Downregulation of carbonic anhydrase IV contributes to promotion of cell proliferation and is associated with poor prognosis in non-small cell lung cancer

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Abstract. The present study aimed to unveil the biological role of carbonic anhydrase IV (CA IV) and its association with clinical pathological characteristics and prognostic significance in non-small cell lung cancer (NSCLC). The relative expression level of CA IV was measured by reverse transcription-quantitative polymerase chain reaction in 114 resected NSCLC tumors and matched adjacent normal tissues and NSCLC cell lines. Overexpression and cell proliferation were assessed in human NSCLC cell lines transfected with CA IV mRNA by lentivirus-mediated technology. The association of CA IV expression with clinical pathological features and overall survival in 114 cases of NSCLC patients was analyzed. It was demonstrated that CA IV expression was significantly downregulated in NSCLC tumors and six cell lines. Reduced expression of CA IV was significantly correlated with lymph node metastasis. The overall survival of NSCLC patients with low CA IV expression was significantly shorter compared with the high expression group. Overexpression of CA IV suppressed cell proliferation in A549 and NCI-H1299 cells. The results indicate that low expression of CA IV promotes cell proliferation and serves as an indicator for poor prognosis in NSCLC.

Introduction

Lung cancer is the most common incident cancer and the leading cause of cancer death (1,2). In 2015, and approximately 733,300 new lung cancer cases are diagnosed and 610,200 patients died of lung cancer in China1. Non-small cell lung cancer (NSCLC) accounts for approximately 87% of lung cancer cases (3). Although the mainstays of treatment for lung cancer (such as surgery, radiotherapy, chemotherapy and targeted therapy) have made considerable progress, NSCLC remains an aggressive lung cancer associated with a poor prognosis. Long-term survival of lung cancer is less than 10% (4-6). Thus, finding new biomarkers for predicting progression and prognosis of NSCLC is warranted and urgently needed to improve clinical management of patients with NSCLC.

Carbonic anhydrase IV is one of twelve active human isozymes and one of four expressed on the extracellular surfaces of certain endothelial and epithelial cells, which catalyzes the reversible hydration of CO_2 to HCO_3- and H+ (7,8). Carbonic anhydrase IV (CA IV) was found in human normal tissues like kidney and lung, with the remarkable diversity in tissue distribution, subcellular location, and biological function (9,10). Several CAs are reportedly involved in NSCLC tumorigenesis, progression, regulation of cell proliferation, target therapy and prognosis, excepted CA IV. For example, CA IX, CAXII, and CA I are upregulated in NSCLC tumor tissues and appear to act as oncogenes or prognostic factors (11-14). CA-RP VIII and CA IX expression in NSCLC are related to NSCLC cell invasion and proliferation (15,16). CA IX serves as a target for anticancer therapy (17). A new study found that CA IV is frequently silenced in colorectal cancer and the silencing of CA IV is regulated by promoter hyper-methylation (18). Moreover, CA IV is a novel tumor suppressor in CRC which was found to be associated with the inhibition of the Wnt/β-catenin signalling pathway. The normal lung expression of CA IV was found to be developmentally regulated in the luminal side of the alveolar capillary endothelium cells. We hypothesize that, like many other CAs, CA IV might be associated with NSCLC. Our preliminary studies using high-throughput microarrays and quantitative real-time RT-PCR (RT-qPCR) revealed that the expression of CA IV was downregulated in NSCLC tissues (1). However, the mechanism involved in the influences of CA IV on biological functions in NSCLC is very complicated and the exact clinical roles of CA IV are still remained unclear, making further validation is necessary.

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In the present study, the relative expression of CA IV is estimated by RT-qPCR in 114 resected NSCLC tissues compared to levels in their paired NT. The relationship between expression levels of CA IV, clinical pathological features and overall survivals in NSCLC patients is investigated. We overexpress CA IV mRNA basing on NSCLC A549 and NCI-H1299 cell lines by lentivirus-mediated technology.

Materials and methods

Patient samples. The 114 NSCLC tissues and corresponding adjacent normal tissues (NT) were obtained from patients who underwent surgery at the First Affiliated Hospital of Wenzhou Medical University, China, from August 2013 to October 2015. This study was approved by the Ethical Committee of the First Affiliated Hospital of Wenzhou Medical University, and all patients signed informed consent for the collection and use of their tissues for this study. The clinical pathological features of patients (Table I) were assessed according to the World Health Organization classification (19) and the TNM staging system. The NSCLC and matched NT samples were snap-frozen in liquid nitrogen immediately after resection. Patients were regularly followed up by telephone or other means, the longest follow-up time was 31 months. To investigate the association of CA IV expression with prognosis, the survival data was divided into high and low groups, a value superior or equal to 2 was defined as CA IV overexpression, based on the $2^{-\Delta\Delta Cq}$ method (20).

Quantitative PCR. Approximately 100 mg tissues from liquid nitrogen were cut off with a high-pressure sterile surgical scissors and then placed in a 4 ml centrifuge tube treated with 0.1% DEPC water. Take 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) into the centrifuge tube, then extracted the total RNA follow the reagent instructions. cDNA was reverse-transcribed from total RNA samples using an RT Reagent kit (Takara Bio, Dalian, China), based on the manufacturer's instructions. The measurement of CA IV and β-actin mRNA was performed by RT-qPCR with SYBR Premix Ex Taq in ABI 7000 instrument. CA IV forward primer: 5'-CTGGTGCTACGAGGTTCAA-3' and reverse primer: 5'-GCCTTGGTGGTGACGAT-3'; β-actin sense primer: 5'-CCTGGCACCC AGCACAAT-3', antisense primer: 5'-GCTGATCCACATCTGCTGGAA-3'. 2 μg of total RNA were transcribed into cDNA and PCR reaction was performed in a final volume of 20 μl, containing 10 μl of SYBR Premix (2x), 2 μl of cDNA template, 1 μl of each primer (10 mM), and 6 μl of double-distilled water. The quantitative real-time PCR reaction consisted of an initial denaturation step of 10 min at 95˚C, 40 cycles of 5 sec at 95˚C, 30 sec at 60˚C, and a final extension step of 5 min at 72˚C. Each sample was performed in triplicate and the median was used to calculate the relative concentrations with β-actin as an internal control gene (ΔCt=Ct median CA IV-Ct median β-actin), and $2^{-\Delta\Delta Cq}$ in expression was calculated (20).

Cell culture. Six human NSCLC cell lines (SPCA-1, NCI-H1975, LTEP-a2, NCI-H1299, NCI-H441 and A549) and normal human bronchial epithelial BEAS-2B were all purchased from the Cell Bank of the Chinese Academy of Sciences and maintained with complete medium (containing 10% fetal bovine serum and 90% RPMI1640) at 37°C, 5% CO_2, complete medium was changed at least once every two days.

Lentivirus-mediated overexpression vector transfection. A549 and NCI-H1299 cells were transfected overexpression vector targeting CA IV as well as a negative control (Genechem, Co., Ltd., Shanghai, China). Transfection was accomplished by seeding $2x10^5$ cells into a six-well plate, and after 24 h, the medium was aspirated and incubated with transfection complex, according to the manufacturer's protocols. The A549 and NCI-H1299 cells were infected with lentivirus for 72 h and the overexpression efficiency was detected by RT-qPCR.

Cell proliferation assay. Cell viability was evaluated by Cell Counting Kit-8 (Corning Inc, Acton, MA, USA) abiding by the manufacturer's protocols. Briefly, 3,000 cells of Stable transduced A549 and NCI-H1299 were suspended and seeded into a 96-well plate with supplemented medium (10% fetal bovine serum) and cell growth was monitored every 24 h for 7 days. The next day, the CA IV overexpression cells were incubated with CCK-8 for 1 h, and the absorbance was measured at 450 nm using a multifunctional microplate reader (Tecan, Männedorf, Switzerland) in the 1, 3, 5, and 7 d. This experiment was done in quadruplicate cells.

Statistical methods. Statistical analysis was performed by SPSS software v19 (SPSS Inc., Chicago, IL, USA). Statistical differences in CA IV mRNA expression levels among different groups were calculated using the non-parametric Kruskal-Wallis H test and Mann-Whitney U test for the skewed distribution or the one-way ANOVA and Dunnett's test for the normal distribution after the Kolmogorov-Smirnov test. The Kaplan-Meier method was used to calculate the overall survival rate, and the prognostic significance was evaluated by the log-rank test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

The expression level of CA IV mRNA in NSCLC and adjacent tissues and its relationship with clinical data. Overall, CA IV mRNA expression level of NSCLC is 0.543 (0.032-0.956) and markedly lower than its adjacent normal tissues (Mann-Whitney U=0.000, P<0.001) (Fig. 1). According to Table I, we observed that the CA IV level of NSCLC with lymph node metastasis group was significantly lower than that of NSCLC without lymph node metastasis group (Mann-Whitney U=1.712, P=0.015). CA IV expression levels among five TMN stages were not different (Kruskal-Wallis H test=147.231, P=0.575). The CA IV *mRNA* expression was also not relative to the histology differentiation (Kruskal-Wallis H test=5.345, P=0.712), smoking habits (Mann-Whitney U=243.12, P=0.312), gender (Mann-Whitney U=235.12, P=0.643), and histological subtype (Kruskal-Wallis H test=5.320, P=0.724).

The relation of CA IV mRNA expression to the prognosis of NSCLC. The overall survival time of low CA IV expression

Table I. Relationship between clinical pathological features and CAIV mRNA expression levels in 114 cases of patients with NSCLC.

Parameter	Cases	$2^{-\Delta\Delta Cq}$ of CA IV mRNA Median (range)	Kruskal-Wallis H or Mann-Whitney test	P-value
Gender	114		235.12	0.643
Male	56	0.445 (0.027-0.945)		
Female	58	0.556 (0.025-0.887)		
TMN stage	114		147.231[a]	0.575
Ia	24	0.532 (0.554-0.942)		
Ib	56	0.511 (0.499-0.856)		
IIa	14	0.359 (0.215-0.656)		
IIb	4	0.224 (0.092-0.535)		
IIIa	16	0.167 (0.077-0.339)		
Histological degree	114		5.345[a]	0.712
Poor	22	0.575 (0.132-0.945)		
Poor-moderate	14	0.414 (0.121-0.945)		
Moderate	34	0.323 (0.082-0.876)		
Moderate-high	18	0.323 (0.097-0.819)		
High	26	0.281 (0.095-0.778)		
Lymph node metastasis	114		1.712	0.015
Yes	28	0.054 (0.025-0.164)		
No	86	0.431 (0.287-0.943)		
Smoking	114		243.12	0.312
Yes	40	0.431 (0.026-0.931)		
No	74	0.421 (0.021-0.923)		
histological subtype			5.320	0.724
LAD	68	0.412 (0.031-0.921)		
SCC	40	0.429 (0.026-0.927)		
Big cell cancer	16	0.412 (0.021-0.908)		

LAD, lung Adenocarcinoma; SCC, squamous cell carcinoma. [a]Kruskal-Wallis H test.

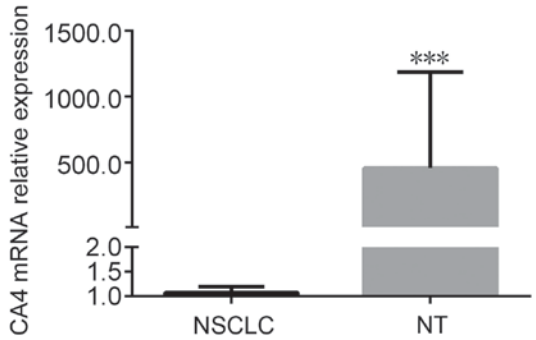

Figure 1. The relative expression levels of CAIV in NSCLC and NT tissues. CAIV expression level of NSCLC is 0.521 (0.025-0.987) and markedly lower than its adjacent normal tissues (P<0.001). ***P<0.001.

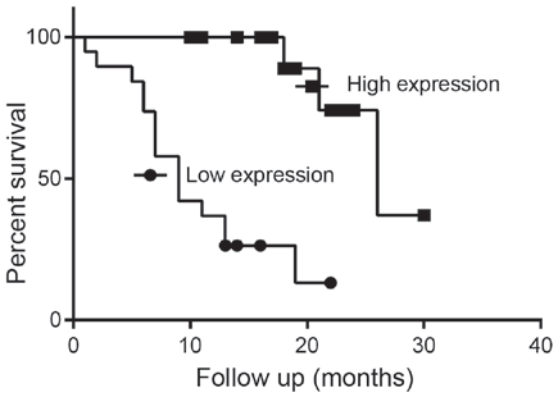

Figure 2. Kaplan-Meier analysis estimates of cumulative overall survival in patients with NSCLC. The overall survival time of low CAIV expression group was marginally lower than that of the high expression (χ^2=18.36, P<0.001).

group (median 9 months) was marginally lower than that of the high expression (median 26 months) (χ^2=18.36, P<0.001) (Fig. 2). Reduced CA IV expression was associated with shorter overall survival in patients with NSCLC. This finding suggests that reduced CA IV *mRNA* expression was a predictive factor of poor survival in NSCLC.

The expression level of CA IV mRNA from six NSCLC cell lines. We detected the expression levels of CA IV from six NSCLC cell lines (including SPCA-1, NCI-H1975, LTEP-a2,

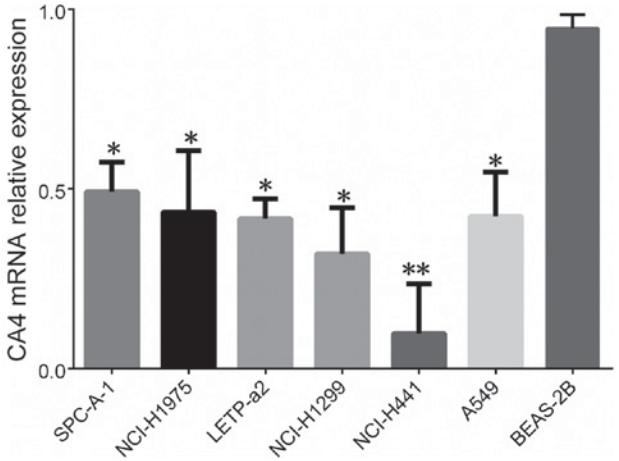

Figure 3. The expression levels of CA IV in six NSCLC cell lines. *P<0.05, **P<0.01, when compared to BEAS-2B.

NCI-H1299, NCI-H441 and A549) by RT-qPCR. It was shown that the expression levels of CA IV from SPCA-1 (P<0.05), NCI-H1975 (P<0.05), LTEP-a2 (P<0.05), NCI-H1299 (P<0.05), A549 (P<0.05) and NCI-H441 (P<0.01) were lower, compared to normal human bronchial epithelial BEAS-2B cell line (Fig. 3).

CA IV can regulate ability of cell proliferation. Growth curves of untreatedA549 and NCI-H1299 cells (control), cell stably transduced with non-targeting negative control (NC) and a vector for overexpression of CA IV (CA IV overexpression) were gradually increased with the change of time (Fig. 4A and B). Compared with the 1d, the OD450 nm of the 3, 5, and 7 d in control group were significantly increased (P<0.05, P<0.01 and P<0.001), the same results were also found in the NC group and CA IV overexpression group, respectively. Compared with corresponding days of control group or NC group, the OD450 nm of 1, 3 d in CA IV overexpression group were no statistically significant difference (P>0.05), while that of the 5 d (P<0.05) and the 7 d (P<0.01) were significantly reduced, it indicates that cell proliferation ability of A549 and NCI-H1299 lines was significantly suppressed by CA IV overexpression (Fig. 4).

Discussion

In the present study, we first demonstrated that CA IV mRNA expression was significantly decreased in NSCLC tissues compared with their adjacent normal tissues via RT-qPCR. Compared to normal human bronchial epithelial BEAS-2B cell line, it was shown that the expression levels of CA IV were also reduced in all six NSCLC cell lines. These results suggest an aberrant downregulation of CA IV in NSCLC and hint CA IV gene may be a potent tumor suppressor molecule. Notably, other members of the carbonicanhydrase family were hypoxia-inducible molecules in various types of solid cancers with a more aggressive phenotype and upregulated by hypoxia-inducible factor 1 (HIF-1) (21-23). In order to further study the mechanism of CA IV, we established CA IV overexpression of A549 and NCI-H1299 cell lines by lentivirus-mediated technology. After CA IV was overexpressed, cell proliferation ability of A549 and NCI-H1299 remarkably

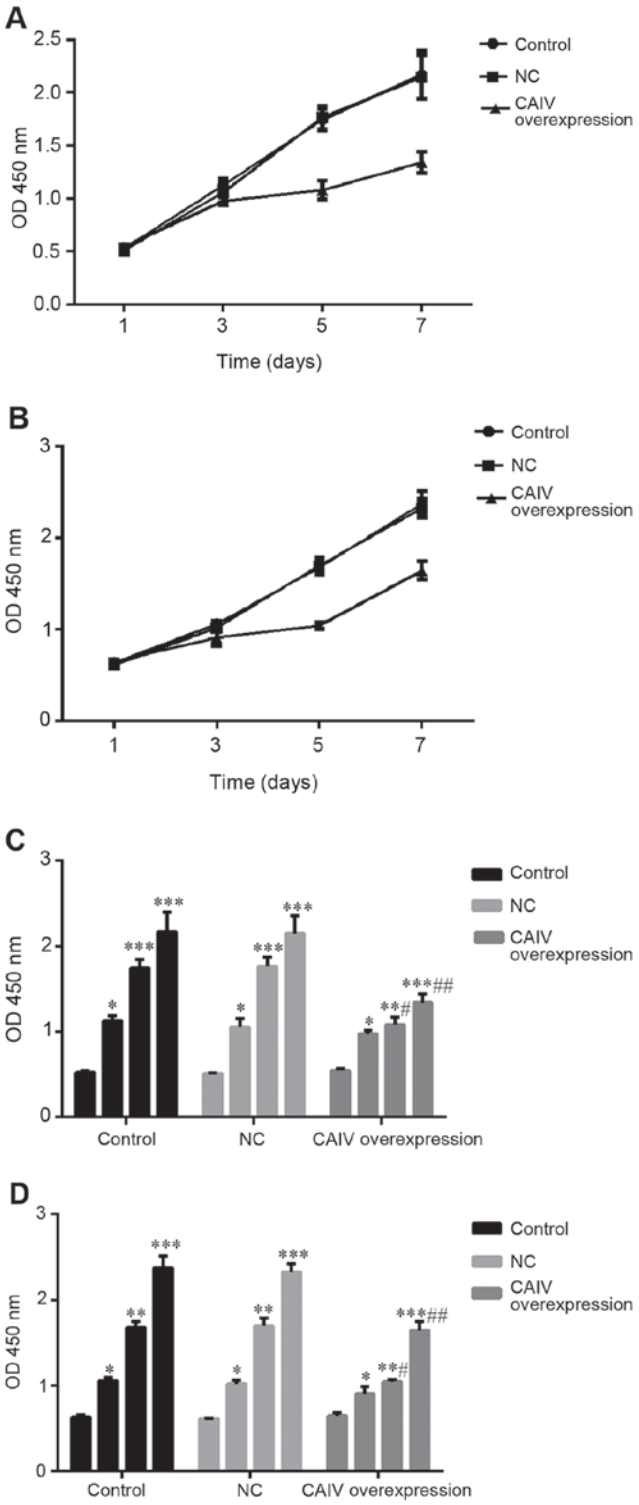

Figure 4. The cell proliferation results of different A549 and NCI-H1299 groups. (A) Growth curves of four days in different A549 groups. (B) Growth curves of four days in different NCI-H1299 groups. Growth curves show that the absorbance of each cell at 450 nm gradually increased over time, respectively. (C) The line boxplots of different A549 groups at four time points. (D) The line boxplots of different NCI-H1299 groups at four-time points. Compared with corresponding days of the control group or NC group, the OD450 nm of 1d, 3d in CA IV overexpression group were no statistically significant difference, while that of the 5d (P<0.05) and the 7d (P<0.01) significantly reduced. *P<0.05, **P<0.01, ***P<0.001; #P<0.05, ##P<0.01.

decreased. Based on these observations, we conclude that low expression of CA IV promotes cell proliferation and CA IV

acts as a tumor suppressor gene. To the best of our knowledge, this is the first identified that CA IV expression associated with tumor suppressor potential in NSCLC. Zhang shown that CA4 was silenced in all nine colon cancer cell lines and 92.6% of colon cancer. The re-expression of CA4 inhibited cell proliferation, induced apoptosis and cell cycle arrest in the G1 phase. CA4 inhibited the activity of the Wnt signalling pathway and mediated the degradation of β-catenin. CA4 interacted with Wilms' tumour 1-associating protein (WTAP) and induced WTAP protein degradation through polyubiquitination. Moreover, CA4 promoted the transcriptional activity of Wilms' tumour 1 (WT1), an antagonist of the Wnt pathway, which resulted in the induction of transducin β-like protein 1 (TBL1) and the degradation of β-catenin (18), we will carry out an in-depth study about the mechanism of CA IV in the NSCLC.

The diagnostic and prognostic value of carbonic anhydrases were proved in many solid tumors. It is generally acknowledged that overexpression of CAs can predict poor survival of patients, containing those with NSCLC (14,24). However, clinical properties and prognostic significance of CA IV in NSCLC remain unclear. In our study, the CA IV expression in NSCLC with lymph node metastasis group was significantly lower than that of NSCLC without lymph node metastasis group, while it was not relative to TMN stages, histology differentiation, gender, and smoking. Survival analysis showed that survival time of low expression CA IV group was significantly shorter than high expression CA IV group in NSCLC patients. Low CA IV expression is associated with cell proliferation and lymph node metastasis, which means that low CA IV expression accelerates tumor growth and aggressiveness, resulting in a poor prognosis. Those findings suggest that reduced CA IV expression is a predictive factor of poor survival in NSCLC.

To summarize, our studies ascertain for the first time that the expression of CA IV is downregulated in NSCLC and associated with promoting cell proliferation and lymph node metastasis. The low expression of CA IV may serve as an indicator of poor prognosis in NSCLC.

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