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Up-Regulation of Angiotensin-Converting Enzyme (*ACE*) Enhances Cell Proliferation and Predicts Poor Prognosis in Laryngeal Cancer

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: The angiotensin-converting enzyme (ACE, CD143) gene plays a crucial role in the pathology of many cancers. Previous studies mostly focused on the gene polymorphism, but the other functions of ACE have rarely been reported. The purpose of this study was to investigate the expression of ACE and its biological function, as well as its prognostic value, in laryngeal cancer.

Material/Methods: The expression of ACE was detected by quantitative real-time polymerase chain reaction (qRT-PCR) analysis in 106 patients with laryngeal cancer and 85 healthy people. Then the cell proliferation was estimated after the cell lines Hep-2 were transfected with pGL3-ACE and empty vector, respectively. In addition, the relationship between ACE expression and clinicopathologic characteristics was analyzed. Finally, Kaplan-Meier analysis was used to evaluate the overall survival of patients with different ACE expression, while Cox regression analysis was conducted to reveal the prognostic value of ACE in laryngeal cancer.

Results: Our results demonstrate that ACE is over-expressed in laryngeal cancer and thus promotes cell proliferation. The up-regulation of ACE was significantly influenced by tumor stage and lymph node metastasis. Patients with high ACE expression had a shorter overall survival compared with those with low ACE expression according to Kaplan-Meier analysis. The ACE gene was also found to be an important factor in the prognosis of laryngeal cancer.

Conclusions: Our study shows that the ACE gene was up-regulated, which promoted the cell proliferation, and it could be an independent prognostic marker in laryngeal cancer.

MeSH Keywords: Angiotensin-Converting Enzyme Inhibitors • Cell Proliferation • Prognosis

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Background

Laryngeal cancer is one of the most common malignancies in the head and neck [1] and accounts for 2.4% of new malignancies worldwide each year [2]. According to GLOBOCAN 2012, there were an estimated 156 877 new cases and 83 376 deaths due to laryngeal cancer per year worldwide [3]. Approximately 40% of patients have advanced (stage III or IV) disease when first evaluated and the lack of reliable and early diagnostic bio-markers lead a poor prognosis of laryngeal cancer [4,5]. Therefore, it is of great importance to look for appropriate and effective prognostic markers in laryngeal cancer.

The angiotensin-converting enzyme (*ACE*, *CD143*) gene is located on chromosome 17q23 and is a member of the renin-angiotensin system (RAS) [6–8]. It is characterized in humans by a major polymorphism exhibiting presence (I allele, insertion) or absence (D allele, deletion) of a 287-base pair Alu repeat sequence in intron 16 of this gene [9]. Hence, previous researchers usually focussed on the association between *ACE* gene polymorphism and the occurrence risk of diseases such as gastric cancer, colorectal cancer, coronary artery disease, and digestive cancer [10–13]. Wacker et al. [14] studied the relationship between *ACE* I/D polymorphism and body mass index, and other studies investigated *ACE* methylation [9,15] and *ACE* inhibitor (ACEI) [16,17]. For example, Ganz et al. [15] reported there was an increased risk of recurrence in patients taking ACEI the year before and after a breast cancer diagnosis. Michael et al. [17] suggested that ACEI use alone or coupled with significant weight loss predisposed patients to acute kidney injury during chemoradiation for head and neck cancer. However, the effects of *ACE* on laryngeal cancer are incompletely understood.

In the present study we measured the expression of ACE at mRNA and protein levels via qRT-PCR and ELISA analysis, and we also estimated its function in cell proliferation and its association with clinicopathologic characteristics. The prognostic value of ACE was evaluated through Kaplan-Meier and Cox regression analysis.

Material and Methods

Patients and samples

The study was conducted in Liaocheng People's Hospital and EENT Hospital and was approved by the Ethics Committee of the hospital. We enrolled 106 patients who were diagnosed as having laryngeal cancer during 2009–2010; none of them had ever received radiotherapy or chemotherapy before sampling. We enrolled 85 healthy people as healthy controls. All participators signed written informed consent in advance.

We extracted 3~4 ml of peripheral venous blood from all patients and healthy controls under the standardized phlebotomy procedures. Then the blood samples were put into BD Vacutainer spray-coated K2EDTA tubes (BD, Franklin Lakes, USA) and stored at room temperature for 30 min. After centrifuging at 3000×g for 15 min at 4°C, the supernatant was transferred to the centrifugal tube for another centrifugation at 13 000×g for 10 min at 4°C. Finally, the plasma samples were transferred to fresh tubes and stored at -80°C until the cell debris were all removed. The clinicopathologic characteristics including age, sex, tumor stage, smoking, drinking, differentiation, lymph node metastasis, and tumor depth were recorded in a database. Five-year follow-up was carried out with the laryngeal cancer patients. Patients who died from unexpected events or other diseases were excluded from our study.

Cell culture and treatment

The human laryngeal cancer cell line Hep-2 was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 100 μg/ml penicillin/streptomycin at 37°C with 5% CO_2.

ACE gene were cloned from human genomic DNA into the pGL3 basic vector (Promega, E1751). The cells were seeded in 96-well plates. When the concentration reached 80~90%, the cells were transfected with plasmids pGL3 containing *ACE* and empty plasmids pGL3 using Lipofectamine 2000 (Invitrogen 11668027) according to the manufacturer's instructions. Moreover, in order to reinforce the role of *ACE* on the progression of laryngeal cancer, some of the Hep-2 cells transfected with empty plasmids pGL3 were treated with Fosinopril (Sigma) at the concentration of 10 μmol/L for 48 h. The experiment was performed in triplicate.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the plasma of patients and healthy controls with TRIzol (Invitrogen, Carlsbad, CA, USA). Reverse transcription was conducted to synthesize the first chain of cDNA with TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). RT-PCR reaction was performed in the Applied Biosystems 7900 Fast Real-Time PCR system (Applied Biosystems, Foster City, California, USA). GAPDH was taken as the endogenous control for *ACE*. The relative quantification of *ACE* at mRNA level was evaluated by comparative cycle threshold (CT) method. Samples were analyzed in triplicate.

ELISA analysis

Total protein was extracted from all plasma samples of patients and healthy controls. The protein expression of *ACE* in

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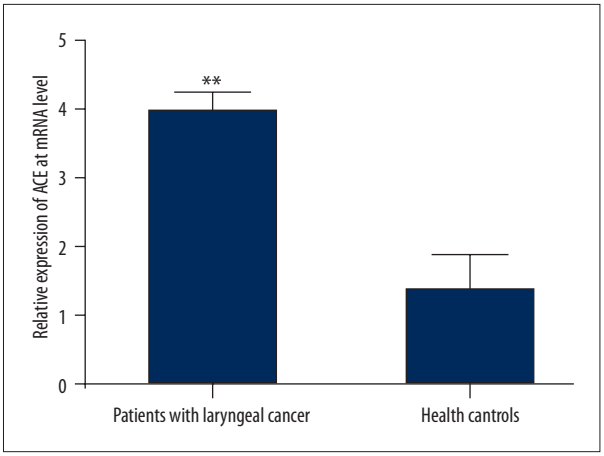

Figure 1. The expression of *ACE* at mRNA level in patients with laryngeal cancer. *ACE* expression was higher in the plasma of patients with laryngeal cancer than in healthy controls ($P<0.05$).

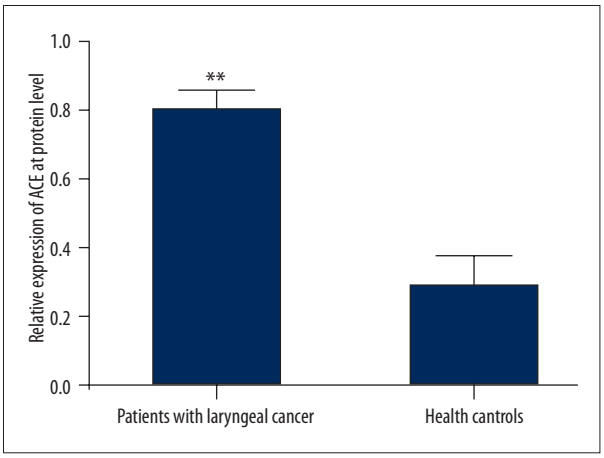

Figure 2. The expression of *ACE* at protein level in patients with laryngeal cancer. *ACE* protein expression was increased in the plasma of patients with laryngeal cancer compared with that in healthy controls ($P<0.05$).

plasma samples was measured by ACE (human) ELISA kits (DSA00-R&D systems) according to the manufacture's protocol. The absorbance was measured using an automated ELISA reader (ChroMate 4300 Microplate Reader; Palm City, FL, USA) at 450 nm. Each experiment was performed in triplicate.

Cell proliferation analysis

Cell proliferation ability was analyzed via MTS assay using the MTS Cell Proliferation Assay Kit (Colorimetric 197010) according to the manufacturer's instructions. After transfection and ACEI treatment, viability of Hep-2 cells (pGL3-ACE and *ACE* empty) at different time points (0, 24, 48, and 72 h) was measured at 450 nm with an enzyme immunoassay analyzer (Bio-Rad, Hercules, CA, USA). Each sample was detected in triplicate.

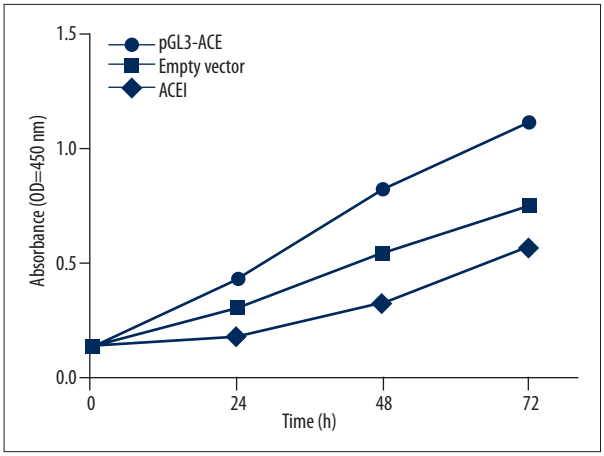

Figure 3. The effects of *ACE* on the cell proliferation of laryngeal cancer cells. The up-regulation of *ACE* promoted cell proliferation in laryngeal cancer and ACEI treatment reduced the proliferation.

Statistical analysis

Statistical analysis was performed using SPSS version 13.0 software (SPSS Inc, IL, USA). All quantified data were presented as mean ±SD. The differences between 2 groups were analyzed via the *t* test, while one-way ANOVA was used to compare the variances among 3 or more groups. The relationship between ACE expression and clinicopathologic characteristics was analyzed using the chi-square test. The prognostic value of *ACE* was estimated through Kaplan-Meier and Cox regression analysis. Differences were considered to be significant at $P<0.05$.

Results

ACE was over-expressed in the plasma of patients with laryngeal cancer

The expression of *ACE* at mRNA level was detected by qRT-PCR analysis. As shown in Figure 1, *ACE* was increased in the plasma of patients with laryngeal cancer compared with that in healthy controls ($P<0.05$).

ACE protein expression in the plasma of patients with laryngeal cancer and healthy controls

ELISA analysis was used to measure the expression of ACE protein. The result demonstrated that ACE protein expression was higher in patients with laryngeal cancer than that in healthy controls (Figure 2, $P<0.05$). Combined with expression at the mRNA level, we inferred that the *ACE* gene is related to laryngeal cancer and it might act as an oncogene.

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Table 1. Association between ACE expression and clinicopathological characteristics in 106 patients with laryngeal cancer.

Clinicopathological characteristics	n	ACE expression		χ^2	*P*
		High	Low		
Age				0.359	0.549
<60	40	17	23		
≥60	66	32	34		
Gender				3.036	0.081
Female	28	9	19		
Male	78	40	38		
Tumor stage				17.320	0.000
I–II	49	12	37		
III–IV	57	37	20		
Smoking				2.035	0.154
Nonsmokers	49	19	30		
Current smokers	57	30	27		
Drinking				0.000	0.988
Drinker	52	24	28		
Nondrinker	54	25	29		
Differentiation				3.518	0.172
Well	26	8	18		
Moderate	39	21	18		
Poor	41	20	21		
Lymph node metastasis				7.986	0.005
Absent	43	27	16		
Present	63	22	41		
Tumor depth (pT)				1.683	0.641
T1	19	9	10		
T2	27	12	15		
T3	31	12	19		
T4	29	16	13		

The function of ACE gene in cell proliferation in laryngeal cancer

As *ACE* was up-regulated in laryngeal cancer, we further explored its effects on cell proliferation via MTS assay. The cell line Hep-2 was transfected with pGL3-*ACE* and empty vector. The outcome showed the proliferation of cells transfected with pGL3-*ACE* was induced compared with cells transfected with empty vector, but the proliferation of cells treated with Fosinopril was worse than in the empty vector group. The results indicate that the increase of *ACE* could promote Hep-2 cell proliferation and ACEI could reverse the role of ACE to a certain degree (Figure 3).

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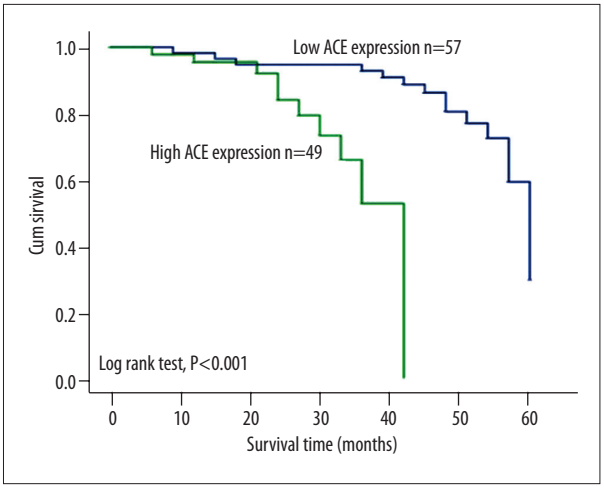

Figure 4. Kaplan-Meier analysis was conducted for patients with laryngeal cancer. Patients with high *ACE* expression had a shorter overall survival than those with low ACE expression (log-rank test, *P*<0.001).

The relationship between ACE expression and clinicopathologic characteristics

To investigate whether *ACE* was correlated with the development of laryngeal cancer, the relationship between its expression and clinicopathologic characteristics was analyzed. And tumor stage (*P*=0.000) and lymph node metastasis (*P*=0.005) were shown to influence ACE expression (Table 1). These findings show that *ACE* plays a role in the progression of laryngeal cancer.

The association between ACE expression and overall survival of patients with laryngeal cancer

To study the prognostic value of *ACE* gene in laryngeal cancer, we performed a 5-year follow-up. During the follow-up, 26 patients were censored and the median follow-up time was 34.8±15.9 months. Kaplan-Meier analysis showed that patients with high expression of *ACE* had a shorter overall survival than those with low *ACE* expression (Figure 4). After adjusting for all clinicopathologic characteristics, Cox regression analysis was carried out to estimate the prognostic value of the *ACE* gene. Results showed that lymph node metastasis (HR=2.610, 95CI%=1.165–5.848, *P*=0.020) and high *ACE* expression (HR=8.190, 95CI%=2.362–28.393, *P*=0.001) were both important prognostic factors in laryngeal cancer (Table 2).

Discussion

The human larynx is an organ essential for breathing, sound production, and protecting the trachea against food aspiration; cancer of the larynx affects quality of life and causes serious disease [3]. Laryngeal cancer is caused by many factors, including alcohol and tobacco abuse, exposure to hard-alloys dust, chlorinated solvents, and familial genetic patterns [18–20]. Invasion and metastasis are the main causes of cancer mortality [21]. Although surgery, radiation therapy, or chemotherapy alone or combination therapy have been widely used and developed in the treatment of laryngeal cancer, the overall survival of patients is still low [4]. The survival rate is only about 30–40% for laryngeal cancer if it metastasizes [22]. It is therefore necessary to explore effective markers for predicting the prognosis of laryngeal cancer.

Accumulated evidence had verified some bio-markers in the prognosis of laryngeal cancer. For instance, stanniocalcin 2 (STC2) was found to be increased and led to a poor prognosis in laryngeal cancer according to the study of Zhang et al. [23]. A meta-analysis by Liu et al. reported that matrix metalloproteinase 2 (MMP2) protein is a prognostic factor due to its overexpression in laryngeal cancer [24]. Zhang et al. detected the expression of miR-23a and analyzed its function, reporting that it is an independent prognostic factor for laryngeal cancer [25]. Serum HMGB1 was significantly up-regulated in patients with laryngeal cancer and it was reported to be a potential diagnostic and prognostic marker [26]. Other studies showed that lncRNA AC026166.2-001 and RP11-169D4.1-001, Dicer, Combined expression of serum exosomal miR-21, and HOTAIR, as well as miR-152, were all important prognostic indicators in laryngeal cancer through [27–30]. However, the role of *ACE* in laryngeal cancer was rarely reported.

Every organ and tissue is related with blood vascular systems and angiogenesis is of great importance. A previous study has shown that sodium nitrite can induce angiogenesis *in vitro* and *in vivo* [31]. *ACE* has been studied in a variety of diseases; for example, Sliva et al. [32] reported that low expression of *ACE* can be used as a biomarker to identify an endothelial cell subpopulation that is more capable of driving neovascularization. Sorich et al. [33] reported that angiotensin system inhibitors can improve the survival of patients with metastatic renal cell carcinoma. Babacan et al. [34] reported that *ACE* inhibitor/ARBs were effective and reduced the risk of recurrence

Table 2. Cox regression analysis for the prognostic value of *ACE* in laryngeal cancer.

Parameter	HR	95% confidence interval	*P*
Lymph node metastasis	2.610	1.165–5.848	0.020
High-ACE expression	8.190	2.362–28.393	0.001

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in breast cancer patients. Therefore, the effect of *ACE* in laryngeal cancer progression and prognosis attracted our interest.

In this study, we detected the expression of *ACE* gene at mRNA and protein levels, and we confirmed the up-regulation of *ACE*. Moreover, we showed that the expression of *ACE* was significantly regulated by tumor stage and lymph node metastasis. This reveals that *ACE* is related to laryngeal cancer and might participate in the development of this disease.

We further explored the function of *ACE* in laryngeal cancer. The development of cancers is closely linked with cell proliferation. For example, Chen et al. [35] showed that p21 plays a key role in the cellular response to UVB-induced DNA damage in skin cancer development. Fong et al. [36] studied the effect of mTOR inhibitors through transplant HEC-1A cells or Ishikawa cell in nude mice. We therefore investigated the influence of *ACE* on proliferation of Hep-2 cells. Our results demonstrate that *ACE* is a positive factor in cell proliferation. Kaplan-Meier and Cox regression analysis were performed to determine the prognostic value. The overall survival of patients with high *ACE* expression was relative shorter than in those with low expression. High *ACE* expression and lymph node metastasis were proven to be vital factors in the prognosis of laryngeal cancer.

Conclusions

The *ACE* gene is up-regulated in laryngeal cancer and its expression is related to tumor stage and lymph node metastasis. Cell proliferation is regulated by *ACE* expression and *ACE* could be an independent prognostic indicator in laryngeal cancer. Based on the above results, further studies with larger sample sizes are warranted to develop effective therapies for laryngeal cancer patients.

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