Capn4 is a marker of poor clinical outcomes and promotes nasopharyngeal carcinoma metastasis via nuclear factor-κB-induced matrix metalloproteinase 2 expression

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Key words
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Nasopharyngeal carcinoma (NPC) is a common head and neck malignant tumor, particularly in southern China.1–3 Recent advances in diagnosis and treatment have lead to great expectations for long-term survival of patients with early NPC.3 However, patients with advanced NPC, characterized by extensive invasion and metastasis, still exhibit poor prognosis and high mortality. Like many other types of malignancies, NPC progresses via a process involving multiple steps, including initiation, local progression, and metastasis, which are likely associated to a wide variety of genetic aberrancies.4,5 However, the precise mechanism(s) responsible for NPC metastasis remain to be defined. Thus, identification and characterization of novel molecules involved in progression of NPC are urgently needed to provide biomarkers for prognosis and therapeutic targets for treatment.

Calpain represents a family of calcium-dependent cytosolic cysteine proteases. So far, there are 14 calpain isoforms identified in human, most of which are found at focal adhesions and are involved in cell spreading and migration, proliferation, cell cycle control, and apoptosis.6–11 Capn4 (also known as CapnS1) is a small regulatory subunit of the calpain proteolytic system and plays a critical role in regulation of calpain stability and activity.12 Several studies have demonstrated that deficiency of Capn4 leads to dysfunction of calpain-1 and calpain-2, which is embryonic lethal.11–15 Knockdown of Capn4 results in decreased migration and focal adhesion of fibroblasts cells.14 Recently, overexpression of Capn4 was found in hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC).16–18 Moreover, whereas Capn4 overexpression is closely correlated with prognosis of patients with HCC or ICC, siRNA-mediated silencing of the Capn4 gene results in marked inhibition of invasion and metastasis in HCC and ICC cells. Therefore, Capn4 may play a critical role in migration and adhesion of metastatic cancer cells.

However, it remains unknown whether Capn4 plays a functional role in pathogenesis of NPC. To address this question, we examined expression of Capn4 in NPC tissues and analyzed its correlation with clinicopathologic features of patients
with NPC. We also investigated the biological function of Capn4 in progression of NPC using NPC cell lines. Here we report that Capn4 is highly expressed in both primary NPC tumor tissues and NPC cell lines, compared to normal tissues and cells. Moreover, Capn4 plays an important role in invasion and metastasis of NPC, most likely through regulation of matrix metalloproteinase 2 (MMP2) via nuclear factor-κB (NF-κB) activation. Collectively, these findings suggest that Capn4 may represent an independent prognostic factor and a potential therapeutic target in NPC.

Materials and Methods

Ethics statement. The clinical processes were approved by the Ethics Committees of the Fujian Medical University and patients provided informed consent. Animal studies were performed with the approval of the Institutional Committee for Animal Research and in conformity with national guidelines for the care and use of laboratory animals.

Primary tissue specimens. Paraffin-embedded blocks of 153 NPC tumors resected surgically between March 1999 and September 2010 at the Fuzhou General Hospital, Fuzhou, China, and Guangzhou General Hospital, Guangzhou, China, were examined in the present study. Thirty blocks of non-tumoral tissues were obtained from these patients with NPC or with other diseases, who underwent surgery. Clinical and pathological data were obtained from the Surgical Pathology Files, including age, gender, race, tumor size, tumor location, and TNM stage. H&E-stained slides from each case of primary NPC were screened by light microscopy, and representative sections was performed as previously described. The slides were reviewed and scored independently by two pathologists blinded to the clinical data, using the scoring standard described before. For analysis of Capn4 expression in NPC versus non-tumoral tissues, the scores of 0, 1−2, 3−4, and 5−6 were considered to be negative, low, medium, and strong, respectively. For analysis of correlation between Capn4 expression and clinical features or prognosis of NPC patients, the scores of 0−4 and 5−6 were considered to be low and high, respectively.

RNA and proteins and transfection. The pSilencer3.1 (Ambion, Austin, TX, USA) plasmid was used as a negative control of the constructs encoding siRNA targeting human Capn4, according to the manufacturer’s protocol. Two separate pairs of specific oligonucleotide were used, including Capn4 siRNA1 (sense, 5'-UCAAUUGAUAUGAGUCCCG-3'; antisense, 5'-GCAUCUAUCAUAGCG-3') targeting GGCGCTCTAC ATATCAATGACG, and Capn4 siRNA2 (sense, 5'-UGAAUUGUCAUCAUCCU-3'; antisense, 5'-GAUGAAC GUUGACAUCAAAA-3') targeting AAGATGATATGGG CTATCTAAA. The constructs in pcDNA3.1 (+) (Invitrogen) encoding human Capn4 and MMP2 were used for ectopic expression of these genes. Transfection was performed using Lipofectamine2000 (Invitrogen), as per the manufacturer’s instructions.

Gelatin zymography of MMP activity. To detect specific MMP2 enzyme activity, serum-free media was collected from culture medium of cells 24 h after transfection with Capn4 siRNA1 and Capn4 siRNA2, and then analyzed using a gelatin zymography assay kit (Applygen, Beijing, China). Matrix metalloproteinase activity was determined by SDS-PAGE containing 1% gelatin and 30% acrylamide under non-reducing condition. Briefly, electrophoresis was carried out at 4°C. After washing with buffer A (containing 2% Triton X-100), the gels were incubated in 37°C with buffer B (containing the necessary metal ion: 5 mmol/L CaCl2, 1 mmol/L ZnCl2). The active form of MMP was then visualized by staining with Coommasie Blue R-250.

In vitro assays of migration and invasion. To measure cell migration and invasion, 24-well transwell plates (8-mm pore size, Corning Inc., Corning, NY, USA) were used. For transwell migration analysis, 2.5 × 104 cells suspended in medium with serum or growth factors were plated in the top chamber underlined with a non-coated membrane. For invasion analysis, chamber inserts were coated with 200 μg/mL Matrigel and dried overnight under sterile conditions, after which 5 × 104 cells in medium with serum or growth factors were plated in the top chamber. For both assays, medium containing serum was added in the lower chamber as a chemo attractant. After incubation at 37°C for 24 h, the top chambers were wiped with cotton wool to remove non-migratory or non-invasive cells. Invading cells on the underside of membrane were fixed on behalf of Japanese Cancer Association.
in 100% methanol for 10 min, air-dried, stained in 0.1% crystal-violet, and counted under microscopy. Values represent the means ± SD for three independent experiments.

**In vivo analysis of metastasis.** All animal experiments were conducted in accordance with a protocol approved by the Fujian Medical University Institutional Animal Care and Utilization Committee. Five-week-old BALB/c-\(\nu\)-\(\nu\) nude mice obtained from the Shanghai Laboratory Animal Center of China, and maintained in a sterile animal facility. To evaluate metastasis, \(1 \times 10^6\) cells were resuspended in 0.1 mL of PBS and injected via the lateral tail vein. After 10 weeks, mice were euthanized, and metastatic nodules in lung and liver were quantified using dissecting microscopy after H&E staining.

**Statistical analysis.** Statistical analysis was performed using the spss software (SPSS Standard version 17.0, SPSS Inc., Chicago, IL, USA). Correlation of Capn4 levels with patients’ clinicopathological features were analyzed by either the \(\chi^2\) test or the Fischer’s exact test. The Kaplan–Meier analysis and the log-rank test was performed analyze survival of patients with NPC and to compare the different survival curves, respectively. \(P < 0.05\) was considered significant.

**Results**

**Overexpression of Capn4 in nasopharyngeal carcinomas.** To examine expression of Capn4 in primary NPC tumor and normal nasopharyngeal tissues, RT-PCR and Western blot analysis were performed to detect mRNA and protein levels. The results revealed that the Capn4 mRNA and protein expression level was significantly upregulated in NPC tissues \((n = 7)\) compared with normal nasopharyngeal tissues \((n = 2; \text{Fig. 1a,b})\).

In parallel, upregulation of Capn4 was also observed in three NPC cell lines, 5-8F, CNE2, and 6-10B, when compared to immortalized normal human nasopharyngeal epithelial cell lines NP69 (Fig. 1c,d).

To confirm the RT-PCR and Western blot results, we next detected expression of Capn4 protein in archived paraffin-embedded NPC tumor and non-tumoral nasopharyngeal specimens by immunohistochemical staining. Whereas Capn4-specific staining was observed in both non-cancerous and malignant tissues, primarily localized in the cytoplasm of cells (Fig. 2a), expression levels of Capn4 in NPC samples were significantly higher than that in non-tumoral tissues \((P < 0.001; \text{Table 1})\).

**Capn4 expression is correlated with NPC progression.** We then analyzed the potential correlation between Capn4 expression levels and clinicopathological implications. As shown in Table 2, expression of Capn4 in NPC tumor tissues was not correlated to patients’ gender, age, smoking status, family history, and disease recurrence in a total of 153 cases. However, protein levels of Capn4 in tumors were significantly higher in patients with positive Epstein-Barr (EB) virus infection, advanced tumors (T3–T4), lymph node metastasis (N2–N3), distant metastasis (M1), or TNM stage III/IV, compared to patients with negative EB virus infection \((P < 0.001)\), early tumors (T1–T2; \(P < 0.001)\), no or a few lymph node metastasis (N0–N1; \(P < 0.001)\), no distant metastasis (M0; \(P < 0.001)\), or TNM stage I/II \((P = 0.007)\). Moreover, Kaplan–Meier survival analyses demonstrated that patients with higher expression of Capn4 had a significantly shorter overall survival (OS), compared to those with lower levels of Capn4 \((P = 0.002; \text{Fig. 2b})\). Last, high levels of Capn4 in NPC tumor were significantly correlated with reduced progression-free survival (PFS, \(P = 0.003\), Fig. 2c). Together, these findings

![Fig. 1. Capn4 expression in nasopharyngeal carcinoma (NPC) biopsy tissues and cell lines. (a) mRNA levels of Capn4 in tumor tissues from seven patients with NPC and two normal tissues were determined by RT-PCR. The results were normalized against mRNA levels of β-actin in each sample. N, normal tissues; T, tumor tissues. (b) Western blot analysis on Capn4 expression was performed in NPC tumor tissue samples and normal tissues. Total proteins were extracted from tissues and subjected to immunoblotting probed by antibodies against Capn4. β-actin was probed as control. (c, d) Capn4 mRNA and protein levels were monitored in the NPC cell lines (5-8F, CNE2, and 6-10B) and the immortalized human nasopharyngeal epithelial cell line NP69, respectively. *\(P < 0.05\).](https://www.wileyonlinelibrary.com/journal/cas)
indicate that Capn4 expression was closely associated with progression and metastasis of NPC, thereby probably representing marker for poor prognosis.

Knockdown of Capn4 reduces migration and invasion of NPC cells in vitro and in vivo. To determine the functional role of Capn4 in metastasis of NPC, we first generated two clones of 5-8F cells stably transfected with Capn4 siRNA (Capn4/siRNA-1 and Capn4/siRNA-2). RT-PCR and Western blot analyses demonstrated that both protein and mRNA levels of Capn4 were substantially downregulated in Capn4/siRNA-1 and Capn4/siRNA-2 cells, compared to siRNA control cells (Fig. 3a,b). Then, the transwell assays were performed to assess the effects of Capn4 knockdown on capabilities of invasion and migration in NPC cells. Notably, Capn4/siRNA cells displayed significantly lower rate of invasion and migration than the parental cells and siRNA control cells (Fig. 3c). In contrast, there was no change observed in expression of MMP9, N-cadherin, or β-catenin. Among the proteins that were downregulated in Capn4 siRNA cells, MMP2 plays an important role in multiple steps of cancer progression, including tumor invasion and metastasis, angiogenesis, as well as extracellular matrix remodeling in NPC. We then tested whether MMP2 contributes to Capn4-mediated regulation of NPC cell invasion. First, RT-PCR analysis confirmed a reduction in MMP2 mRNA after Capn4 was knocked down in NPC cells (Fig. 4b). Second, MMP2 enzyme activity was determined using the gelatin zymography in media obtained from 5-8F cells and CNE2 cells transfected with Capn4 siRNA1 and siRNA2. The results showed lower enzyme activity in these cells than siRNA control cells, manifested by a band at 72 kDa (Fig. 4c). Last, knockdown of MMP2 by siRNA significantly suppressed invasion capability of 5-8F cells and CNE2 cells in transwell assays. Conversely, overexpression of MMP2 in cells, in which Capn4 was silenced by siRNA, rescued the functional effect of Capn4 on cell invasion and metastasis.

Table 1. Overexpression of Capn4 protein in nasopharyngeal carcinoma (NPC) compared to NP epithelium tissues

| Group          | Protein expression (n) | P-value |
|----------------|------------------------|---------|
|                | Total | Low | High |         |
| Normal epithelium | 30    | 28  | 2    | <0.001 |
| Cancer         | 153   | 81  | 72   |         |

**Fig. 2.** Expression of Capn4 is correlated with overall survival of patients with nasopharyngeal carcinoma (NPC). (a) Capn4 expression, reflected by staining intensity, in tumor and normal tissues were evaluated by IHC. 1 and 2, normal tissues; 3 and 4, NPC tumor tissues. (b, c) Kaplan–Meier analysis of overall survival (b) or progression-free survival (c) in 153 patients with NPC, stratified by Capn4 levels.
Calpains have been implicated in a wide variety of biological functions, including signal transduction, cell proliferation and differentiation, apoptosis, membrane fusion, platelet activation, and tumor progression.\(^{6,11,34,35}\) As a regulatory subunit of calpains, Capn4 is essential for calpain stability and activity. Previous studies have demonstrated that Capn4 regulates integrin-mediated cell migration and thus plays a pivotal role in pathogenesis of HCC and ICC.\(^{12,36,37}\) However, it has not yet been defined whether Capn4 also involves progression of NPC, and if so by what mechanism(s). In the present study, we found that Capn4 was highly expressed in NPC tumor tissues, as well as multiple human NPC cell lines. Of note, that expression of Capn4 was significantly higher in NPC cells with high metastatic potential (e.g., 5-8F cells) than those with low metastatic potential (e.g., CNE2 and 6-10B cells).\(^{19,20}\) These results suggest that Capn4 upregulation might be associated with progression of NPC. In this context, previous studies have revealed that Capn4 expression is correlated with tumor metastasis. For example, elevated Capn4 mRNA has been found within stances of liver metastasis when comparing with primary colorectal cancer.\(^{38}\) Moreover, SV40-transformed Capn4–/– mouse embryonic fibroblasts display elevated protein levels of retinoblastoma, a tumor suppressor gene.\(^{39}\) Further, overexpression of Capn4 leads to tumor invasion and metastasis in HCC and ICC.\(^{18,36}\) To this end, we found that Capn4 expression was positively correlated with worse TNM classification, distance metastasis, and advanced stages of NPC, suggesting that Capn4 might contribute to the aggressive phenotype of NPC. We also found that high levels of Capn4 were closely correlated with poor clinical outcome and survival of NPC patients. Therefore, these findings support a notion that Capn4 might represent a novel, independent marker for prognosis of patients with NPC.

Tumor metastasis involves multiple sequential steps, including dysregulation of intercellular adhesion, remodeling and degradation of extracellular matrix (ECM), and increasing cell motility.\(^{10,41}\) Capn4 has been reported to play a role in invasion and migration of HCC and ICC cells.\(^{17,18,56}\) In the present study, we found that downregulation of Capn4 by siRNA dramatically reduced ability of invasion and migration in vitro, as well as distant metastasis in vivo of 5-8F cells, a human NPC cell line exhibiting high metastatic potential.\(^{19}\) These findings strongly suggest that high expression of Capn4 might functionally contribute to metastatic phenotypes of NPC. Among numerous genes that are associated with tumor metastasis, MMP2, MMP9, Snail, Vimentin, E-cadherin, H-cadherin, and β-catenin are substrates for proteolysis mediated by calpains, of which Capn4, as a regulatory subunit, plays a critical role in stability and activity.\(^{23-27,42}\) The present results showed that Capn4 knockdown by siRNA led to downregulation of Snail, Vimentin and MMP2, but upregulation of E-cadherin. Among these targets, calpains can cleave one or more of the focal adhesion components (e.g., Vimentin, E-cadherin, and H-cadherin), resulting in disassembly of focal adhesions during cell migration.\(^{26,33,44}\) Snail induces EMT, one of the most important events in tumor metastasis,\(^{45}\) and MMPs involves tumor metastasis by regulation of tumor microenvironment.\(^{46}\) Thus, the present findings suggest that Capn4 promotes NPC metastasis likely via these multiple downstream events. However, a possibility exists that other proteins might also contribute to Capn4-mediated promotion of NPC metastasis.

Matrix metalloproteinase 2 plays a critical role in pathogenesis of various types of cancer.\(^{47}\) Notably, MMP2 is associated with metastasis of NPC.\(^{30,48}\) In the present study, we found that mRNA level and enzyme activity of MMP2 was markedly decreased after knockdown of Capn4 by siRNA in 5-8F cells and CNE2 cells, consistent with a recent report that Capn4

**Table 2. Correlation between the clinicopathologic characteristics and expression of Capn4 protein in nasopharyngeal carcinoma (NPC)**

| Characteristics                      | n | Capn4 |
|-------------------------------------|---|-------|
|                                     |   | High expression | Low expression | p  |
| Gender                              |   |                 |               |    |
| Male                                | 104| 46   | 58     | 0.3072 |
| Female                              | 49 | 26   | 23     |     |
| Age                                 |   |                 |               |    |
| >50                                 | 69 | 30   | 39     | 0.4211 |
| <50                                 | 84 | 42   | 42     |     |
| Smoking                             |   |                 |               |    |
| Yes                                 | 43 | 18   | 25     | 0.4217 |
| No                                  | 110| 54   | 56     |     |
| Family tumor history                |   |                 |               |    |
| Yes                                 | 4  | 1    | 3      | 0.5400 |
| No                                  | 149| 71   | 78     |     |
| EB Viral                            |   |                 |               |    |
| Positive                            | 138| 72   | 66     | <0.0001*|
| Negative                            | 15 | 0    | 15     |     |
| Recurrence                          |   |                 |               |    |
| Yes                                 | 35 | 21   | 14     | 0.0814 |
| No                                  | 118| 51   | 67     |     |
| T classification                    |   |                 |               |    |
| T1-T2                               | 101| 29   | 72     | <0.0001*|
| T3-T4                               | 52 | 43   | 9      |     |
| N classification                    |   |                 |               |    |
| N0-N1                               | 83 | 20   | 63     | <0.0001*|
| N2-N3                               | 70 | 52   | 18     |     |
| Distant metastasis                  |   |                 |               |    |
| Yes                                 | 17 | 15   | 2      | <0.0001*|
| No                                  | 136| 57   | 79     |     |
| TNM clinical stage                  |   |                 |               |    |
| I–II                                | 53 | 17   | 36     | 0.0072*|
| III–IV                              | 100| 55   | 45     |     |

*P < 0.05.
Fig. 3. Knockdown of Capn4 reduces nasopharyngeal carcinoma (NPC) cell migration and invasion in vitro and in vivo. (a, b) 5-8F cells and CNE2 cells were stably transfected with constructs encoding siRNA specifically targeting Capn4 or scrambled sequence as siRNA control, after which semi-quantitative RT-PCR and Western blot analyses were performed to monitor mRNA and protein levels of Capn4 in Capn4 siRNA and control siRNA cells, as well as parental 5-8F cells and CNE2 cells. β-actin was probed as internal control. (c) Transwell assays were performed to measure in vitro migration and invasion of Capn4 siRNA and control siRNA cells. (d) To evaluate in vivo metastasis of Capn4 siRNA and control siRNA cells (n = 10 per group), $1 \times 10^5$ cells were resuspended in 0.1 mL of PBS and injected via the lateral tail vein. After 10 weeks, mice were euthanized, and metastatic nodules in lung and liver were quantified using dissecting microscopy after H&E staining. Representative H&E staining images of lungs and livers were shown.
upregulates MMP2 in ICC cells, suggesting that Capn4 might promote invasion and metastasis of NPC cells by upregulating MMP2. Indeed, like knockdown of Capn4, downregulation of MMP2 by siRNA inhibited invasion of 5-8F cells. Importantly, overexpression of MMP2 in Capn4 siRNA 5-8F and Capn4 siRNA CNE2 cells restored their invasion capability. Thus, these results argue that MMP2 represents one of the downstream targets in Capn4-mediated regulation of NPC metastasis.

The NF-κB signaling pathway plays important roles in various biological processes such as inflammation, apoptosis, cell migration, and cell cycle control, and is often dysregulated in a variety of malignancies. Of note, NF-κB is capable of inducing production of MMPs by tumor cells as well as surrounding mesenchymal cells, events contributing to degradation of extracellular matrix during tumor metastasis. Interestingly, NF-κB is commonly activated in NPC, which acts as an activator of the invasion process to promote tumor progression. The present results showed that downregulation of Capn4 by siRNA significantly reduced phosphorylation of the NF-κB p65 subunit, while ectopic expression of Capn4 induced p65 phosphorylation in NPC cells. Moreover, the NF-κB inhibitor helenalin markedly prevented MMP2 expression induced by ectopic expression of Capn4. Thus, these findings suggest that Capn4 regulates NPC metastasis through a process involving NF-κB-dependent expression of MMP2. However, it could not be excluded that other mechanisms might also contribute to Capn4-promoted NPC metastasis. For example, the EBV-encoded multifunctional oncoprotein latent membrane protein 1 (LMP1) is essential for EBV-induced B-cell proliferation and transformation in vitro, probably by acting as an inducer of NF-κB activation. Thus, as Capn4 also induced activation of the NF-κB pathway, it is possible that LMP1 and Capn4 may cooperate in the pathogenesis of NPC.

Table 3. Incidence of metastasis in mice that injected with different cell lines

| Metastasis | 5-8F/control | 5-8F/siRNA1 | P-value ($\chi^2$ test) |
|------------|--------------|-------------|------------------------|
| Liver      | 7/10         | 1/10        | 0.006                  |
| Lung       | 7/10         | 2/10        | 0.025                  |

Fig. 4. Matrix metalloproteinase 2 (MMP2) functionally contributes to Capn4-mediated nasopharyngeal carcinoma (NPC) cell migration and invasion. (a) Western blot analysis was performed to monitor protein levels of genes associated with cell migration and invasion in Capn4 siRNA and control siRNA 5-8F cells. (b) mRNA levels of MMP2 were determined by RT-PCR analysis in Capn4 siRNA and control siRNA cells. (c) MMP2 enzyme activity was measured by gelatin zymography in Capn4 siRNA and control siRNA cells. (d) 5-8F and CNE2 cells were transfected with constructs encoding Capn4 or MMP2 siRNA, or full-length MMP2, respectively. Western blot analysis was then performed to monitor knockdown or overexpression of target genes. Data is presented as means ± SD for three independent experiments (*P < 0.05).
In summary, the present findings provide first evidence that Capn4 is upregulated in NPC tumor tissues, representing a marker for poor prognosis of NPC. Moreover, they demonstrate that Capn4 promotes in vivo invasion and migration as well as in vitro metastasis of NPC cells. Furthermore, they also unveil a potential mechanism involving upregulation of MMP2 via an NF-kB-dependent mechanism, underlying Capn4-mediated promotion of NPC progression. In the light of these findings, it would be of interest to validate whether Capn4 could serve as a biomarker for predicting prognosis of patients with NPC, and/or a potential target in treatment of NPC.

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Disclosure Statement

The authors have no conflict of interest.
Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Knockdown of Capn4 inhibited NPC cell proliferation in vitro.