

Original Article

Oncogenic roles of carbonic anhydrase IX in human nasopharyngeal carcinoma

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Abstract: Carbonic anhydrase IX (CA IX), a hypoxia-inducible protein in tumors, has been shown to be valuable for the prognosis of nasopharyngeal carcinoma (NPC). However, the function and mechanism of CA IX has been not explored in NPC. Here, we found that CA IX was detected at higher levels in NPC cells and tissues than their corresponding partners. Furthermore, the cell growth, migration and invasion *in vitro* were altered with shRNA or overexpression of CA IX in NPC cells. More importantly, the metastatic ability of NPC cells stably expressing CA IX was significantly enhanced using the hepatic metastasis model of nude mice *in vivo*. Finally, the mTOR pathway was indicated to be involved in such effects of CA IX on NPC. This is the first evidence that CA IX may promote the NPC metastasis to potentially be a therapeutic target for NPC, and that the inhibitory molecules of CA IX and/or the mTOR pathway alone or combination with both may be worth to have a clinical trial for the patients with NPC.

Keywords: CA IX, NPC, growth, metastasis, mTOR

Introduction

Nasopharyngeal carcinoma (NPC), originating from the nasopharynx, is highly prevalent with an incidence rate of 15-50/100,000 in Southern China and Southeast Asia [1-3]. NPC, also often undifferentiated and highly sensitive to radiotherapy, has a high rate of distant metastasis, which makes it very different from other head and neck cancers [4, 5]. But the molecular mechanisms of NPC is still poorly understood, although some progressions have been made recently [2, 6-9]. So it is necessary to clarify the pathogenesis of NPC and to find new targets for the treatment of nasopharyngeal carcinoma.

Carbonic anhydrase IX (CA IX), a member of the carbonic anhydrase family, was first identified as an endogenous HeLa cells antigen [10], and it catalyzes the reversible conversion of carbon dioxide to bicarbonate and protons and is thus involved in ion transport and pH control [11, 12]. It has been reported that CA IX were higher in tumor tissues, including clear cell renal cell carcinoma and breast cancer, than in normal

tissues, and over-expression of CA IX predicts a poor clinical outcome in most cancer types including NPC [13-15]. But the function and mechanism of CA IX in NPC has not been explored, furthermore, whether CA IX can be used as a potential therapeutic target for NPC needs to be confirmed. The present study focuses on the expression and roles of CA IX in NPC. It has been found that CA IX is up-regulated in NPC cells. This study may provide new ideas and methods suitable for the treatment of nasopharyngeal carcinoma.

Materials and methods

Cell culture and sample collection

Six human NPC cell lines (Hone1, CNE1, CNE2, Sune1, 6-10B, 5-8F) were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (HyClone). The nasopharyngeal epithelial cell line (NP69) was grown in defined-KSFM medium supplemented with epidermal growth factor (EGF) (Invitrogen, Carlsbad, USA). All cell lines were incubated in a humidified chamber with

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5% CO₂ at 37°C. 10 fresh primary NPC tissues and 4 fresh NP tissues were obtained at the time of diagnosis before any therapy from Sun Yat-sen University cancer center (Guangzhou, China). The clinical processes were approved from the Ethics Committees of Sun Yat-sen University and the informed consent was collected from each patient.

Construction of stable lines overexpressing CA IX

Full-length human CA IX cDNA was cloned into pSin-puro vector, and CA IX was verified by DNA sequencing. The primers were as follows: 5'-GGAATTCATATGACCATGGCTCCCCTGTGCC-CCAG-3' (forward), 5'-GGAATTCATATGACCATGGCTCCCCTGTGCCCCAG-3' (reverse). pSin-puro delivering CA IX or empty vector were co-transfected with pMD.2G and psPAX2 into HEK-293T cells for 48 hours. The recombinant virus were collected and added to 6-10B and Hone1 cells cultured with 8 µg/ml polybrene for 24 hours. The stable lines were selected with 1 µg/ml of puromycin for two weeks.

Construction of stable lines silencing CA IX

The vectors expressing either CA IX short hairpin RNAs (shRNAs) or a scrambled shRNA were generated using the Sigma shRNA system according to the manufacturer's instructions. The targets of human CA IX shRNA#1, #2, #3 and #4 are 5'-TGCTGCCTCGCCTCTAGATAT-3', 5'-CCTTAACTTCTGTGCCAACAA-3', 5'-GCTGAAC-CATGCCTCCATCAT-3', 5'-CATGCTGAAGAGAGGC-ATCTT-3' and respectively. CA IX shRNAs or scrambled shRNA were co-transfected with pMD.2G and psPAX2 into HEK-293T cells for 48 hours. The recombinant virus were collected and added to 5-8F cells cultured with 8 µg/ml polybrene for 24 hours. The stable lines were selected with 1 µg/ml of puromycin for two weeks.

RNA extraction and quantitative real-time RT-PCR (qRT-PCR)

Quantitative real-time RT-PCR was performed as described previously [16, 17]. Total RNA of tissue specimens was isolated using Trizol (Invitrogen) according to the manufacturer's protocol. First-strand cDNA was synthesized using PrimeScript® RT reagent Kit with gDNA Eraser. And quantitative PCR was performed

for detection of CA IX mRNA using SYBR® Premix Ex Taq™ II (Takara). The sequences of primers were as follows: for CA IX: 5'-GGATCTACCTACTGTTGAGGCT-3' (forward), 5'-CATAGCGCCAATGACT CTGGT-3' (reverse); for GAPDH: 5'-ACAGTCAGCCGCATCTTCTT-3' (forward), 5'-GACAAGCTTCCCCTTCTCAG-3' (reverse). The PCR condition was: 95°C for 10 minutes, followed by 40 cycles of 95°C for 20 s, 60°C for 20 and 70°C for 30 s.

CCK8 assay

The cell viability *in vitro* was assessed using CCK8 assay. Cells were seeded in 96-well plates at the density of 1,000 cells/well, the cells were incubated for 1, 2, 3, 4, or 5 days. Ten microliters of CCK8 (Cell Counting Kit-8, Beyotime, China) was added to each well and incubated for 1.5 hours. The absorbance value (OD) of each well was measured at 450 nm. For each experimental condition, 6 wells were used. Experiments were performed three times.

Colony formation assay

Cells were plated in the 6-well culture plates at 250 cells per well. Each group had 3 wells. After incubation for 15 days at 37°C, cells were washed twice with PBS and stained with Giemsa solution. The number of colonies containing ≥50 cells was counted under a microscope.

Wound-healing assay

Cell motility was assessed by measuring the movement of cells into a scraped, acellular area created by a 200 µl pipette tube, and the spread of wound closure was observed after 24 h and 48 h and photographed under a microscope.

Transwell assay

For the transwell migration assay, 3.5×10⁴ cells in 200 µl of serum-free DMEM were added to the cell culture inserts with an 8-µm microporous filter without extracellular matrix coating (Becton Dickinson Labware, Bedford, MA). The DMEM medium containing 10% FBS was added to the bottom chamber. After 24 hours of incubation, the cells in the lower surface of the filter were fixed and stained followed by microscopic examination. The number of migrated cells in

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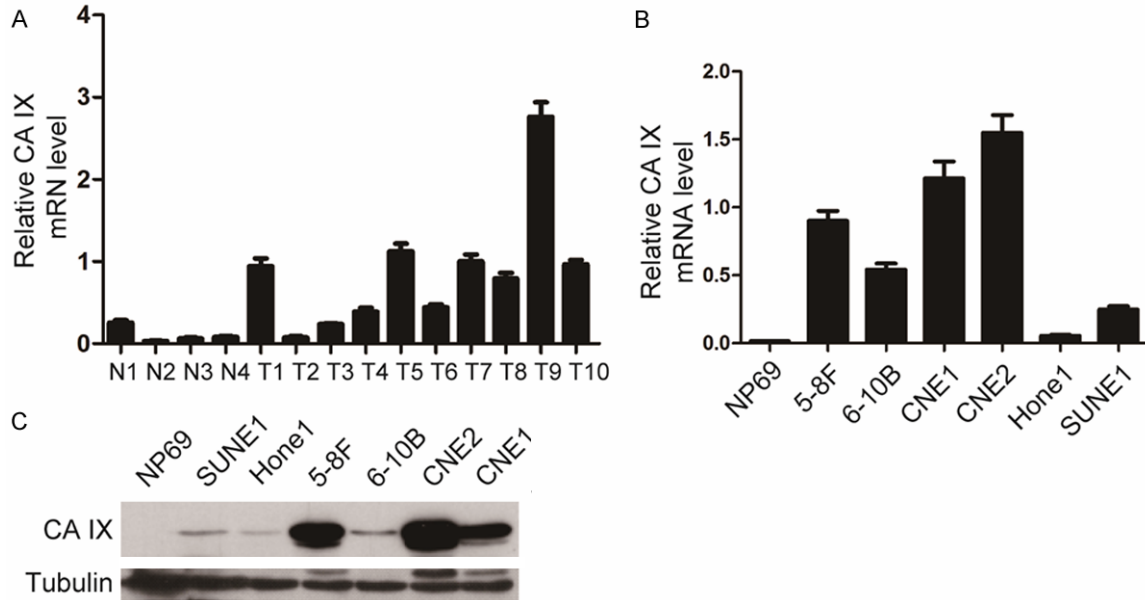


Figure 1. Up-regulation of CA IX was detected in NPC cell and tissues. A: The CA IX mRNA was detected in 10 human NPC primary tissues (T) and 4 normal nasopharyngeal tissues (N) by qRT-PCR. GAPDH was used as the internal control. Statistical analysis (Mann-Whitney test) was performed ($*P < 0.05$). B: The mRNA levels of CA IX were determined in 6 NPC cell lines by qRT-PCR. GAPDH was used as the internal control. C: The protein levels of CA IX were analyzed in NPC cell lines. Tubulin was used as the internal control.

three random optical fields ($\times 100$ magnification) for each filter from triplicate filters was averaged. For the invasion assay, the inserts of the chambers to which the cells were seeded were coated with Matrigel (Becton Dickinson Labware, Bedford, MA). The number of invading cells in three random optical fields ($\times 100$ magnification) for each filter from triplicate inserts was averaged.

Western blotting

Western blotting was performed as described previously [16, 17]. Briefly, cells were collected and lysed by RIPA buffer (150 mM NaCl, 0.5% EDTA, 50 mM Tris, 0.5% NP40) and centrifuged for 20 min at 12000 rpm at 4°C . Fifty micrograms of harvested total protein was loaded, separated in 8% sodium dodecyl sulfate-polyacrylamide gradient gels and transferred onto PVDF membranes followed by blocking with 5% non-fat milk for 2 hours at room temperature. Membranes were incubated with primary antibody and horseradish peroxidase-conjugated secondary antibody, and then detected using the ECL chemiluminescence system (Pierce, Rockford, USA). Antibodies against CA IX, mTOR, p-mTOR (Ser2448) were from Cell Signaling Technology. Antibody against Tubulin was from Bioworld Technology.

Animal experiments

All animal work was performed in accordance with protocols approved by Research Animal Resource Center of Sun Yat-sen University. Male athymic mice between 5 and 6 weeks of age were obtained from Shanghai Institutes for Biological Sciences (Shanghai, China). All the animal studies were conducted in accordance with the principles and procedures outlined in the guidelines of Institutional Animal Care and Use Committee at Sun Yat-sen University Cancer Center. The hepatic metastasis model of nude mice has been published previously [7]. Briefly, the total of 3×10^5 cells in $30 \mu\text{l}$ were injected into spleens of laparotomized mice using insulin syringes (Becton Dickinson). After tumor cell inoculation for 32 days, the experiment was terminated. The metastatic nodules in each liver were counted.

Results

CA IX is up-regulated in NPC cell lines and tissues

CA IX was reported to be expressed at higher levels in tumor tissues than in normal tissues and associated with prognosis in various can-

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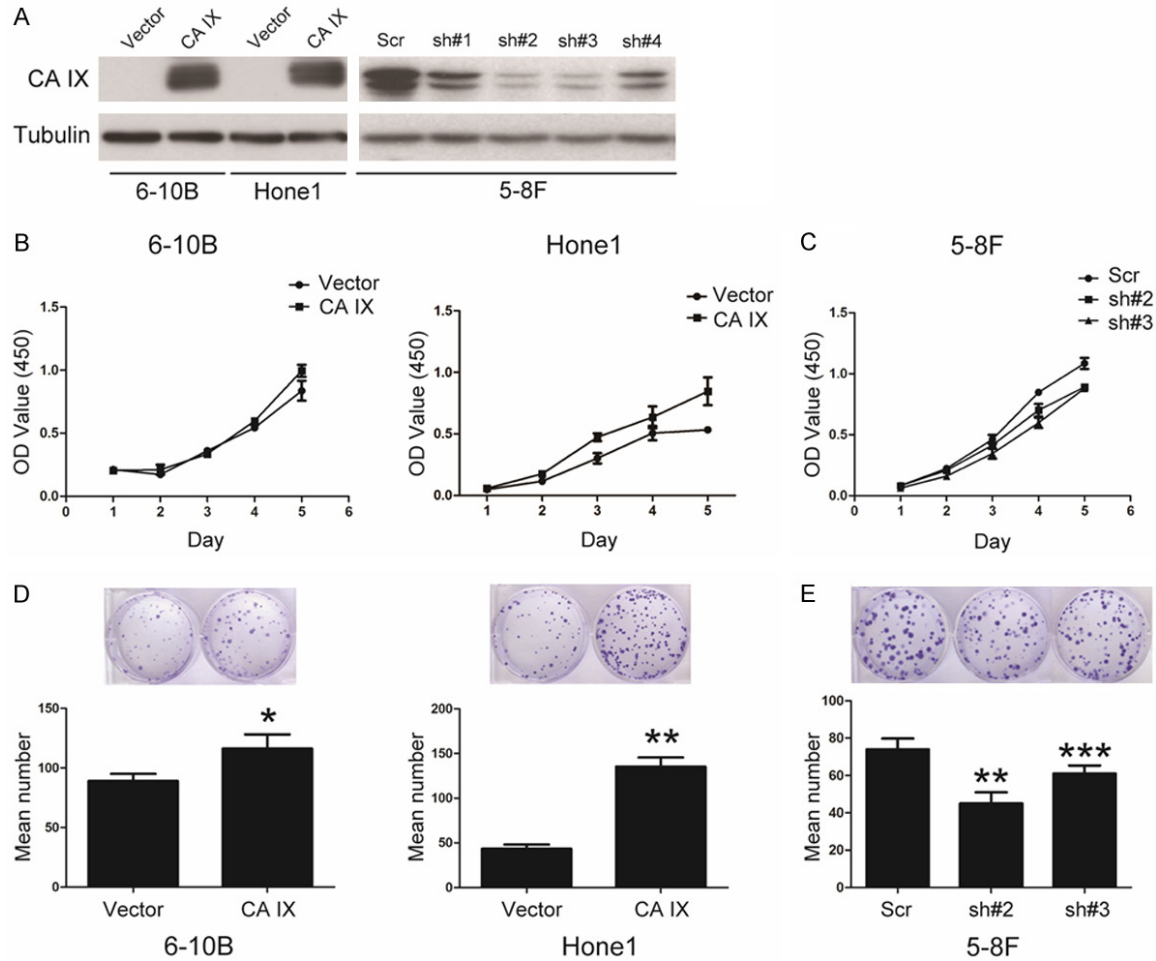


Figure 2. CA IX promotes cell growth and colony formation in NPC cells. A: The generation of stable cell lines in 6-10B, Hone1, and 5-8F cells overexpressing or knocked down of CA IX, as indicated, and the protein level was analyzed by western blotting. B, C: Cell growth of the indicated stable cell lines *in vitro* was measured in different time points as indicated by CCK8 assay. D, E: Colony formation of the indicated stable cell lines *in vitro* was measured for 14 days, as described in "Materials and methods". Bars correspond to mean + standard error, with *P* value calculated using Student's *t*-test. **P*<0.05, ***P*<0.001, ****P*<0.0001.

cers [13-15, 18]. To evaluate the expression level in NPC, we detected the mRNA level of CA IX in NPC tissues and normal nasopharyngeal tissues using qRT-PCR. As shown in **Figure 1A**, the mRNA level of CA IX was significantly higher in 8 out of 10 tumor tissues than that in 4 normal nasopharyngeal tissues. Likewise, we found that the mRNA and protein levels of CA IX were higher in 4 out of 6 and all of 6 NPC cell lines tested, respectively, than in nasopharyngeal epithelial cell line (NP69) (**Figure 1B, 1C**). Notably, both the mRNA and protein levels of CA IX were much higher in 5-8F than in 6-10B, which is a pair of cell lines derived from the NPC Sune1 cells with high and low metastatic abilities, respectively. These results indicate

that CA IX is up-regulated in NPC and may be related to the NPC progression and metastasis.

CA IX increases the cell growth and colony formation of NPC cells

In order to explore the function of CA IX in NPC, we generated the stable cell lines expressing ectopic CA IX in Hone1 and 6-10B cells (**Figure 2A**). On the other hand, four different shRNAs specifically targeting different CA IX coding regions were used in 5-8F cells, and sh#2 and sh#3 were more efficient to silence CA IX (**Figure 2A**). Then we evaluated the effect of CA IX on cell growth using CCK8. As shown in

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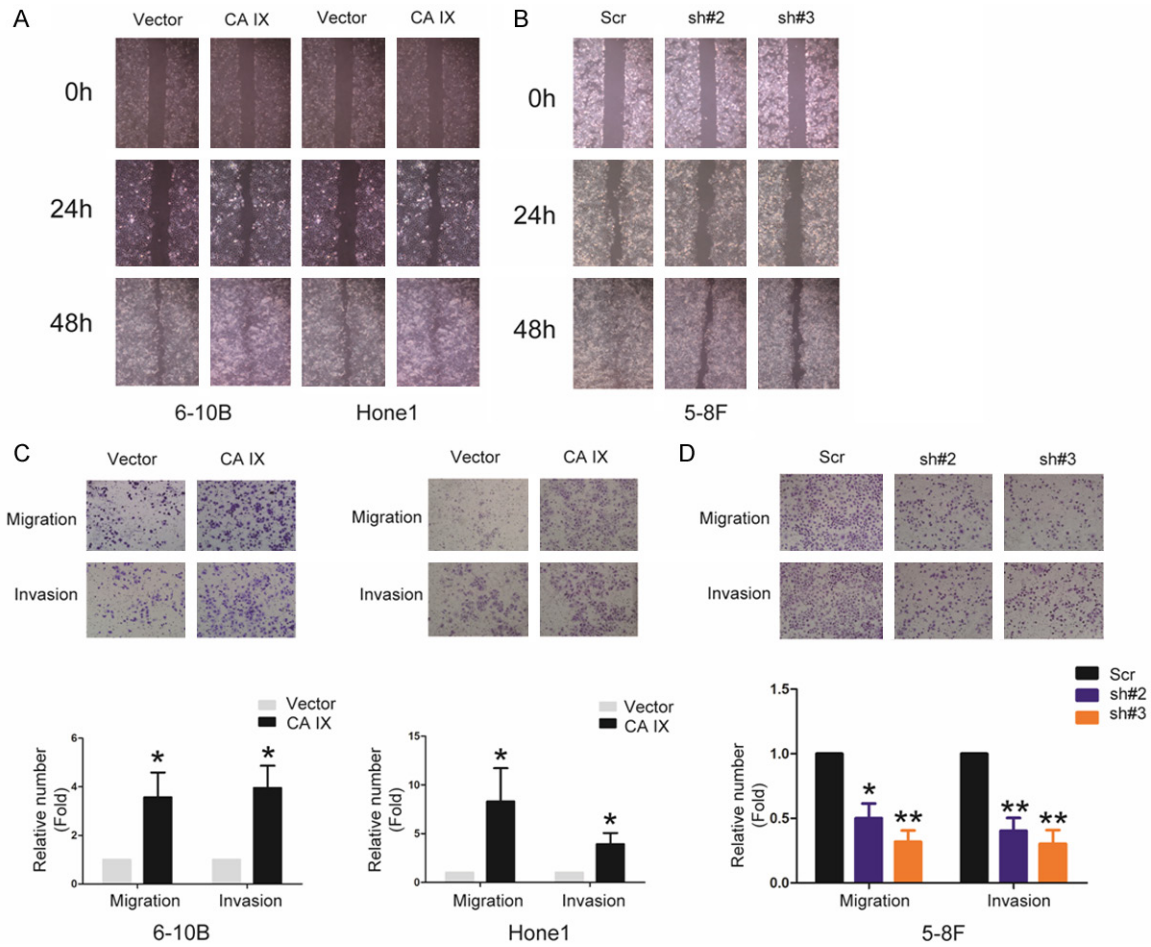


Figure 3. CA IX strongly promotes the cell migration, invasion of NPC *in vitro*. A, B: The cell ability of wound gap closure were dramatically enhanced and suppressed by both overexpressing and knocking down CA IX, respectively. C, D: Migratory and invasive abilities of the indicated stable cell lines *in vitro* were measured by Transwell assay as described in “Materials and methods”. Bars correspond to mean + standard error, with *P* value calculated using Student’s *t*-test. **P*<0.05, ***P*<0.001.

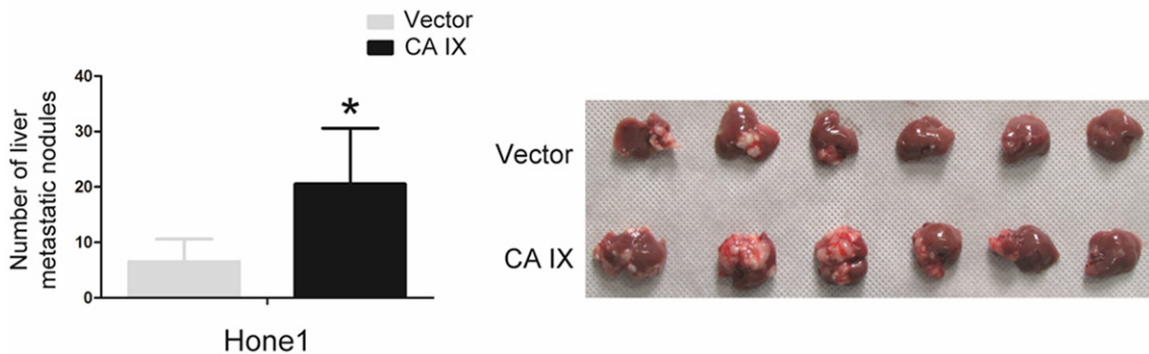


Figure 4. CA IX strongly promotes metastasis of NPC *in vivo*. The liver metastatic ability of Hone1 cells expressing CA IX or empty vector *In vivo* was evaluated in nude mice. Bars correspond to mean + standard error, with *P* value calculated using Student’s *t*-test. **P*<0.05.

Figure 2B, overexpression of CA IX in 6-10B and Hone1 cells increased the cell growth com-

pared with the control cells. On the contrary, knockdown of CA IX in 5-8F cells decreased the

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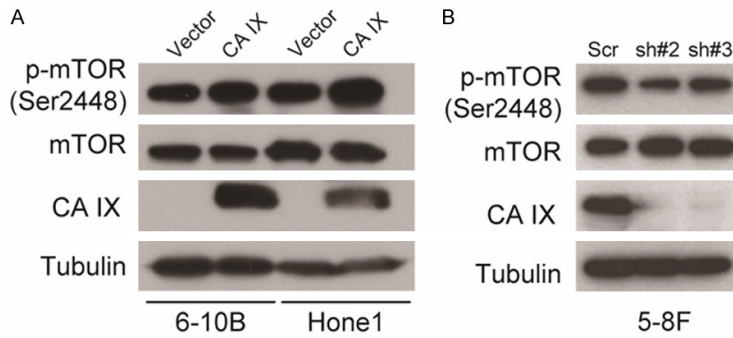


Figure 5. CA IX may activate the mTOR pathway in NPC cells. A, B: Immunoblots of whole-cell lysates from the indicated stable cell lines using the indicated antibodies, as described in “Materials and methods”.

cell growth compared with the control cells (**Figure 2C**). In addition, overexpression of CA IX increased the ability of colony formation of 6-10B and Hone1 cells (**Figure 2D**), whereas knockdown of CA IX decreased the ability of colony formation of 5-8F cells (**Figure 2E**). Taken together, these results indicate that CA IX play an important role in regulation of the cell growth and colony formation of NPC cells.

CA IX promotes the cell migration and invasion of NPC cells in vitro

Our results showed CA IX expression higher levels in NPC cells than normal nasopharyngeal cells. Furthermore, CA IX expression higher levels in high metastatic cells 5-8F than low metastatic cells 6-10B. So we evaluated the effect of CA IX on the abilities of migration and invasion in NPC cells using wound-healing and transwell assay. As shown in **Figure 3A, 3B** the cell ability of wound-healing was dramatically enhanced and suppressed by both overexpressing and knocking down CA IX, respectively. And the transwell assays showed the abilities of migration and invasion were dramatically enhanced and suppressed by both overexpressing and knocking down CA IX, respectively (**Figure 3C, 3D**). These results suggest that CA IX play a crucial role in regulation of the cell migration and invasion of NPC cells *in vitro*.

CA IX promotes metastasis of NPC cells in vivo

Based on these findings that CA IX dramatically enhanced the abilities of migration and invasion of NPC cells *in vitro*, we detected the metastatic ability through the hepatic metastasis model of nude mice *in vivo*. Strikingly,

Overexpression of CA IX markedly increases metastasis of NPC cells *in vivo* (**Figure 4**). These results indicate that CA IX strongly enhances metastasis of NPC cells *in vivo*, consistent with the previous reports showing that CA IX takes part in pH regulation facilitating acidification of the microenvironment, enhancing cell growth and migration in most cancer cells [19, 20].

CA IX may activate the mTOR pathway in NPC cells

Given that mTOR is a key regulator for cancer progression and metastasis including NPC [21, 22], As showed in **Figure 5A**, both the p-mTOR (Ser2448) level was increased, while the total mTOR protein level was constant, in both Hone1 and 6-10B cells stably expressing of ectopic CA IX. On the other hand, knockdown of CA IX decreased the p-mTOR (Ser2448) level in 5-8F cells (**Figure 5B**). These results suggest that CA IX may activate the mTOR pathway in NPC cells.

Discussion

Although it has been reported that tumor hypoxia can induce CA IX expression and the high level of CA IX predicts a poor outcome in NPC [15, 18], its function and mechanism in NPC has not been characterized. Furthermore, whether it can be used as a potential therapeutic target for NPC needs to be confirmed. In this study, we demonstrated that CA IX plays an oncogenic role in NPC probably through the mTOR pathway.

We demonstrated that CA IX is higher in NPC cell lines and tissues than in its counterpart's (**Figure 1**). This is constant with the literature showing that CA IX is expressed at higher levels in tumor tissues than in normal tissues in various cancer types [13-15, 18]. Furthermore, we provided the first evidence that CA IX can promote NPC cells growth and colony formation but dramatically enhances NPC cells migration and invasion *in vitro* and metastasis *in vivo* (**Figures 2-4**). These results indicate that CA IX may play oncogenic roles in NPC progression, especially in NPC metastasis, providing a reasonable explanation that the high level of CA IX predicts a poor outcome in NPC.

Notably, CA IX has been proposed to be an attractive therapeutic target [23], as some inhibitory molecules, such as BAY 79-4620, for CA IX have been developed for anticancer therapies [24]. Our results indicated that CA IX can activate the mTOR pathway in NPC (**Figure 5**), and the preclinical animal models have been showed that targeting the mTOR pathway is an efficient strategy for NPC [25-28]. Therefore, we speculate that the inhibitory molecules of CA IX and/or the mTOR pathway alone or combination with both may be worth to have a clinical trial for the patients with NPC.

Disclosure of conflict of interest

None.

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