcription of genes involved in regulating proliferation and tumorigenesis.^{12,13} CKAP4 has been reported to be a molecule that promotes multiple cancers to progress, such as pancreatic cancer (PC), renal cancer, lung cancer, and esophageal cancer.^{14–17} However, contrary studies have reported that CKAP4 can resist tumor development in different tumor types, such as hepatocellular carcinoma (HCC), intrahepatic cholangiocellular carcinoma (ICC), and glioma.^{18–20} It is still necessary to determine whether CKAP4 can promote or resist cancer progression. Furthermore, up to now, researches fail to examine CKAP4 expression as well as its role in NPC.

In the study, immunohistochemistry (IHC) and immunoblot (Western blot) methods served for examining CKAP4 expression in primary and metastatic NPC tissues, thereby exploring the effect of CKAP4 on NPC. Second, analysis was conducted on the association of CKAP4 expression with clinical factors and NPC patients' outcomes. At last, *in vivo* and *in vitro* experiments were carried out for evaluating how CKAP4 affected NPC cell metastasis and invasion.

Materials and methods

Patients and clinical tissue samples

The study included 557 NPC specimens from the Department of Pathology, Sun Yat-Sen University Cancer Center (SYSUCC), Guangzhou, China, from January 2001 to

December 2003, adopting the same inclusion criteria as the previous literature.²¹ All the patients underwent normal curative RT, accompanied or not accompanied by chemotherapy. A total of 23 lung, liver, or lymph node (LN) metastatic tissues from NPC patients and samples from the corresponding primary tumors were obtained from above department. The study had obtained the approval of the Institute Research Medical Ethics Committee of Sun Yat-Sen University.

IHC and evaluation

CKAP4 received IHC staining as previous description and vimentin and E-cadherin, paraffin-embedded tissues were cut into 5 mm sections.²¹ In brief, the sections underwent one night of incubation in a rabbit anti-human CKAP4 polyclonal antibody (Cat. No.: HPA000792, Sigma-Aldrich; working dilution 1:500), mouse anti-human E-cadherin monoclonal antibody (Abcam, working dilution 1:500), or rabbit anti-human vimentin monoclonal antibody (Cat. No.: ab92547, Abcam; working dilution 1:500) at 4°C. An EnVision kit (DAKO) assisted in the detection of the primary antibodies, followed by the visualization of 3,3'-diaminobenzidine substrate and hematoxylin counterstaining. The IHC results were evaluated and scored as described previously.¹⁹ The pathologists' evaluation results exhibited a concordance rate of 87.3%, demonstrating the high reproducibility of such scoring method.

Cell line and viral transduction

Cells of four NPC cell lines, namely, CNE2, SUNE1, 5-8F, and 6-10B, underwent culturing in RPMI 1640 with 10% fetal bovine serum (FBS). Cells of two additional NPC cell lines (S18 and S26) received culturing in DMEM (Dulbecco's Modified Eagle Medium) added with 10% FBS. Cells of NP69 received culturing in keratinocyte serum-free medium with fetal calf serum (FCS) (5%), bovine pituitary extract (25 µg/mL), and recombinant epidermal growth factor (0.2 ng/mL), as per the instruction of the manufacturer. All cell lines underwent incubation in humidified atmosphere at 37°C with 5% CO₂.

A BLOCK-iT Lentiviral Pol II miRNAi system served for establishing cell lines with stably expressed CKAP4 short hairpin RNA (shRNA) or a scrambled non-target shRNA, as per the instruction of manufacturer. The targets of human CKAP4 shRNAs were 5'-GGATAGGTTGTTTGTGAAA-3' and 5'-GGAGCCAGAAGGAGATCAA-3'.

Immunoblot analysis

RIPA buffer was adopted for harvesting and lysing cells. SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis) assisted in separating identical amounts of the denatured protein samples, which then were transferred to polyvinylidene difluoride (PVDF) membranes to receive immunoblot analysis. The immunoblot analysis adopted antibodies against CKAP4, E-cadherin, vimentin, N-cadherin, fibronectin, and β-catenin antibody, with anti-GAPDH mouse monoclonal antibody as a loading control. An improved chemiluminescent Western blotting kit served for the visualization of all protein bands.

ImageJ software (version 1.44) served for normalizing the gray value possessed by target bands under the detection of corresponding antibodies to those under the detection of GAPDH (loading control), thereby quantifying the protein level. We repeated each experiment no less than three times.

Wound-healing, Transwell migration, and invasion assays

Wound healing assays together with Transwell assays served for the evaluation of cell migration and invasion. For the Transwell assays, after 24 h, cells on the inner side of the membrane were cleared, and cells on the outer side received crystal violet dyeing and were counted microscopically. For the wound healing assay, cells were photographed at 0, 24, and 36 h after wounding, and the migrated areas were measured. We repeated all experiments three times.

Animals and spontaneous LN and lung metastasis assay

Five- to six-week-old male athymic BALB/c nu/nu mice were provided by Shanghai Institutes for Biological Sciences. All the animal studies conformed to the principles and procedures in the guidelines of the Institutional Animal Care and Use Committee of SYSUCC. Spontaneous LN metastasis analysis was carried out based on previous description.²² In brief, each mouse was injected with 1×10^5 cells into the left hind footpad for generating a primary tumor. Seven to eight weeks later, we collected the popliteal LN of the left hind foot before experiment terminated for the regular tissue processing. In the lung metastasis assay, the lateral vein of athymic BALB/c nude mice was injected with 1×10^6 CKAP4 stable knockdown or scrambled cells in 100 μ L phosphate buffered saline (PBS), and 7–8 weeks later, the mice received euthanization. The split lungs were photographed and weighed, followed by one night of fixation in 4% paraformaldehyde (PFA), cryoprotection in 30% sucrose in PBS, and freezing treatment in embedding media under optimal cutting temperature (OCT). Serial sections of the LN and lungs received H&E staining, and a light microscope served for counting the number of pulmonary colonies.

Statistical analysis

SPSS (version 25.0) served for statistical analysis. Monthly calculation of the survival time was conducted, and the calculation of OS started from the initial diagnosis date to the death date or the last follow-up. The χ^2 test or Fisher's exact probability method assisted in the comparisons between groups. The Kaplan–Meier and log-rank methods served for the analysis of the survival rate. The Cox regression model served for multivariate analysis. $P < 0.05$ reported statistical significance.

Results

Patient characteristics

Male patients occupied 74.5% (415) of the total number of patients (557), with an average age of 46 years (in the range of

Table 1. Associations between CKAP4 expression and clinicopathological characteristics in NPC patients.

Characteristics	CKAP4 IHC staining		P value
	Low expression (n=244)	High expression (n=313)	
Age, years			0.238
<46	130	151	
≥46	114	162	
Sex			0.417
Male	179	236	
Female	65	77	
Tumor stage			0.012
T1 + T2	109	107	
T3 + T4	135	206	
Lymph node (N) status			0.171
N0	71	77	
N1–3	173	236	
Recurrence or metastasis			<0.001
No	195	154	
Yes	49	159	
TNM clinical stage			0.04
I + II	75	72	
III + IV	169	241	
Histological type			0.045
NKUC	190	212	
NKDC	49	90	

CKAP4, cytoskeleton-associated protein 4. NPC, nasopharyngeal carcinoma. NKUC, non-keratinizing undifferentiated carcinoma; NKDC, non-keratinizing differentiated carcinoma.

19–78 years). Thereinto, 139 patients (25.0%) developed non-keratinizing differentiated carcinoma (NKDC), 402 patients (72.2%) developed non-keratinizing undifferentiated carcinoma (NKUC), and the rest 2.8% developed keratinizing squamous cell carcinoma (KSCC). The number of patients in stage I and stage II, and stage III and stage IV took up 26.4% (147) and 73.6% (410) of the total number of patients, respectively. The follow-up lasted for 58.63 months on average (in the range of 2–99 months). During the 5-year follow-up period, of all the patients, 184 (33.0%) died, and disease progressed in 208 (37.3%) patients. Supplemental Table 1 lists patients' clinical information in detail.

CKAP4 is expressed at the invasive front of NPC tissues and is a poor prognostic indicator

For investigating the CKAP4 expression status in NPC biopsies, CKAP4 in a set of tissue microarrays that involved 557 NPC samples underwent IHC staining (Table 1; Figure 1(a)). IHC staining revealed that 484 of 557 (86.36%) paraffin-embedded samples showed positive staining of CKAP4 in a majority of tumor cells (Figure 1(a), red arrow); adjacent non-cancerous epithelial cells presented no positive staining (Figure 1(a), black arrow). As shown in Figure 1(b), CKAP4 mainly presented expression in the tumor cell cytolymph and were preferentially distributed at the tumor invasive front (Figure 1(b), red arrow).

The expressions and the cutoff value were taken into account for separating NPC samples into group with low

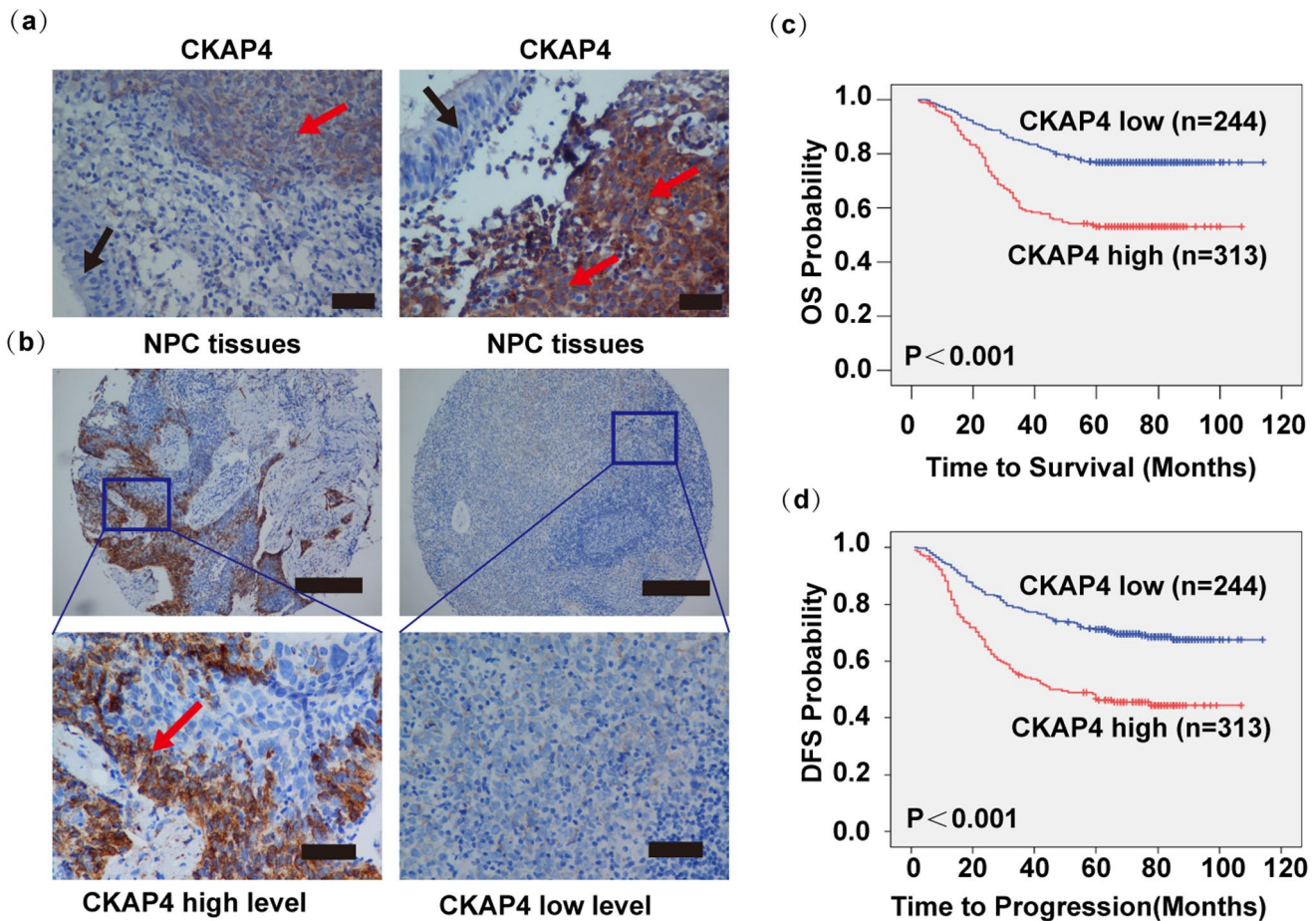


Figure 1. Elevated CKAP4 levels reported poor OS and DFS of NPC patients. (a) Immunohistochemical analysis demonstrated certain staining of CKAP4 in NPC tumor nests (red arrows) instead of in adjacent normal NPE cells (black arrow, scale bar is 100 μm). (b) High and low CKAP4 protein expression levels in the TMA are displayed with low and high magnifications under a light microscope (the top scale bar is 500 μm , and the bottom is 100 μm). (c, d) The low CKAP4 group presented remarkably higher OS rate and DFS rate.

CKAP4 expression and group with high CKAP4 expression. In all, 313 of 557 (56.2%) NPC tissues presented high CKAP4 expression. χ^2 test analysis confirmed the positive relevance of CKAP4 expression to the tumor T classification (Pearson's $R=0.107$; $P=0.012$), recurrence or metastasis (Pearson's $R=0.314$; $P<0.001$), clinical stage (Pearson's $R=0.087$; $P=0.04$), and histological type (Pearson's $R=0.0085$; $P=0.045$) (Table 1); comparatively, CKAP4 expression was not obviously relevant to other clinicopathologic features (patient sex, age, and N classification) ($P>0.05$, Table 1).

The cohort of 557 NPC patients presented a 5-year OS rate of 67.0% and disease-free survival (DFS) rate of 61.0%. According to Kaplan–Meier and log-rank test analyses, high CKAP4 expression reported poor OS (5-year survival rates, 55% vs 86.4%, $P<0.001$, Figure 1(c)) and DFS (5-year survival rates, 49.2% vs 79.8%, $P<0.001$, Figure 1(d)). According to multivariate analyses, a high CKAP4 expression could independently and negatively indicate patients' OS and DFS or the distant metastasis-free survival (Table 2). Furthermore, the CKAP4 expression was positively associated with recurrence and metastasis (Table 2). Accordingly, a high CKAP4 expression in NPC presented an obvious relevance to

unfavorable outcomes of patient, the localization at the invasive front indicated that it could promote tumor invasion, and CKAP4 protein levels might show a positive relevance to NPC metastasis.

CKAP4 is upregulated in liver, lung, and LN metastatic tissues from NPC patients and in highly migratory/invasive NPC cells

For evaluating the clinical relevance of the hypothesis in the study that CKAP4 was capable of regulating NPC metastasis, archival tissues of NPC patients' liver, lung, and LN metastatic tissues were collected to be compared with primary NPC tissues ($n=23$). IHC staining determined the obviously higher protein levels of CKAP4 in the liver, lung, and LN metastatic tissues relative to the primary tissues (Figure 2(a) and (b)). Relative to NP69 cells, NPC cell lines including 6-10B, 5-8F, SUNE1, S26, S18, and CNE2 NPC cells presented remarkably increased CKAP4, according to the Western blot analysis ($P<0.001$, Figure 2(c) and (d)). CKAP4 was also highly expressed in the highly metastatic S18 ($P<0.05$) and 5-8F cells ($P<0.01$), and lowly expressed in the metastatic potential S26 and 6-10B cells.^{23,24}

Table 2. Univariate and multivariate Cox proportional hazards regression analysis for CKAP4.

	5 year OS	95% CI	HR	P value	5 year DFS	95% CI	HR	P value
Univariate Cox regression								
Age								
<46	75.4%	Reference			66.4%	Reference		
≥46	62%	1.261–2.316	1.709	0.001	59.2%	1.046–1.809	1.375	0.022
Sex								
Male	67.9%	Reference			59.3%	Reference		
Female	71.%	0.626–1.268	0.891	0.52	67.4%	0.550–1.071	0.768	0.12
Clinical stage								
I + II	92.5%	Reference			85.7%	Reference		
III + IV	60.1%	3.535–11.993	6.511	<0.001	54.3%	2.532–6.245	3.976	<0.001
Tumor stage								
T1 + T2	84.7%	Reference			78.7%	Reference		
T3 + T4	58.5%	2.187–4.67	3.196	<0.001	52.4%	1.939–3.736	2.692	<0.001
Lymph node (N) status								
N0	80.1%	Reference			72.6%	Reference		
N1–3	64.6%	1.333–2.959	1.986	<0.001	59.%	1.178–2.349	1.664	0.004
WHO histological classification								
NKUC	69.8%	Reference			61.6%	Reference		
NKDC	67.6%	0.768–1.522	1.081	0.656	61.9%	0.918–2.083	1.383	0.121
KSCC	53.8%	0.91–2.056	1.368	0.132	61.5%	0.748–4.113	1.755	0.196
CKAP4 expression								
Low	86.4%	Reference			79.8%	Reference		
High	55%	2.879–6.151	4.208	<0.001	49.2%	2.343–4.452	3.23	<0.001
Multivariate Cox regression								
Age								
<46	75.4%	Reference			66.4%	Reference		
≥46	62.%	1.16–2.15	1.58	0.003	59.2%	1.01–1.75	1.33	0.04
Clinical stage								
I + II	92.5%	(Reference)			85.7%	(Reference)		
III + IV	60.1%	1.98–8.75	4.16	<0.001	54.3%	1.35–4.48	2.46	0.003
Tumor stage								
T1 + T2	84.7%	(Reference)			78.7%	(Reference)		
T3 + T4	58.5%	0.88–2.19	1.39	0.16	52.4%	0.95–2.24	1.46	0.08
Lymph node (N) status								
N0	80.1%	(Reference)			72.6%	(Reference)		
N1–3	64.6%	0.99–2.24	1.49	0.06	59%	0.968–1.976	1.38	0.07
CKAP4 expression								
Low	86.4%	(Reference)			79.8%	(Reference)		
High	55%	2.64–5.68	3.87	<0.001	49.2%	2.2–4.21	3.04	<0.001

CKAP4, cytoskeleton-associated protein 4; CI, confidence interval; WHO, World Health Organization; NKUC, non-keratinizing undifferentiated carcinoma; NKDC, non-keratinizing differentiated carcinoma; KSCC, keratinizing squamous cell carcinoma.

CKAP4 knockdown suppresses NPC cell migration and invasion

For examining the causal effect of CKAP4 on NPC cell motility, we engineered S18 and 5-8F cell lines stably expressing either shRNAs targeting CKAP4 (CKAP4 KD1 and CKAP4 KD2) or a scrambled non-target shRNA. Immunoblotting confirmed that CKAP4 expression was inhibited at the protein level (Figure 3(a)). The loss of CKAP4 led to an obvious suppression of S18 cell invasion and migration (Figure 3(b) to (d)). Similar results were observed in 5-8F cells after we knocked down the expression of CKAP4. These results indicated that CKAP4 could facilitate NPC cell migration and invasion, in cells which were examined in culture.

CKAP4 knockdown suppresses the NPC metastasis rate *in vivo*

LN and lung metastasis models assisted in determining the impact of CKAP4 *in vivo*.²⁰ CKAP4 knockdown cells presented an obviously lower metastasis rate (Figure 4(a) and (b)). Furthermore, CKAP4 silencing prevented lung metastasis in mice in a lateral vein lung metastasis tumor model (Figure 4(c) and (d)). Accordingly, CKAP4 could impact NPC metastasis in LN and lung metastasis models.

CKAP4 mediates NPC cell metastasis and is related to EMT

For more deeply exploring how CKAP4 promoted NPC metastasis, we evaluated the protein levels of many

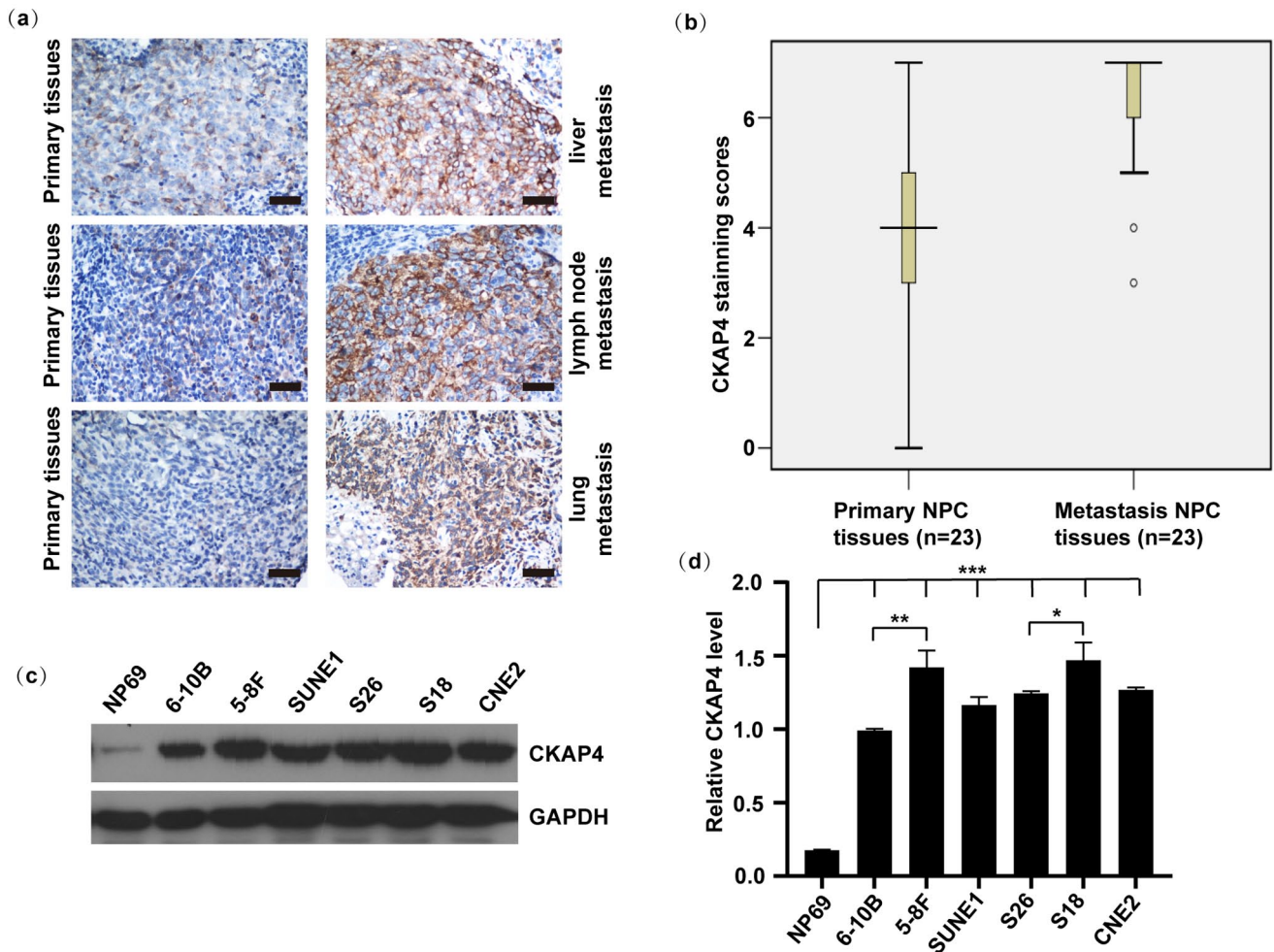


Figure 2. CKAP4 expression presented upregulation in liver, lung, and LN metastases and in highly metastatic NPC cell lines. (a) IHC staining served for evaluating the CKAP4 protein expression in primary NPC tissues and compare liver, lung, and LN metastases from NPC (scale bar is 100 μ m). Metastatic lesions show obvious staining. (b) Statistical analysis (Mann–Whitney test) discovered remarkably increased CKAP4 expression in NPC metastatic tissues relative to primary NPC tissues ($P < 0.001$). (c and d) The Western blot examined the CKAP4 protein level. (c) ImageJ software served for normalizing the gray value possessed by target bands under the detection of corresponding antibodies to those under the detection of GAPDH (loading control), thereby quantifying the protein level. (d) We repeated each experiment at least three times. Data are in the form of the mean \pm SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. One-way analysis of variance.

epithelial-mesenchymal transition (EMT) markers in S18 and S26 cells. The mesenchymal markers vimentin, fibronectin and β -catenin were more significantly expressed in the S18 cells relative to the S26 cells, while epithelial protein E-cadherin and N-cadherin presented lower expression in the S18 cells ($P < 0.001$, Figure 5(b) and (c)). High CKAP4 expression was accompanied by increased vimentin level and decreased E-cadherin level in the highly metastatic cells (Figure 5(a)). CKAP4 suppression led to lower vimentin levels and higher E-cadherin levels (Figure 5(a)). To confirm this finding, we examined the relationship between CKAP4 and EMT and carried out IHC staining (in 102 primary human NPC tissue samples) analysis of CKAP4, vimentin and E-cadherin. Accordingly, the CKAP4 expression level exhibited a positive relation to vimentin expression, and an inverse relation to E-cadherin expression (Figure 5(a), (d), (e), and (f)). These data suggest that CKAP4 modulates vimentin level, thereby impacting the EMT.

Discussion

Distant organ metastasis is currently the primary reason for treatment failure in NPC patients.^{2,25} Effective targeted therapy can improve the clinical treatment efficacy in patients with locally advanced and advanced NPC.²⁶ Hence, it is required to identify effective molecular targets for enhancing the NPC treatment. The present study conducted immunohistochemical staining, finding the high CKAP4 expression in NPC tumor tissues, instead of in normal NPE tissues. Moreover, we found that CKAP4 was specifically highly expressed at the invasion front of NPC tissues. It presented an obviously higher expression in liver, lung, and LN metastatic tissues than in matched primary NPC tissues. High expression of CKAP4 could increase the risk of metastasis, recurrence, and death for NPC patients. Hence, CKAP4 expression showed a close relevance to NPC metastasis. Cell function experiments showed that interference

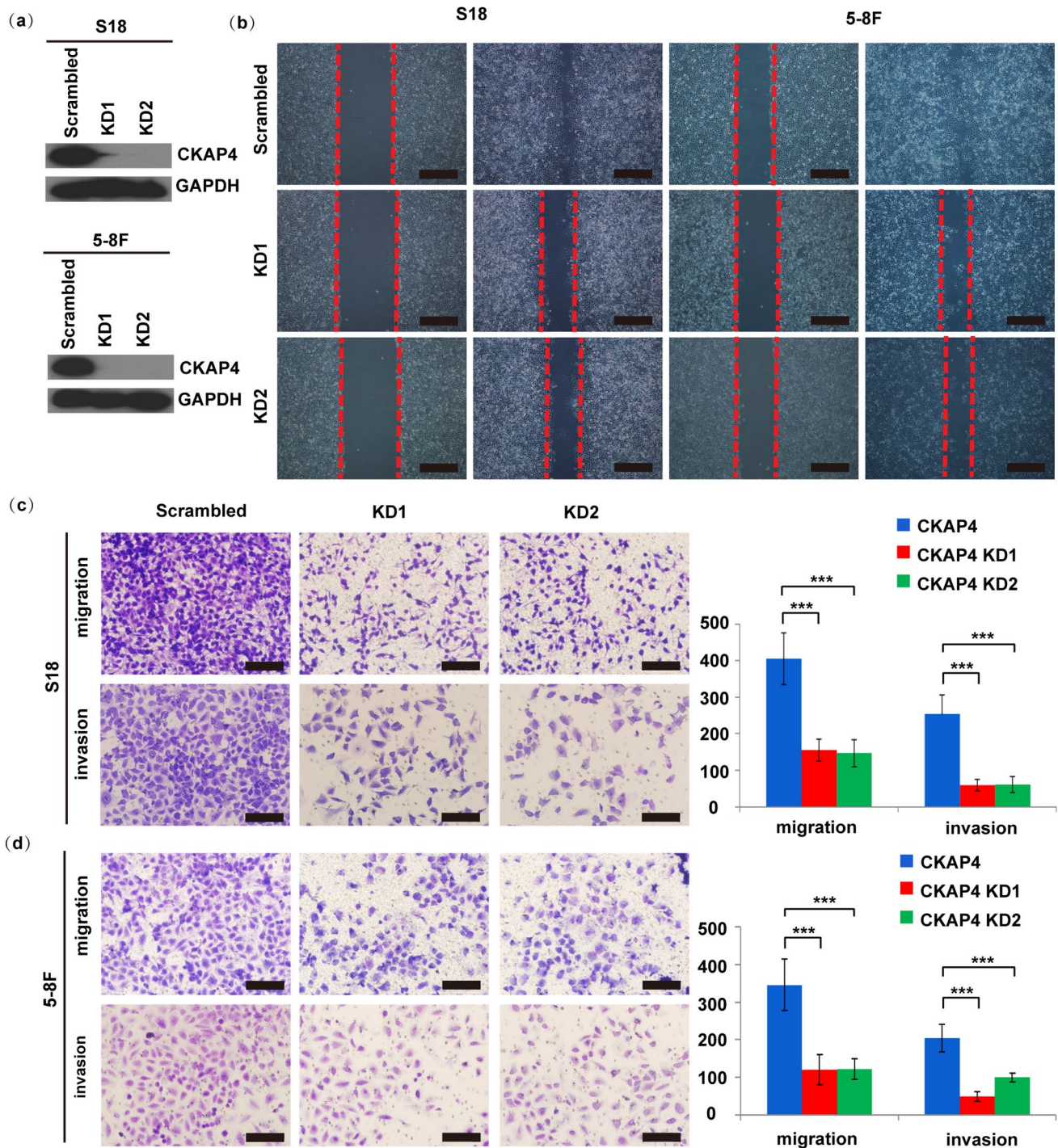


Figure 3. Suppression of CKAP4 inhibits S18 and 5-8F cell migration and invasion. (a) Immunoblotting determined the CKAP4 protein levels. CKAP4 suppression in KD1 and KD2 cells led to a complete elimination of CKAP4 protein expression. (b) Suppression of CKAP4 resulted in remarkably decreased migration ability of S18 and 5-8F cells, as determined by a wound healing assay (scale bar is 100 μm). The red dashed lines represent the wound margins. (c, d) A two-chamber assay served for further evaluating S18 and 5-8F cell migration and invasion (scale bar is 100 μm). Columns, mean; bars, SE (from triplicates). Student's *t*-test served for the statistical analyses. ***, $P < 0.001$ versus the scrambled shRNA group; ***, $P < 0.001$ for the KD1 and KD2 groups versus the scrambled shRNA group.

with CKAP4 expression could significantly reduce NPC cell migration and invasion ability *in vitro*. Furthermore, similar results were obtained in animal experiments: in plantar-popliteal LN and tail vein-lung metastasis mouse models, the ability of NPC cells metastasis to the LNs and lungs was significantly reduced after stable CKAP4 knockdown.

These results strongly indicate that CKAP4 promotes NPC metastasis.

CKAP4 can significantly help to stabilize the ER structure, and is associated with tumorigenesis.²⁷ Many studies have found that CKAP4 can impact the development of a variety of tumors as a cancer-promoting factor.^{14,27} Kimura *et al.*¹⁴

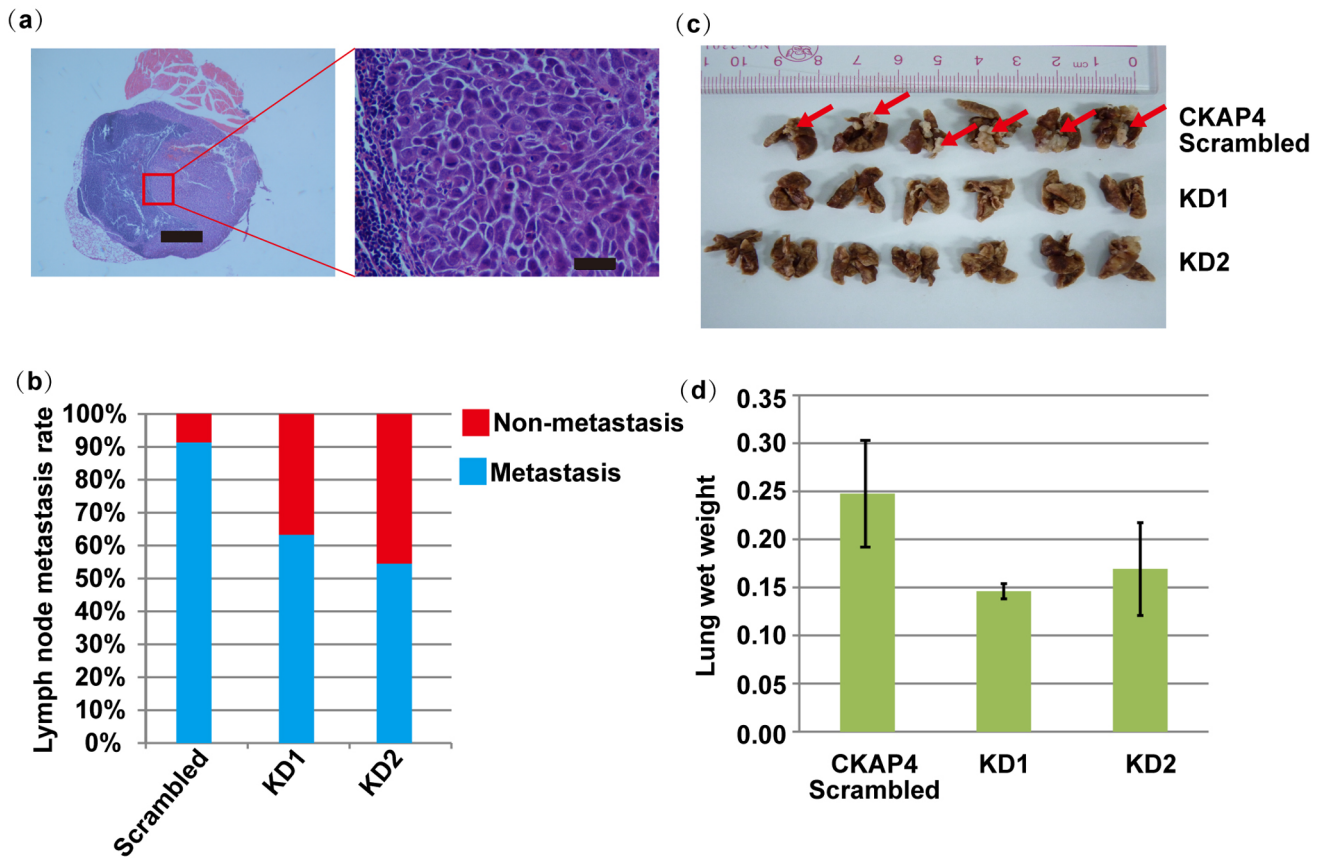


Figure 4. *In vivo* LN and lung metastasis rates of CKAP4 knockdown cells. (a) Image of hematoxylin-stained sections of popliteal LNs (left scale bar is 1000 μm , right scale bar is 100 μm). (b) The proportion of popliteal LN metastasis following the inoculation. CKAP4 knockdown in S18 cells considerably lowered the metastasis rate from 90.9% (10 of 11) to 63.6% (7 of 11) in the KD1 group and 54.5% (6 of 11) in the KD2 group. (c) Images of lungs harvested from scrambled and CKAP4 shRNA mice. The red arrows indicate metastatic foci. (d) The mean lung wet weight in scrambled shRNA ($n=6$) and CKAP4 KD1 and KD2 shRNA group mice ($n=6$ and 7, respectively). Bars represent the means and upper 95% confidence intervals; $P < 0.001$, Student's *t*-test.

found that CKAP4 highly expressed on the plasma membrane can promote the cell proliferation regarding PC and lung cancers by activating the phosphoinositol 3-kinase-Akt pathway. Shinno *et al.*¹⁷ reported that CKAP4 was upregulated in approximately 40% of patients who had esophageal squamous cell carcinoma (ESCC) and promoted ESCC cell proliferation by binding to the receptor DKK1 and activating the AKT pathway. CKAP4 overexpression promotes the proliferation, invasion, and migration of renal clear cell carcinoma through the cyclin B signaling pathway during the G2/M phase of the cell cycle.¹⁵ Another study showed that CKAP4 are capable of mediating PC cell invasion and migration by binding endogenous $\beta 1$ integrin in the cell surface and cytoplasm and recycling of $\alpha 5\beta 1$ integrin.⁶ On the other hand, CKAP4 plays an anticancer role in various tumors such as HCC, ICC, and glioma.^{18–20} For example, CKAP4 suppresses the EGFR signaling, thereby restricting HCC growth and metastasis.²⁸ Therefore, CKAP4 exerts various impacts on tumor biology, which may be associated with various signaling pathways due to the binding of massive ligands in different microenvironments.

Epithelial cells are capable of obtaining mesenchymal features during the process of EMT. In cancer, during EMT, E-cadherin level is declined and vimentin level is increased, thus promoting metastasis and invasion of tumor cells and

making cells more easily metastasized to target tissues or organs, which are related to tumor initiation, invasion, metastasis, and treatment resistance.²⁹ In NPC cell lines, knocking down endogenous CKAP4 significantly reduced vimentin expression and increased E-cadherin expression. The IHC results of 102 cases of NPC tissues also showed the positive relevance of CKAP4 expression to vimentin expression and the obviously negative relevance of CKAP4 expression to E-cadherin expression. Hence, interfering with CKAP4 can make NPC cell lose interstitial properties but acquire epithelial properties. The correlation between CKAP4 and the expression of vimentin and E-cadherin also suggests that CKAP4 may facilitate NPC cells in terms of the migration, invasion, and metastasis by inducing EMT. Additional studies on the specific mechanism by which CKAP4 promotes NPC metastasis need to be carried out.

In summary, this study revealed that the cytoskeleton protein CKAP4 is specifically expressed in NPC tumor tissues, especially in metastatic tissues and NPC cells, but is not expressed in normal NPE tissues. Furthermore, high CKAP4 expression indicates a higher risk of metastasis, recurrence, and death in NPC patients. After stable knockdown of CKAP4 expression, the invasion and migration ability of NPC cells and tumor LN and lung metastasis were significantly decreased in nude mice. Moreover, after CKAP4

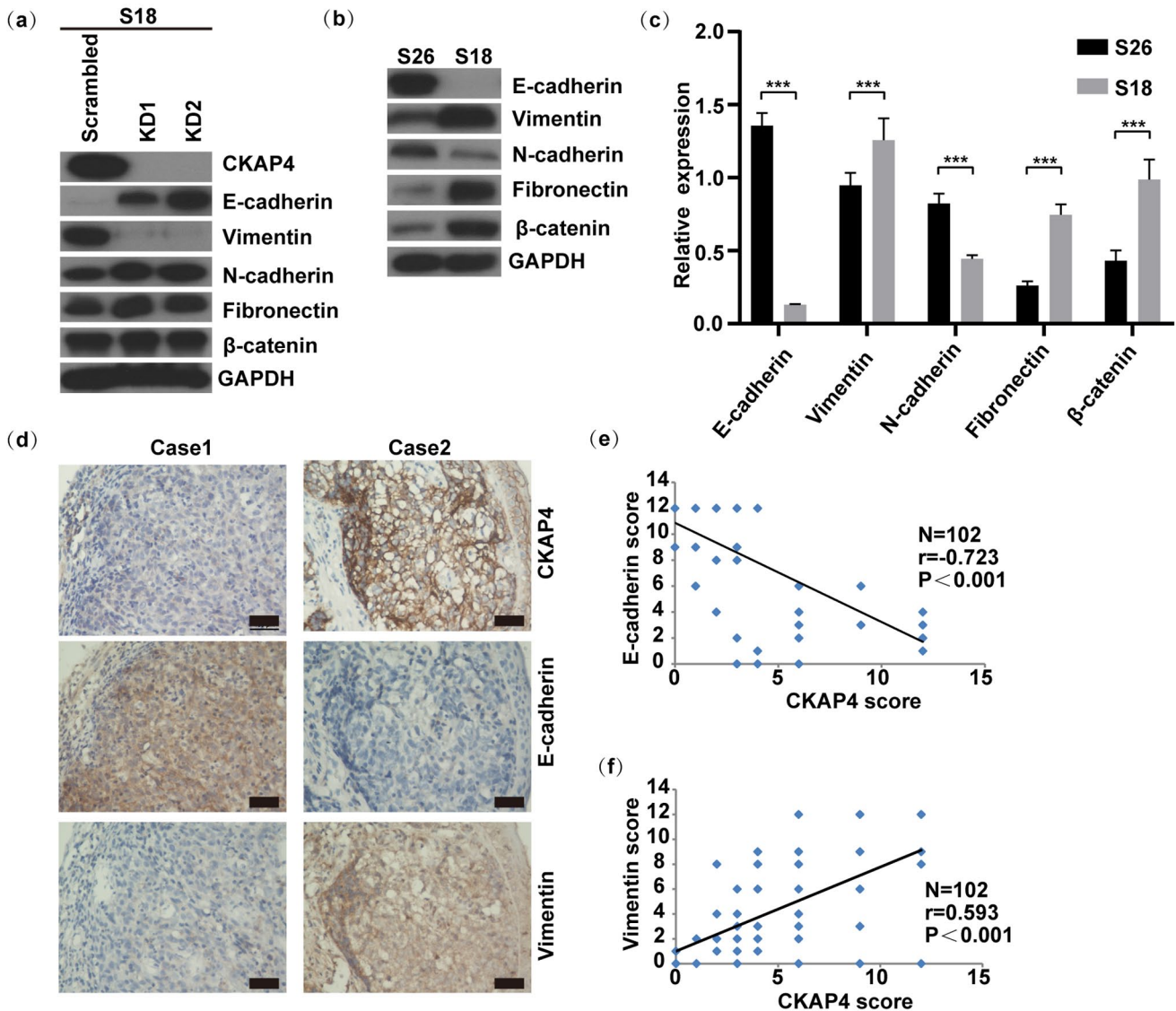


Figure 5. CKAP4 expression modulates mesenchymal marker expression in NPC cells. (a) Impact of stable CKAP4 knockdown on the EMT-associated markers. (b and c) Western blot served for examining the expression of EMT-associated proteins in S18 and S26 cells (b) ImageJ software served for normalizing the gray value possessed by target bands under the detection of corresponding antibodies to those under the detection of GAPDH (loading control), thereby quantifying the expression of these proteins (c). We repeated each experiment no less than three times. Data are in the form of the mean \pm SEM. ***, $P < 0.001$. One-way analysis of variance. (d) Continuous sections of human NPC tissue received IHC staining using antibodies against CKAP4, vimentin and E-cadherin (scale bar is 100 μ m). Low CKAP4 expression in the tumor tissue in case 1 resulted in lost vimentin and increased E-cadherin. Comparatively, high CKAP4 expression in case 2 resulted in increased vimentin levels and lost E-cadherin. (e) Same methods were adopted for scoring and plotting 102 primary NPC tissues that received the staining of antibodies against E-cadherin and CKAP4. A significant negative relevance of E-cadherin to CKAP4 levels was illustrated. (f) For vimentin staining, 102 NPC tissues underwent qualification for analysis. A markedly positive relevance of vimentin to CKAP4 levels was illustrated.

knockdown, the EMT marker E-cadherin protein level presented an obvious upregulation, while the level of vimentin presented an obvious downregulation, which demonstrated that CKAP4 might promote NPC cell metastasis through inducing EMT.

AUTHORS' CONTRIBUTIONS

MB conducted all the experiments, wrote the initial draft, and revised the manuscript. WJ, SL, and QY helped to perform some of the molecular biology experiments. HB, HY, JX, and QS performed animal and IHC work. JY and YX participated in the study design and interpretation of the results. JJ contributed to the study coordination and revisions. All authors read and approved the final manuscript.

ACKNOWLEDGEMENTS

We thank Prof. Chao-Nan Qian from Sun Yat-Sen University Cancer Center for providing the N-cadherin, β -catenin, and fibronectin antibodies.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this

article: This study was supported partially by the Natural Science Foundation of Hunan Province, China (2020JJ4539 and 2021JJ30610); and the Key Guiding Project of Hunan Provincial Health Commission (202104101985); the Science and Technology Innovation Project of Hengyang City (202150064015); the University of South China Teaching Reform Research Project (2020YB-XJG127); and the National High Technology Research and Development Program of China (863 Program) (2012AA02A206).

ORCID ID

Manbo Cai  <https://orcid.org/0000-0003-0235-0111>

SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

REFERENCES

- Chen YP, Chan ATC, Le QT, Blanchard P, Sun Y, Ma J. Nasopharyngeal carcinoma. *Lancet* 2019;**394**:64–80
- Liu SL, Sun XS, Lu ZJ, Chen QY, Lin HX, Tang LQ, Bei JX, Guo L, Mai HQ. Nomogram predicting the benefits of adding concurrent chemotherapy to intensity-modulated radiotherapy after induction chemotherapy in stages II-IVb nasopharyngeal carcinoma. *Front Oncol* 2020;**10**:539321
- Zhang LL, Xu F, He WT, Huang MY, Song D, Li YY, Deng QL, Huang YS, Wang T, Shao JY. Development and validation of a prognostic nomogram for the pre-treatment prediction of early metachronous metastasis in endemic nasopharyngeal carcinoma: a big-data intelligence platform-based analysis. *Ther Adv Med Oncol* 2020;**12**:0978132
- Laskar SG, Gurram L, Gupta T, Budrukkar A, Murthy V, Agarwal JP. Outcomes in nasopharyngeal carcinoma: results from a nonendemic cohort. *Indian J Cancer* 2016;**53**:493–8
- Schweizer A, Ericsson M, Bächi T, Griffiths G, Hauri HP. Characterization of a novel 63 kDa membrane protein. Implications for the organization of the ER-to-Golgi pathway. *J Cell Sci* 1993;**104**:671–83
- Osugi Y, Fumoto K, Kikuchi A. CKAP4 regulates cell migration via the interaction with and recycling of integrin. *Mol Cell Biol* 2019;**39**:e00073–10019
- Razaq TM, Bass R, Vines DJ, Werner F, Whawell SA, Ellis V. Functional regulation of tissue plasminogen activator on the surface of vascular smooth muscle cells by the type-II transmembrane protein p63 (CKAP4). *J Biol Chem* 2003;**278**:42679–85
- Gupta N, Manevich Y, Kazi AS, Tao JQ, Fisher AB, Bates SR. Identification and characterization of p63 (CKAP4/ERGIC-63/CLIMP-63), a surfactant protein A binding protein, on type II pneumocytes. *Am J Physiol Lung Cell Mol Physiol* 2006;**291**:L436–46
- Conrads TP, Tocci GM, Hood BL, Zhang CO, Guo L, Koch KR, Michejda CJ, Veenstra TD, Keay SK. CKAP4/p63 is a receptor for the frizzled-8 protein-related antiproliferative factor from interstitial cystitis patients. *J Biol Chem* 2006;**281**:37836–43
- Barbier M, Martínez-Ramos I, Townsend P, Albertí S. Surfactant protein A blocks recognition of *Pseudomonas aeruginosa* by CKAP4/P63 on airway epithelial cells. *J Infect Dis* 2012;**206**:1753–62
- Kimura H, Yamamoto H, Harada T, Fumoto K, Osugi Y, Sada R, Maehara N, Hikita H, Mori S, Eguchi H, Ikawa M, Takehara T, Kikuchi A. CKAP4, a DKK1 receptor, is a biomarker in exosomes derived from pancreatic cancer and a molecular target for therapy. *Clin Cancer Res* 2019;**25**:1936–47
- Planey SL, Keay SK, Zhang CO, Zacharias DA. Palmitoylation of cytoskeleton associated protein 4 by DHHC2 regulates antiproliferative factor-mediated signaling. *Mol Biol Cell* 2009;**20**:1454–63
- Zacharias DA, Mullen M, Planey SL. Antiproliferative factor-induced changes in phosphorylation and palmitoylation of cytoskeleton-associated protein-4 regulate its nuclear translocation and DNA binding. *Int J Cell Biol* 2012;**2012**:150918
- Kimura H, Fumoto K, Shojima K, Nojima S, Osugi Y, Tomihara H, Eguchi H, Shintani Y, Endo H, Inoue M, Doki Y, Okumura M, Morii E, Kikuchi A. CKAP4 is a Dickkopf1 receptor and is involved in tumor progression. *J Clin Invest* 2016;**126**:2689–705
- Sun CM, Geng J, Yan Y, Yao X, Liu M. Overexpression of CKAP4 is associated with poor prognosis in clear cell renal cell carcinoma and functions via cyclin B signaling. *J Cancer* 2017;**8**:4018–26
- Kajiwara C, Fumoto K, Kimura H, Nojima S, Asano K, Odagiri K, Yamasaki M, Hikita H, Takehara T, Doki Y, Morii E, Kikuchi A. P63-dependent Dickkopf3 expression promotes esophageal cancer cell proliferation via CKAP4. *Cancer Res* 2018;**78**:6107–20
- Shinno N, Kimura H, Sada R, Takiguchi S, Mori M, Fumoto K, Doki Y, Kikuchi A. Activation of the Dickkopf1-CKAP4 pathway is associated with poor prognosis of esophageal cancer and anti-CKAP4 antibody may be a new therapeutic drug. *Oncogene* 2018;**37**:3471–84
- Li MH, Dong LW, Li SX, Tang GS, Pan YF, Zhang J, Wang H, Zhou HB, Tan YX, Hu HP, Wang HY. Expression of cytoskeleton-associated protein 4 is related to lymphatic metastasis and indicates prognosis of intrahepatic cholangiocarcinoma patients after surgery resection. *Cancer Lett* 2013;**337**:248–53
- Li SX, Tang GS, Zhou DX, Pan YF, Tan YX, Zhang J, Zhang B, Ding ZW, Liu LJ, Jiang TY, Hu HP, Dong LW, Wang HY. Prognostic significance of cytoskeleton-associated membrane protein 4 and its palmitoyl acyltransferase DHHC2 in hepatocellular carcinoma. *Cancer* 2014;**120**:1520–31
- Lu GF, You CY, Chen YS, Jiang H, Zheng X, Tang WW, Wang XY, Xu HY, Geng F. MicroRNA-671-3p promotes proliferation and migration of glioma cells via targeting CKAP4. *Oncotargets Ther* 2018;**11**:6217–26
- Liu LN, Huang PY, Lin ZR, Hu LJ, Liang JZ, Li MZ, Tang LQ, Zeng MS, Zhong Q, Zeng BH. Protein tyrosine kinase 6 is associated with nasopharyngeal carcinoma poor prognosis and metastasis. *J Transl Med* 2013;**11**:140
- Li XJ, Ong CK, Cao Y, Xiang YQ, Shao JY, Ooi A, Peng LX, Lu WH, Zhang ZF, Petillo D, Qin L, Bao YN, Zheng FJ, Chia CS, Gopalakrishna Iyer N, Kang TB, Zeng YX, Soo KC, Trent JM, Teh BTe Qian CN. Serglycin is a theranostic target in nasopharyngeal carcinoma that promotes metastasis. *Cancer Res* 2011;**71**:3162–72
- Qiang YY, Li CZ, Sun R, Zheng LS, Peng LX, Yang JP, Meng DF, Lang YH, Mei Y, Xie P, Xu L, Cao Y, Wei WW, Cao L, Hu H, Yang Q, Luo DH, Liang YY, Huang BJ, Qian CN. Along with its favorable prognostic role, CLCA2 inhibits growth and metastasis of nasopharyngeal carcinoma cells via inhibition of FAK/ERK signaling. *J Exp Clin Cancer Res* 2018;**37**:34
- Li F, Zhao X, Sun R, Ou J, Huang J, Yang N, Xu T, Li J, He X, Li C, Yang M, Zhang Q. EGFR-rich extracellular vesicles derived from highly metastatic nasopharyngeal carcinoma cells accelerate tumour metastasis through PI3K/AKT pathway-suppressed ROS. *J Extracell Vesicles* 2020;**10**:e12003
- Lee AWM, Tung SY, Ng WT, Lee V, Ngan RKC, Choi HCW, Chan LLK, Siu LL, Ng AWY, Leung TW, Yiu HHY, O'Sullivan B, Chappell R. A multicenter, phase 3, randomized trial of concurrent chemoradiotherapy plus adjuvant chemotherapy versus radiotherapy alone in patients with regionally advanced nasopharyngeal carcinoma: 10-year outcomes for efficacy and toxicity. *Cancer* 2017;**123**:4147–57
- Tang LL, Chen YP, Chen CB, Chen MY, Chen NY, Chen XZ, Du XJ, Fang WF, Feng M, Gao J, Han F, He X, Hu CS, Hu DS, Hu GY, Jiang H, Jiang W, Jin F, Lang JY, Li JG, Lin SJ, Liu X, Liu QF, Ma L, Mai HQ, Qian JY, Shen LF, Sun Y, Wang PG, Wang RS, Wang RZ, Wang XS, Wang Y, Wu H, Xia YF, Xiao SW, Yang KY, Yi JL, Zhu XD, Ma J. The Chinese Society of Clinical Oncology (CSCO) clinical guidelines for the diagnosis and treatment of nasopharyngeal carcinoma. *Cancer Commun* 2021;**41**:1195–227
- Li XJ, Li J, Dong LW, Guo ZY. Cytoskeleton-associated protein 4, a promising biomarker for tumor diagnosis and therapy. *Front Mol Biosci* 2021;**7**:552056
- Li SX, Liu LJ, Dong LW, Shi HG, Pan YF, Tan YX, Zhang J, Zhang B, Ding ZW, Jiang TY, Hu HP, Wang HY. CKAP4 inhibited growth and metastasis of hepatocellular carcinoma through regulating EGFR signaling. *Tumour Biol* 2014;**35**:7999–8005
- Pastushenko I, Blanpain C. EMT transition states during tumor progression and metastasis. *Trends Cell Biol* 2019;**29**:212–26