Alpha B-crystallin promotes the invasion and metastasis of gastric cancer via NF-κB-induced epithelial-mesenchymal transition

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Abstract
Alpha B-crystallin (CRYAB) is overexpressed in a variety of cancers. However, little is known about its specific function and regulatory mechanism in gastric cancer. Here, we first explore the role of CRYAB in gastric cancer progression and metastasis. The expression of CRYAB was determined by western blot and immunohistochemistry in gastric cancer tissues. Besides, methods including stably transfected against CRYAB into gastric cancer cells, western blot, migration and invasion assays in vitro and metastasis assay in vivo were also conducted. The expression of CRYAB is up-regulated in gastric cancer tissues compared with matched normal tissues. High expression level of CRYAB is closely correlated with cancer metastasis and shorter survival time in patients with gastric cancer. Additionally, CRYAB silencing significantly suppresses epithelial-mesenchymal transition (EMT), migration and invasion of gastric cancer cells in vitro and in vivo, whereas CRYAB overexpression dramatically reverses these events. Mechanically, CRYAB facilitates gastric cancer cells invasion and metastasis via nuclear factor-κ-gene binding (NF-κB)-regulated EMT. These findings suggest that CRYAB expression predicts a poor prognosis in patients with gastric cancer. Besides, CRYAB contributes to gastric cancer cells migration and invasion via EMT, mediated by the NF-κB signalling pathway, thus possibly providing a novel therapeutic target for gastric cancer.

KEYWORDS
CRYAB, epithelial-mesenchymal transition, gastric cancer, NF-κB

1 | INTRODUCTION

Gastric cancer is the fifth most common malignancy\(^1\) and is the third leading cause of cancer-related mortality worldwide.\(^2\) Despite advances in early detection, surgical skills and conventional chemotherapy over the past few decades, metastasis remains a crucial impediment to effective treatment for gastric cancer.\(^3\) However, the molecular mechanisms responsible for gastric cancer metastasis are still poorly characterized. Thus, identification of novel metastases-related biomarkers and investigation of the underlying mechanisms driving gastric cancer metastasis may provide potential targets useful for improving outcomes.
Emerging evidences have revealed that EMT functions importantly in cancer metastasis via the transdifferentiation of epithelial cells into motile mesenchymal cells. During EMT, the hallmark is a decrease in E-cadherin expression, accompanied by an increase in N-cadherin or vimentin expression. Conversely, mesenchymal-epithelial transition indicates the reverse process. As already reported, the switch in EMT process is controlled by transcription factors such as Slug, Snail, ZEB1, ZEB2 and Twist, as well as certain signalling pathways, including Notch, Wnt, TGF-β and NF-κB. Therefore, a further exploration of gene regulation mechanism of EMT in cancer metastasis has great significance.

CRYAB, a member of the small heat shock protein family, was first identified as one of major structural proteins of the ocular lens. A widely accepted function of CRYAB is molecular chaperoning, allowing the prevention of aggregation and degradation of denatured proteins in response to cellular damage including oxidative stress, radiation, heat shock and other factors, thereby promoting cell survival. More recently, the influence of CRYAB on cell invasion and metastasis has received increasing attention in several types of cancer, including head and neck squamous cell carcinoma, brain, breast cancer and colorectal cancer. However, the role and the underlying mechanisms of CRYAB in gastric cancer invasion and metastasis have not yet been elucidated.

Thus, this study aimed to explore the functional role of CRYAB in the invasion and metastasis of gastric cancer, as well as the molecular mechanisms responsible for CRYAB function.

2 | METHODS

2.1 | Patient samples

Matched cancerous and normal tissues were obtained from 92 patients with gastric adenocarcinoma who underwent radical gastrectomy without preoperative treatment, at the Department of General Surgery of our hospital. Among them, fresh tissues of 40 cases were also determined by western blot for CRYAB protein. Informed consent was obtained from each patient, and the experimental protocols were approved by the Ethics Committee of the Fifth Affiliated Hospital of Nantong University.

2.2 | Immunohistochemistry

Immunohistochemistry (IHC) analysis of CRYAB expression in specimens was performed as described previously. We used antibody against CRYAB (1:200; Abcam, UK). Sections were observed by 2 independent pathologists under the microscope. The evaluation of staining result was graded as described previously. The sum scores <3 points were indicative of negative staining, while the sum scores ≥3 points were regarded as positive staining.

2.3 | Cell lines and cell culture

The human gastric cancer cells (MKN45, MGC803, SGC7901, KATO-III, AGS and MGC823) and normal gastric epithelial GES-1 cell were obtained from American Type Culture Collection (Manassas, VA, USA). All cells were cultured according to the manufacturer’s instructions, in a humidified atmosphere of 37°C containing 5% CO₂.

2.4 | Western blot assay

Western blot analysis was carried out as described previously. The following primary antibodies were used: CRYAB rabbit mAb (1:1000; Abcam), E-cadherin rabbit mAb (1:1000; CST), Slug rabbit mAb (1:1000; CST), N-cadherin rabbit mAb (1:1000; CST), vimentin rabbit mAb (1:1000, CST), p-NF-κB p65 mouse mAb (1:500; Santa Cruz, USA), NF-κB p65 mouse mAb (1:500, Santa Cruz) and GAPDH mAb-HRP (1:5000; Bioworld Technology, USA). All the blots were visualized using the enhanced chemiluminescence detection kit (Thermo Scientific, USA).

2.5 | Lentivirus infection

Transient and stable transfection of CRYAB was performed with the Lipofectamine 2000 reagent (Invitrogen) as described previously. The sequence of short hairpin RNA (shRNA) oligonucleotides specifically targeting the CRYAB transcripts was as follows: 5'-GCACCTGTTGGAGTCTGAT-3'. Lentiviral vector encoding the human CRYAB gene was generated using pcDNA3.1 as described previously. An empty vector was employed as the negative control. The CRYAB expression was determined by western blot.

2.6 | Wound-healing assay

Cells were grown to full confluence in a 6-well plate, and a cell-free wound area was scratched using a sterile plastic tip. To evaluate wound closure, images were captured at indicated time (0 and 48 hours) after the wound was created. The wound healing = (0 hour width – 48 hours width)/0 hour width × 100%. Transwell assay was performed in accordance with a previous protocol. For cell migration assay, cells were added to the upper chamber (8 mm pore size; Corning, USA). For cell invasion assay, cells were seeded into the upper chamber containing a Matrigel-coated membrane (BD Biosciences, USA). The medium with 20% foetal bovine serum was added to the lower chamber as a chemoattractant. After 24 hours of incubation, migrated or invaded cells on the bottom surface were fixed, stained and counted under a microscope.

2.8 | Proliferation assay

Cells were seeded in a 6-well plate at a density 4 × 10⁴ cells per well. At the indicated times (0, 1, 2, 3 and 4 days after seeding), the viable cells were counted with a hemocytometer after trypan blue staining. Cell viability was determined using the cell proliferation reagent MTS (Promega) following the manufacturer’s protocol.
2.9 | In vivo metastasis assay

Cells were injected intravenously into the tail vein of 6-week-old male BALB/c nude mice. At 30 days after the injection, mice were killed, and the lungs were resected for the count of metastatic nodules. Subsequently, harvested lungs were further processed for haematoxylin-eosin (H&E) staining and western blot analysis. All animal procedures were approved by the Ethics Committee of the Fifth Affiliated Hospital of Nantong University and were performed in accordance with institutional guidelines.

2.10 | Statistical analysis

Statistical analyses of clinicopathological features were performed by chi-square. Survival curves were obtained using the Kaplan-Meier method, and statistical assess for survival was analysed by log-rank method. The data were presented as mean ± SD and were analysed by Student’s t test. Statistical analyses were performed using SPSS 21.0 software (SPSS Inc, USA). P < .05 was considered statistically significant.

3 | RESULTS

3.1 | CRYAB is overexpressed in human gastric cancer tissues and is closely correlated with clinical outcomes

To investigate the function of CRYAB in gastric cancer progression, we first evaluated CRYAB protein expression in 40 pairs of tumour samples and matched normal tissues by western blot.
analysis. As shown in Figure 1A, tumour tissues exhibited significantly higher level of CRYAB protein than that in corresponding normal tissues. We then examined CRYAB expression in 92 pairs of tumour samples and matched normal tissues by IHC staining (Figure 1B). Consistently, the result revealed that CRYAB expression was markedly increased in tumour tissues compared with normal tissues (Figure 1C), and its expression was higher in metastatic tumour tissues (Figure 1B). Besides, a clinicopathological association analysis demonstrated that CRYAB expression in tumour tissues significantly correlated with T stage, pTNM stage and lymph node metastasis, respectively ($P < .05$; Table 1). Interestingly, compared with patients without lymph node metastasis, those who developed metastasis exhibited significantly higher staining scores for CRYAB ($P < .001$; Figure 1D). Furthermore, Kaplan-Meier survival curve indicated that patients with positivity for CRYAB expression had a shorter survival time than those with negative for CRYAB expression ($P < .05$; Figure 1E). Taken together, these findings suggest that CRYAB is aberrantly up-regulated in tumour tissues and closely correlated with poor prognosis in patients with gastric cancer, and more remarkably, CRYAB might function crucially in metastasis.

3.2 CRYAB induces EMT in gastric cancer cells

To characterize the biological role of CRYAB in gastric cancer cells, we initially examined the expression level of CRYAB in 6 gastric cancer cell lines (MKN45, MGC803, SGC7901, KATO-III, AGS and MGC823) and normal gastric epithelial cell line (GES-1). As shown in Figure 2A, CRYAB expression was higher in gastric cancer cells compared with GES-1 cells, especially in MKN45 and SGC7901 cells, and was decreased in AGS and KATO-III cells. Then, we established CRYAB-silencing MKN45 cells and SGC7901 cells and CRYAB-overexpressing AGS cells and KATO-III cells by retroviral transduction. A western blot analysis indicated that CRYAB expression was significantly silenced by shCRYAB (Figure 2B) and markedly up-regulated by CRYAB overexpression (Figure 2C). Recently, mounting evidence suggests that EMT was correlated with cancer invasion and metastasis.4 Regarding to the effect of CRYAB expression on EMT in gastric cancer cells, we found that silencing CRYAB expression in MKN45 cells and SGC7901 cells increased E-cadherin expression and decreased the expression levels of N-cadherin and vimentin (Figure 2D). Conversely, CRYAB overexpression in AGS cells and KATO-III cells exerted the opposite effect (Figure 2E). However, the expression of Slug was not significantly changed in above-mentioned
cells (Figure 2D,E). Collectively, these data suggest that CRYAB is a regulator of EMT in gastric cancer cell lines.

### 3.3 CRYAB promotes migration and invasion of gastric cancer cells in vitro

Migration and invasion are hallmarks for cancer metastasis and related to EMT. To evaluate the effects of CRYAB on migration and invasion of gastric cancer cells in vitro, the wound-healing assay was first performed. As shown in Figure 3A, CRYAB silencing decreased the wound-healing capability of MKN45 cells, while CRYAB overexpression accelerated the wound closure in AGS cells (Figure 3B). Consistent with these findings, a transwell assay revealed that CRYAB silencing markedly inhibited the migration and invasion of MKN45 cells (Figure 3C). Conversely, CRYAB overexpression demonstrated the opposite effects (Figure 3D). To rule out the possibility that the effects of CRYAB on cell migration and invasion were attributable to potential confounders such as cell death and proliferation, the cell number in 2 groups was compared. As shown in Figures 3E,F, all cells exhibited similar cell number or growth rates under the same conditions, suggesting that CRYAB expression was not associated with potential confounders. Together, these results indicate that CRYAB facilitates the motility and invasiveness of gastric cancer cells in vitro.

### 3.4 CRYAB promotes gastric cancer cells metastasis in vivo

To further assess whether CRYAB regulates gastric cancer cells metastasis in vivo, we established a lung metastasis model through the injection of cancer cells into tail vein of nude mice. We found that down-regulation of CRYAB rescued the decreased incidence of lung metastasis of CRYAB-silencing MKN45 cells (Figure 4A), and H&E staining of the dissected lungs further confirmed the presence of metastases (Figure 4C), whereas CRYAB-overexpressing AGS cells exhibited more lung metastases compared with the vector group (Figure 4B,D). Additionally, consistent with the findings in vitro, CRYAB expression significantly decreased in the metastasis nodules.
of shCRYAB group, together with E-cadherin expression up-regulation, and p-NF-κB p65 expression down-regulation (Figure 4E), and CRYAB overexpression in AGS cells indicated the opposite result (Figure 4F). In summary, these in vivo results verify the promotive role of CRYAB in gastric cancer cells metastasis.

3.5 | CRYAB promotes gastric cancer cells invasion through the NF-κB signal pathway-mediated EMT

Given that NF-κB signalling pathway is involved in cancer migration and invasion,18 we reasoned that CRYAB might contribute to EMT changes via the activation of NF-κB signal pathway in gastric cancer. To test this hypothesis, we initially examined the protein expression levels of NF-κB p65 and phosphorylated NF-κB p65 (p-NF-κB p65) with CRYAB knockdown or overexpression in gastric cancer cells. As shown in Figure 5A,B, the protein expression of p-NF-κB p65 was decreased in CRYAB-silenced MKN45 cells and was increased in CRYAB-overexpressing AGS cells. To demonstrate the involvement of NF-κB signal pathway in CRYAB-induced gastric cancer cells EMT and invasion, helenalin (1 μmol/L for 1 hour), an NF-κB inhibitor, was used.6 The results revealed that helenalin pretreatment enhanced shCRYAB-mediated increase in E-cadherin and decrease in p-NF-κB p65, N-cadherin and vimentin (Figure 5C), and observably potentiated shCRYAB-inhibited cell invasion (Figure 5E). Additionally, helenalin pretreatment resulted in a noticeable reversal of CRYAB-induced EMT and a decrease in p-NF-κB p65 (Figure 5D) and obviously suppressed CRYAB-facilitated cell invasion (Figure 5F). On the whole, these data demonstrate that the NF-κB signal pathway contributes to CRYAB-mediated EMT and invasion of gastric cancer cells.

4 | DISCUSSION

As numerous genetic changes are in relation to cancer cell invasion and metastasis,14,19-22 great efforts have been made in the identification of novel key regulators and the potential mechanisms involved in this process over the years. Interestingly, recent studies have revealed that CRYAB is aberrantly overexpressed in various types of cancer, and its expression could promote cell invasion and metastasis.8-10 For example, Shi et al8 found that CRYAB promoted the invasion and metastasis of colorectal cancer cells via EMT. Consistently, Chantal et al suggested CRYAB as a useful biomarker to help fine-tune treatment in head and neck squamous cell carcinoma,
possibly by targeting CRYAB-mediated cell motility. Obviously, these findings provide evidence delineating the pivotal role of CRYAB in cancer invasion and metastasis. Nevertheless, to the best of our knowledge, the clinical significance and the specific role of CRYAB in gastric cancer remain obscure, and even less is known about the regulatory mechanism of CRYAB-induced gastric cancer cell invasion and metastasis.

In this study, we first found that CRYAB was frequently up-regulated in gastric cancer tissues compared with tumour adjacent normal tissues. Importantly, clinical data analyses showed that high CRYAB expression significantly correlated with T stage, pTNM stage, lymph node metastasis and shorter overall survival, suggesting that CRYAB is involved in gastric cancer progression and metastasis. Next, we further explored the biological function of CRYAB in gastric cancer cells. The results revealed that CRYAB overexpression increased the motility and invasiveness of gastric cancer cells and promoted EMT in vitro, as well as accelerated metastasis in vivo, whereas CRYAB silencing had the opposite effect on gastric cancer cells both in vitro and in vivo.

A well-known function of EMT is thought to emerge as a key regulator of cell invasion and metastasis in multiple types of cancer by conferring an invasive phenotype. In recent years, therefore, the EMT pathway is of great therapeutic interest in cancer treatment and could be targeted to prevent the dissemination of organ-confined cancers in patients at high risk of developing metastatic lesions or to eliminate existing metastatic cells in patients with more advanced disease. In consideration of accumulating evidence indicating EMT as a clinically relevant mechanism for targeting tumour metastasis, it is worth identifying new therapeutic targets. However, key questions remain regarding the targeted reversal of EMT to suppress metastasis to some extent.

Here, a key finding is that we demonstrated NF-κB signalling pathway as a mediator involved in CRYAB-facilitated gastric cancer cells EMT and invasion. As already reported, the NF-κB signalling pathway has been confirmed to function as not only an important player in cancer development but also a key regulator of the EMT-related transcription factors. Mechanistically, we first revealed that CRYAB silencing noticeably decreased the protein level of p-NF-κB p65 in MKN45 cells, suggesting the inactivation of NF-κB pathway. Conversely, CRYAB overexpression markedly activated the NF-κB pathway, which strengthened shCRYAB-inhibited EMT and cell invasion as well as weakened CRYAB-induced EMT and cell invasion. Thus, we could reasonably assume that CRYAB facilitated gastric cancer cells invasion and metastasis via NF-κB-regulated EMT.

In conclusion, our study demonstrates that the high expression of CRYAB is closely correlated with cancer metastasis and shorter survival time in patients with gastric cancer, and CRYAB promotes EMT and metastasis of gastric cancer cells via NF-κB signalling pathway, thus possibly providing a promising candidate for treatment against gastric cancer metastasis.
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CONFLICT OF INTEREST

The authors confirm that there are no conflicts of interest.

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