

Original Article

Down-regulation of *FHL1* is associated with a poor prognosis of patients with oral cancer

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Abstract: Background: The four and a half LIM domains 1 (*FHL1*) gene has been reported to be related to carcinogenesis of some cancers. However, it was aberrant expressed in oral cancer, its prognostic value was never reported. The purpose of this study was to detect the expression of *FHL1* and investigate its prognostic role in patients with oral cancer. Methods: The expression level of *FHL1* at mRNA and protein level were detected by quantitative real-time polymerase chain reaction (qRT-PCR) and western blot analysis in the tumor tissues and adjacent normal tissues of 112 patients, respectively. Then, we analyzed the relationship between the *FHL1* expression and clinicopathological features of patients. Besides, Kaplan-Meier analysis was used to estimate the overall survival of patients while cox regression analysis was taken to evaluate the prognostic value of *FHL1* in oral cancer. Results: The relative expression of *FHL1* was significantly lower in tumor tissues compared with paired adjacent normal tissues both at mRNA and protein level. And the low expression of *FHL1* was correlated with cell differentiated grade, TNM stage, and lymph node metastasis. Kaplan-Meier analysis indicated that patients with low *FHL1* expression had a shorter overall survival than those with high expression. Multivariate analysis showed that the low expression of *FHL1* was an independent predictor for the prognosis of oral cancer. Conclusion: *FHL1* was decreased in oral cancer tissues and participated in the progression of oral cancer. And it may be a novel prognostic indicator and potential target for gene therapy in oral cancer.

Keywords: The four and a half LIM domains 1 (*FHL1*), oral cancer, prognosis

Introduction

Oral cancer is a subtype of head and neck cancer and commonly occurred in some certain tissues such as the floor of the mouth and the tongue [1, 2]. It accounts for about 90% of all the malignancies appearing in the oral cavity [3]. The incidence of oral cancer is extremely high around the world, in sixth place of the whole body malignant tumor (row in the lungs, stomach, breast, colon and rectal cancer, cervical cancer) [4]. As a high degree of malignant tumors, although there are continuous efforts from lots of oncologists and surgeons, and the mortality of oral cancer was declined slightly in the past 20 years, but the 5-year survival rate is only 40%-75% [5-7]. The clinical and histopathological parameters are often regarded as the important reference of therapeutic decisions, but they always fail to predict patient out-

come and therapy success. Therefore, it is essential to search a new prognostic and predictive factors of oral cancer to improve the methods of risk assessment.

Four and a half LIM protein 1 (*FHL1*) is one of the members of *FHL* family which includes *FHL1*, *FHL2*, *FHL3*, *FHL4* and *FHL5* [8]. It is located on chromosome Xq27.2 and encodes four-and-a-half LIM protein-1. *FHL1* was confirmed to be aberrant expressed in skeletal muscle, heart, colon, small intestine, and prostate [9-11]. Previous studies have reported that *FHL1* was a tumor suppressor due to its abnormal expression in various types of tumor and might be a valuable biomarker in some cancers. For example, Ji et al. have showed that the expression of *FHL1* was reduced in ESCC tissues and might be an independent prognostic factor for patients with esophageal cancer. Cao

The prognostic value of FHL1 in oral cancer

Table 1. Relationship between *FHL1* expression and clinicopathologic parameters of oral cancer patients

Variables	Cases (n=112)	<i>FHL1</i> expression		P-value
		High (n=53)	Low (n=59)	
Age				0.296
≥50	67	29	38	
<50	45	24	21	
Gender				0.584
Male	58	26	32	
Female	54	27	27	
Tumor size				0.478
<3	51	26	34	
≥3	61	27	25	
Primary site of tumor				0.694
Buccal	17	8	9	
Musoca	29	16	13	
Tongue	35	17	18	
Alveolus	18	8	10	
Others	13	4	9	
TNM stage				0.004
I-II	58	35	23	
III-IV	54	18	36	
Cell differentiated grade				0.020
Well	48	16	32	
Moderate	35	18	17	
Poor	29	19	10	
Lymph node metastasis				0.001
Yes	60	20	40	
No	52	33	19	

et al. reported that *FHL1* mRNA and protein expressions were decreased in head and neck squamous cell carcinoma and was identified as independent prognostic predictor of patients survival [12-15]. However, the functions of *FHL1* in the diagnosis, treatment and prognosis of oral cancer are still not clear.

The aim of our study was to detect the expression of *FHL1* and investigate the relationship between its expression with clinical factors of patients with oral cancer. What's more, the influences of *FHL1* expression on the overall survival and prognosis of oral cancer patients were estimated.

Material and method

Patients and specimens

112 patients who were diagnosed with oral cancer were collected from State Key Laboratory

Breeding Base of Basic Science of Stomatology (Hubei-MOST) & Key Laboratory of Oral Biomedicine Ministry of Education (KLOBM). All of them had never received any chemotherapy or radiotherapy before sampling. This study was approved by the Ethnic Committee of the hospital and each participant signed a written informed consents in advance.

Tumor tissues and corresponding adjacent normal tissues were extracted from the patients with oral cancer and frozen by liquid nitrogen immediately. Then all samples were stored at -80°C for use. A 5-years' follow-up was conducted with all patients every 3 months. The basic clinicopathological characteristics of each patients were detailed in **Table 1**. Patients who were died from unexpected events or other diseases were excluded from our study.

RNA extraction and qRT-PCR analysis

Total RNA was extracted from the tissue samples using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions, respectively. Reverse transcription was performed using the SuperScript First Strand cDNA System (Invitrogen) following the manufacturer's instructions to synthesize the first chain of cDNA. Then RT-PCR reaction was performed using SYBR® Premix Ex Taq™II (Takara, Dalian, China) according to the manufacturer's instructions in the 7500 real-time RT-PCR system (Applied Biosystems, Foster City). GAPDH was taken as the internal controls. The relative mRNA expression of *FHL1* was calculated by the $2^{-\Delta\Delta CT}$ method. Each sample was in triplicate.

Western blot analysis

Total protein was isolated from the tumor tissues and adjacent normal tissues, respectively. Then the protein was separated by SDS-PAGE and the brands were transferred onto nitrocellulose membranes (Invitrogen). The membranes were blocked by 5% non-fat milk and washed three times with 0.1% Tween-20 in Tris-buffered saline. Subsequently, the membranes were incubated with 0.1 µg/ml rabbit anti-*FHL1* polyclonal antibody (Aviva Systems Biology, San Diego, CA) overnight at 4°C. After being

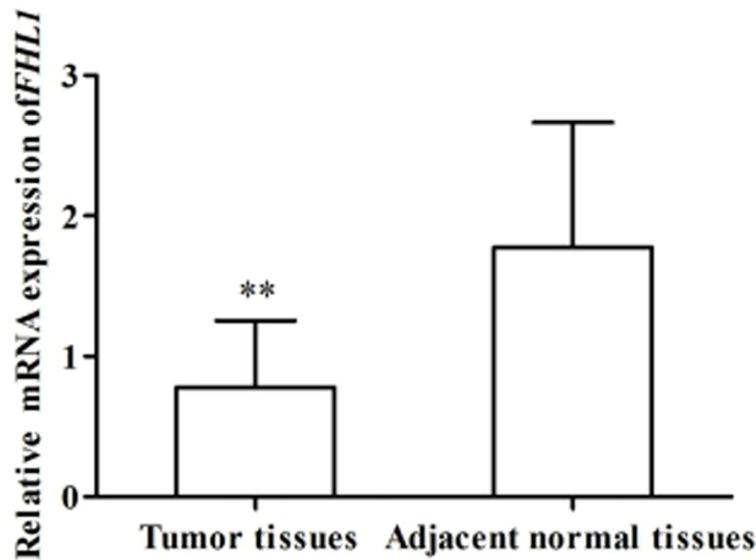


Figure 1. The relative mRNA expression of *FHL1* in oral cancer tissues and corresponding adjacent normal tissues. It was significantly lower in tumor tissues than that in adjacent normal tissues ($P < 0.001$).

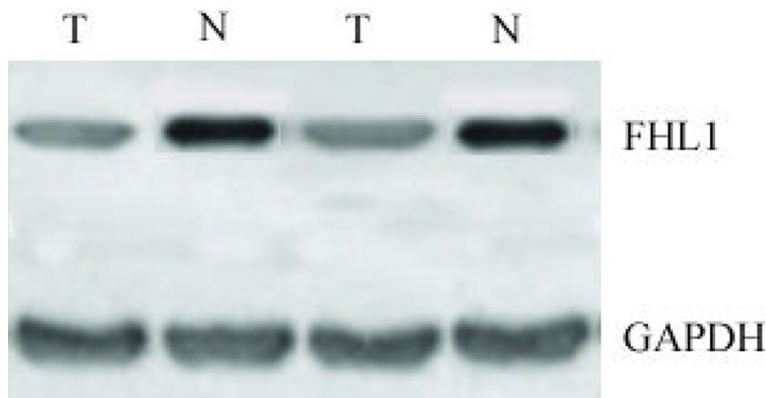


Figure 2. The relative protein expression of *FHL1* in oral cancer tissues and corresponding adjacent normal tissues. It was significantly decreased in tumor tissues compared to that in adjacent normal tissues ($P < 0.001$), T: tumor, N: normal.

washed, the membranes were incubated with a 1:2,500 of anti-rabbit IgG (H+L) horseradish peroxidase (HRP) conjugate (Promega, Madison, WI) as a secondary antibody for 1 h at room temperature. Finally, the proteins were detected by SuperSignal Chemiluminescent substrate (Thermo, Waltham, MA) and the western blot analysis results were visualized by exposing the membrane to a cooled CCD camera system, Light-Capture II (ATTO, Tokyo, Japan). Signal intensities were quantitated using the CS Analyzer version 3.0 software (ATTO).

corresponding adjacent normal tissues ($P < 0.001$).

The relative protein expression of FHL1 was lower in oral cancer tissues than that in adjacent normal tissues

The relative protein expression of *FHL1* in oral cancer tissue and adjacent normal tissues was measured via western blot analysis. The result manifested that it was also lower in tumor tissues compared to that in adjacent normal tissues ($P < 0.001$, **Figure 2**).

Statistical analysis

All statistical analyses were carried out using the SPSS 21.0 statistical software and the figures were designed by GraphPad Prism 5. The data were presented as mean \pm SD. The differences between two groups were analyzed by students' t test. The relationship between *FHL1* expression and clinical factors of patients with oral cancer was estimated by chi-square test. Kaplan-Meier analysis was used to estimate the overall survival of patients with different expression of *FHL1*. The multivariate analysis with cox regression analysis was taken to assess the potential prognostic value of *FHL1* in oral cancer. $P < 0.05$ was considered to be statistical significantly.

Results

The relative mRNA expression of FHL1 was decreased in oral cancer tissues

QRT-PCR was applied to detect the relative mRNA expression of *FHL1* in 112 primary oral cancer tissues and corresponding adjacent normal tissues. As shown in **Figure 1**, the relative mRNA expression of *FHL1* in tumor tissues was significantly lower than that in

The prognostic value of FHL1 in oral cancer

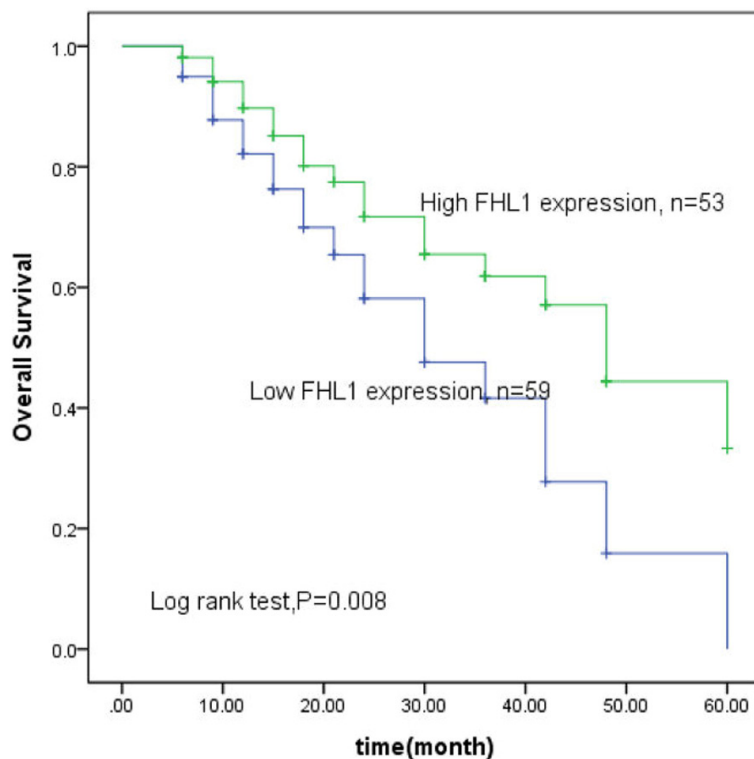


Figure 3. Kaplan-Meier analysis showed the overall survival of patients with oral cancer based on the expression level of *FHL1*. Patients with low expression of *FHL1* had a significant shorter overall survival than those with high expression (log rank test, $P=0.008$).

Table 2. Multivariate analyses adjusted for clinical factors for estimating the prognostic value of *FHL1* in patients with oral cancer

Factor	Beta value	HR	95% CI
<i>FHL1</i>	0.847	2.334	1.016-5.362
Cell differentiation grade	1.919	6.813	1.211-38.322

Footnote: age, gender, Tumor size, Primary site, TNM stage and Lymph node metastasis, all $P>0.05$.

Associations between the expression of *FHL1* and clinicopathological parameters of patients with oral cancer

To investigate whether *FHL1* was involved in the development of oral cancer, we analyzed its association with clinicopathological parameters of patients. The result demonstrated that the low expression of *FHL1* was significantly associated with cell differentiated grade ($P=0.020$), lymph node metastasis ($P=0.001$) and TNM stage ($P=0.004$). However, there was no correlation between *FHL1* expression and other clinicopathological parameters including age ($P=0.296$), gender ($P=0.584$), tumor size ($P=0.478$), and primary site of tumor ($P=0.694$).

The prognostic significance of *FHL1* in oral cancer

To evaluate the prognostic value of *FHL1* in oral cancer, we made a 5-years' follow-up. Based on the data of follow-up, we found the overall survival of patients with low expression of *FHL1* was shorter than those with high expression via Kaplan-Meier analysis (log rank test, $P=0.008$, **Figure 3**). Furthermore, a multivariate analysis adjusted for the clinical factors of patients with oral cancer was performed using cox regression analysis. The outcome showed that the low expression of *FHL1* (HR=2.334, 95% CI=1.016-5.362, $P=0.046$) and cell differentiation grade (HR=6.813, 95% CI=1.211-38.322, $P=0.029$) were related to the prognosis of oral cancer and they might act as independent predictors for the prognosis of patients with this cancer (**Table 2**).

Discussion

Oral cancer is one of the most common malignant tumor of head and neck neoplasm, accounting for more than 10,000 deaths per year [16, 17]. The main factors of oral cancer includes alcohol, tobacco, betel quid chewing and viral infections according the statistics [18-22]. The early detection and treatments of oral cancer can greatly improve the long-term prognosis of patients. However, it is usually at advanced stage when the patients were found, so the 5-year survival rate hovers around 50% [23]. Therefore, it is very emergency to identify some new novel prognostic bio-markers for this disease.

FHL1, consists of four and a half LIM structure domains which can function with a variety of protein interactions and have relevance with cell proliferation, differentiation, transcription regulation, and closely related to cell apoptosis

The prognostic value of FHL1 in oral cancer

[24]. Furthermore, *FHL1* is also a kind of transcription inhibiting factor, and has different functions in a variety of different organizations [25]. Its abnormal expression play an important role in gastrointestinal tumor and myocardial hypertrophy [26, 27]. *FHL1* gene was also play important roles in a variety of diseases. For instance, *FHL1* was considered as a therapeutic target for Duchenne muscular dystrophy which indicated that transgenic *FHL1* expression increased sarcolemmal membrane stability, reduced muscle degeneration, decreased inflammation and conferred protection from contraction-induced injury in mdx mice [28]. Niu et al., suggested that the reduced expression of *FHL1* might play an important role in the development and progression of lung cancer and it might be a useful target for lung cancer gene therapy [29]. Otherwise, the study of *FHL1* interacts with oestrogen receptors and regulates breast cancer cell growth suggested that *FHL1* inhibited anchorage-dependent and anchorage-independent breast cancer cell growth, and *FHL1* might play an important role in ER signalling as well as breast cancer cell growth regulation [30]. But so far, the researches about *FHL1* relations with oral cancer are rarely. These conclusions laid a foundation for us to study the relationship between *FHL1* and oral cancer.

In the present study, we demonstrated that *FHL1* expression in oral cancer tissues was significantly lower than that in normal tissues. This might reveal that *FHL1* was a tumor suppressor in oral cancer. Then we explored its relationship with the development of oral cancer. As it showed that *FHL1* was involved in the progression of oral cancer.

To further explore the prognostic value of *FHL1*, we made a 5 years' follow-up. Followed by that, we estimated the overall survival of patients with oral cancer via Kaplan-Meier analysis. The result manifested that the overall survival of patients with low *FHL1* expression had a much shorter overall survival than those with high expression which indicated that *FHL1* was related to the prognosis of oral cancer. Cox regression analysis showed that the low expression of *FHL1* was an independent predictor for the poor prognosis of oral cancer. To our knowledge, this was the first report which demonstrated the prognostic significance of *FHL1* in oral cancer patients and the first study that investigated its role in oral cancer.

In summary, the principal finding of this study indicates that the expression of *FHL1* is decreased and associated with progression of the oral cancer. Moreover, *FHL1* maybe a good candidate as a molecular prognostic marker in oral cancer. However, the mechanism of *FHL1* function is still unknown and more study on the role of *FHL1* in oral cancer progression and prognosis will need further efforts.

Disclosure of conflict of interest

None.

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