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Expression Analysis of *ALDH1A1* and *ALDH1A3* Genes in Oral Squamous Cell Carcinoma Patients

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Abstract

Oral squamous cell carcinoma (OSCC) is still one of the leading cancer related death, worldwide. Owing to the critical role of ALDH1 isoforms in pathogenesis of cancers and controversies in previous reports, the present study was conducted to determine the expression of two challenging *ALDH1* isoforms in OSCC tissue samples. RNA was isolated from 30 OSCC and normal fresh tissues margins and then was converted to cDNA using Thermo Scientific™ Fermentas cDNA synthesis kit. Reverse Transcription Real time polymerase chain reaction (RT-Real time PCR) was performed on synthesized cDNA using primer pairs specific to *ALDH1A1* and *ALDH1A3* genes. It was found that the *ALDH1A1* mRNA expression was significantly higher in OSCC samples compared to normal margins (P-value= 0.001). *ALDH1A1* and *ALDH1A3* expression was significantly associated with alcohol drinking (P-value= 0.01) and smoking (P-value=0.008), respectively. There was no correlation among ALDH1A1 and *ALDH1A3* expressions and clinicopathological features (p-value>0.05). Confirmation of the over-expression of *ALDH1A1* isoform in OSCC samples maybe indicating that it plays critical role in pathogenesis of OSCC. However, owing to the discrepant findings on the association of *ALDH1A1* expression with clinicopathological features, further studies are required to identify its role as driver or passenger carcinogenic alteration and its exact association with smoking.

Keywords: *ALDH1A1*; *ALDH1A3*; Gene expression; Oral squamous cell carcinoma.

Introduction

Oral cancer is the sixth frequent human cancer and one of the most important leading causes of cancer death worldwide. The incidence of oral cancer has been reported as 270,000 cases per year and has a specific trend to be more prevalent in developing countries [1]. According to the different epidemiological studies performed among Iranian population, the prevalence of oral cancer in Iran is similar to the other populations and

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has higher frequency among men than women, as well. Oral squamous cell carcinoma (OSCC) is the most common form of oral cancer and frequently (30%) seen in the tongue. Tobacco or cigarette smoking followed by alcohol consumption are the most important risk factors of OSCC among Iranians [2]. Although, detection of oral lesions seems to be easier than other body sites, OSCC usually is diagnosed at higher grades and therefore has low prognosis and response to treatment. It was shown that the outcome of OSCC and patient's survival is quite satisfactory if it is diagnosed and managed at the lower stages of progression [3]. In this regards, early biomarkers could have substantial role in not only decreasing the mortality rate but also can alleviate the medical charges associated with advanced cancer therapy protocols.

Multiple genetic and epigenetic alterations have been suggested to be used as early biomarkers in OSCC diagnosis. Identification of hot spots mutations in *CCND1*, *p53*, *Rb*, *FLJ10540*, and *TC21* genes in OSCC patients, have made them as candidates diagnostic and prognostic biomarkers [4, 5]. One of the other molecular carcinogenic alterations which has recently been under the focus of cancer studies is oncogenic protein isoform shift. *Aldehyde dehydrogenases* (*ALDH*) gene family is a good example which have demonstrated differential isoform expression pattern among various types of human cancers [6, 7]. They encode for different ALDH enzymes which have potential roles in intracellular detoxification especially from aldehyde as one of the most important toxic agent for stem cells proliferation and differentiation. ALDH family activity, therefore, have been identified as a valuable cancer stem cells marker. ALDH1 has six isoforms including ALDH1A1, ALDH1A2, ALDH1A3, ALDH1B1, ALDH1L1, and ALDH1L2. The role of two ALDH1A1 and ALDH1A3 isoforms expression has been more assayed in various types of cancers [8].

ALDH1A1 gene was mapped on chromosome 9q21.13 and it is the most copious isoform of aldehyde dehydrogenase which is actively expressed in liver. It was shown that *ALDH1A1* expression was associated with poor cancer prognosis, higher rate of metastasis and chemotherapy resistance [9].

ALDH1A3 gene is located on 15q26.3 and includes 16 exons. In addition to alcohol metabolism, ALDH1A3 has much more physiologic and metabolic activities in conjunction with other ALDH isoforms [10]. It was shown that *ALDH1A3* expression increased in brain tumors, ovarian, colorectal and pancreatic cancers while it was downregulated in prostate, bladder and lung cancers [11-13]. Given that alcohol consumption is a major risk factor of OSCC, in addition to the different alcohol metabolism in various ethnicities, molecular assessment of ALDH1 isoenzymes not only can help to elucidate the molecular pathogenesis of OSCC, but also may describe the priority of smoking on alcohol consumption as the main risk factor among Asian populations as well as Iran.

Owing to the contradictory reports on ALDH1 isoenzymes mRNA and protein expression in various types of human cancers, current work was conducted to determine the mRNA expression of *ALDH1A1* and *ALDH1A3* genes as the most challenging isoforms of ALDH1 in OSCC tissue samples compared to normal margin controls.

Materials and Methods

Thirty fresh frozen OSCC and adjacent normal tissues samples were provided by Iran National Tumor Bank. All the enrolled patients had filled the consent form according to local ethics committee of Tehran University of Medical sciences. RNA was extracted from tissue samples using High Pure RNA Isolation Kit (Roche, Germany) and then was subjected to cDNA synthesis using RevertAID TM Firs Standard cDNA synthesis (Thermo Scientific™ Fermentas, USA) according to the manufacturer's instructions. Reverse transcriptase Real time polymerase chain reaction (RT-Real Time PCR) was performed on Applied Biosystems Real-Time PCR Instrument (Thermo Fisher Scientific, USA) to amplify synthesized cDNA using specifically primer pairs designed for *ALDH1A1* and *ALDH1A3* genes in comparison with *β-actin* as reference gene (Table 1).

The statistical analyses were performed using SPSS statistical software (version 16, SPSS Inc., Chicago, IL, USA). The correlation between *ALDH1A1* and *ALDH1A3* expressions with clinicopathological features was analyzed using Fisher's exact test and Student's-test. The differences were considered to be statistically

Table 1. Primer sequences used to amplify *ALDH1A1* and *ALDH1A3* genes

Gene	Forward Primer (5'->3')	Reverse Primer (5'->3')
ALDH1A1	TGTTAGCTGATGCCGACTTG	TTCTTAGCCCGCTCAACACT
ALDH1A3	TCTCGACAAAGCCCTGAAGT	TATTCGGCCAAAGCGTATTC

significant when the p-value was calculated as less than 0.05. RT-Real time PCR data was analyzed using REST (Qiagen, USA) and SPSS softwares.

Results

The mean of the patients' age was 54.87±11.44. Considering Confidence Interval (CI) of 95%, other patients' characteristics were presented in table 2. Real time RT PCR demonstrated that *ALDH1A1* was significantly overexpressed in cancer tissues compared to their normal margins (p= 0.001). In spite of higher expression among OSCC samples, expression of *ALDH1A3* isoform was not significantly different between two studied samples (p=0.86) (Fig. 1.A). *ALDH1A1* expression was shown to be significantly higher in alcohol drinker patients (P-value= 0.01). Moreover, *ALDH1A3* was meaningfully associated with smoking (P-value=0.008) (Table 2). Association among *ALDH1A1* and *ALDH1A3* expression with tumor grade and stage, previous family history of cancer, vascular and lymph node tumor invasion, site of primary tumor, clinical metastasis, tumor necrosis and the histology of the patients' tumor tissue were not statistically meaningful (p<0.05) (Table 2).

Discussion

To the best of our knowledge, this is the first work on the simultaneous assessment of two A1 and A3 isoforms of *ALDH1* mRNA expressions which have been analyzed with complete clinicopathological features of OSCC patients excluding survival. Oppel F et al. have recently shown significant expression of *ALDH1A1* gene in primary Head and neck squamous cell carcinoma cell line model which is in line with our results [14]. Qian et al. have demonstrated significant overexpression of ALDH1A1 protein expression in poor differentiated oropharyngeal squamous cell carcinoma samples, as well [15]. To the best of our knowledge, simultaneous evaluation of mRNA and protein expression of ALDH1A1 was only performed on squamous cell carcinoma of the head and neck cells and tissues which was demonstrated to be higher in cancerous samples and cells similar to the present study [16]. However, our findings are in contrast with some of the previously reported studies which have exclusively performed on ALDH1A1 protein expression among OSCC patients [17, 18]. It may be due to the low sample size and protein quantification through immunohistochemistry (IHC) as a less sensitive protein quantification method in their study.

ALDH1A3 expression was shown to be significantly increased in tongue squamous cell carcinoma samples in all stages of the disease [19]. Although, it was not significant, it is in line with our finding that *ALDH1A3* expression has been increased among tongue SCC as well as other OSCC patients (p<0.05).

ALDH enzyme isoforms activity in cancer cells has been introduced as important indicator of cancer stem cells (CSCs) activity [20, 21]. ALDH1A1 and ALDH1A3 are the seldom ALDH isoforms which have been shown to be able to catalyze the conversion of BODIPY-aminoacetaldehyde (BAAA) to BODIPY-aminoacetate (BAA). Herein, it was determined that *ALDH1A1* expression was meaningfully associated with

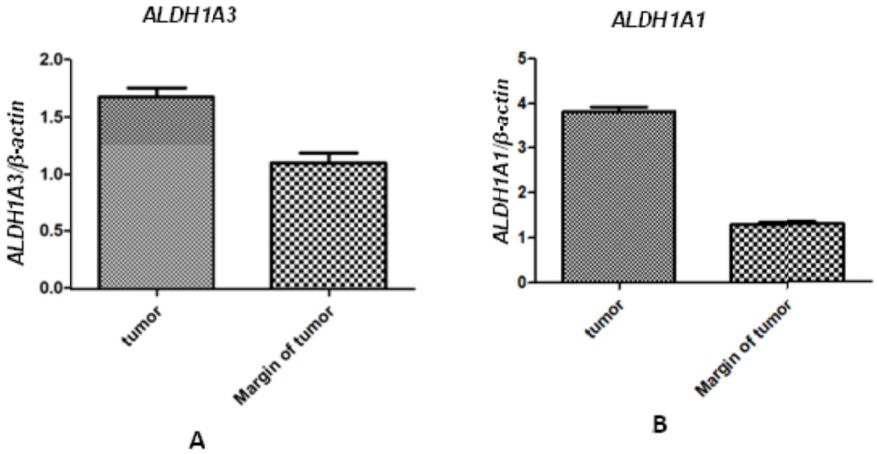

Figure 1. Comparative expression of (A) *ALDH1A3* and (B) *ALDH1A1* genes in OSCC samples compared to normal marginal tissues.

Table 2. Clinicopathological characteristics of the OSCC patients and their correlation with *ALDH1A1* and *ALDH1A3* genes expression

Patients' charactristics	Frequency	*ALDH1A1* expression (P-Value)	*ALDH1A3* expression (P-Value)
Gender			
Male	14	0.62	0.41
Female	16		
Primary tumor site			
Tongue	1	0.49	0.52
Buccal mucosa	7		
Others (lip, gum, etc.)	22		
Histology grade			
Grade I (Well differentiated)	14	0.53	0.34
Grade II (Moderately differentiated)	9		
Grade III (Poorly differentiated)	3		
Grade IV (Undifferentiated)	4		
AJCC staging			
I-II	22	0.14	0.18
III-IV	5		
Lymph node invasion			
Yes	7	0.25	0.12
No	23		
Vascular invasion			
Yes	6	0.9	0.86
No	24		
Familial history of cancer			
Yes	4	0.12	0.17
No	26		
Alcohol drinking			
Non-drinker	25	**0.01**	0.095
Social drinker	3		
Heavy drinker	2		
Smoking			
Smoker	16	0.39	**0.008**
Non smoker	12		
Ex-smoker	2		
Clinical metastasis			
Mx	0	0.23	0.3
M0	26		
M1	4		
Tumor necrosis			
Present	19	0.4	0.67
Absent	11		
Tumor size			
>4 cm	21	0.07	0.28
<4 cm	9		

alcohol drinking. It was shown that the direct effect of alcohol on CSCs is mediated through production of reactive oxygen species (ROS) and stimulation of ErbB2/p38γ MAPK axis [22, 23]. Although CSCs activity was not measured in the present study, owing to the limited number of CSCs marker, *ALDH1A1* expression could be a valuable candidate for OSCC specific CSCs especially among alcohol drinkers. Moreover, in spite of non-significant *ALDH1A3* expression between cancerous and normal samples, it was meaningfully associated with smoking. Various recent studies on *ALDH1A3* have demonstrated that its expression has critical roles in proliferation, progression and invasion of different types of human cancers as well as prostate, breast and brain cancers and, therefore, would be a novel target for cancer diagnosis and treatment [24-26]. Zeynep H. Gümüş et al. have shown that exposure to tobacco smoking led to the

overexpression of multiple genes including *ALDH1A3* [27]. It may explain our finding in such a way that tobacco smoking was the trigger of *ALDH1A3* overexpression. It is in line with previous finding relying on the overexpression of *ALDH1A3* in buccal and nasal epithelium of healthy tobacco smokers [28]. Moreover, in another study on non-small cell lung cancer (NSCLC), it was found that *ALDH1A3* expression was linked to higher overall survival and well differentiated tumor cells [29]. Their findings on the negative association of *ALDH1A3* expression with smoking andcancer patients' gender which were confirmed by protein assays are similar to the findings in the present study. It may indicate the diverse role of ALDH1A3 isoform in different human organs. The latter hypothesis can be enhanced by the previous report which have shown that tobacco smoking reduces the antioxidant effect of saliva. Owing to the role of ALDH family in detoxification, changes in ALDH1A3 isoform maybe dominant beyond the effect of tobacco smoking inside of oral cavity [30].

Taken together, our findings are further confirmation of the role of *ALDH1* in particular A1 isoform in OSCC progression. The major pitfall of the present study was inaccessibility to the survival time of the patients to be analyzed with A1 and A3 expression patterns. In addition, comparing the expression of ALDH1A1 and ALDH1A3 genes and protein among OSCC, healthy controls and benign oral tumors may identify those isoforms as early diagnostic and metastatic markers of oral cancer. Further study is required to exactly determine the mechanism behind the effect of alcohol and smoking on A1 and A3 isoforms expression and their roles in OSCC carcinogenesis. It may open the door toward screening of oral cancer among alcoholic and smoker persons through ALDH1 isoform expression analysis.

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