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Cytokeratins (CK7 and CK20) Genes Expression Association with Clinicopathological Indices in Oral Squamous Cell Carcinoma and Dysplastic Oral Epithelium

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Abstract

Background: High prevalence of oral squamous cell carcinoma (OSCC) demands the additional novel biological markers. Due to the established roles of cytokeratin in the prognosis of metastasis evaluation the relation of expression of both *CK7* and *CK20* in OSCC compared to the dysplastic oral epithelium biopsies with clinicopathological factors were investigated.

Methods: We examined the coordinate mRNA expression of cytokeratin 7 (*CK7*) and cytokeratin 20 (CK20) using quantitative reverse transcription polymerase chain reaction (qRT-PCR) in 110 biopsies of oral squamous epithelium samples including 72 tumoral and 38 dysplastic biopsies. We also collected demographic and pathological data including tumor stage and grade from our patients.

Results: There was a significant difference in CK7 and CK20 gene expression between OSCC and dysplastic samples (p< 0.001). Further, their mean expression in OSCC samples was significantly higher compared to dysplastic samples. Relative mRNA levels of CK7 and CK20 showed that their mean expression in OSCC grade I was significantly lower than other grades (p< 0.01). The relationship between CK7 and CK20 mRNA expression and age or gender was not significant (p> 0.05). Samples in the advanced stage of disease had significantly higher CK7 and CK20 expression compared to early-stage samples of OSCC specimens (p= 0.001).

Conclusions: We found an increase in *CK7* and *CK20* mRNA levels in grade III OSCC samples compared to other grades. This finding suggests a potential role for *CK7* and *CK20* in oral mucosal carcinogenesis and OSCC prognosis.

Keywords: *CK20*, *CK7*, *Clinicopathological Indices*, Cytokeratin, Gene expression, Oral Epithelial Dysplasia, Oral Squamous Cell Carcinoma.

Introduction

Oral squamous cell carcinoma (OSCC) accounts for over 90% of oral neoplasms and is considered the world's top eight cancers in terms of incidence (1). The high mortality seen in cases

associated with OSCC could be attributed to systemic metastasis and local annual recurrence (2). Most oropharyngeal tumors, considered OSCC, arise from previous lesions called oral dysplastic tissues that have the potential to induce malignant disorders (3, 4).

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Received: 30 Nov, 2020; Accepted: 19 Dec, 2020

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The categorization of OSCC patients depending on the prognosis and the pathological findings are indications of therapeutic modalities. Efficacies of new modalities in cancer therapies with the preference of surgical resections have been proven (5-7). However, despite the advances in predictive values from OSCC therapies, additional novel biological markers may be required.

Cytokeratins (CK) are of a group antigenically structural filaments specific to epithelium that are preserved in metastasis and malignant transformations (7, 8). Thus, CK plays an important role in the prognosis of metastasis or malignancies. Previous studies demonstrate that CK7 and CK20 could help to determine the primary site of metastatic carcinomas (9). Other investigations also established the individual role of CK7 and CK20 or their coordinate role (CK7+/CK20+) in various cancers including colorectal, pancreatic, thymic, liver, ovarian, bladder and gastrointestinal adenocarcinomas, bronchoalveolar carcinoma and Merkel cell skin tumors (10).

Some investigations have declared that most squamous cell carcinomas (SCC) exhibit a negative *CK7* or *CK20* expression profile (11), however, there may be differences in the coordinate expression of *CK7* and *CK20* in other epithelial tumors (10).

This study aimed to elucidate the difference in co-mRNA expression of *CK7* and *CK20* between malignant and benign OSCC tumors compared to dysplastic tissues to provide a more accurate diagnostic marker for OSCC tumors.

Materials and Methods

Sample Collection

This retrospective analytical study was conducted on a total of 110 biopsy samples, specifically 72 OSCC tumoral and 38 epithelial dysplastic tissues. Samples were collected from the Pathology Department and Faculty of Dentistry at Mashhad University of Medical Sciences (MUMS) in Iran. Laboratory analyses were completed in the molecular pathology and cytogenetic laboratory within the Faculty of Medicine of MUMS. Patients consent form was signed by all study

participants. Demographic information including age, sex and alcohol, smoke and drug consumption were obtained. Related pathology documents of these samples including the degree of tumor grade and stage of tumor based were also collected. Tumor stage was determined using the tumor-nodemetastasis staging system (12), where the grade I and II was considered early, while grade III and VI were considered advanced (12). All fixed samples in 10% formalin were cut uniformly with a microtome to ensure 5µm thickness and stained with hematoxylin and eosin (H&E) for histopathological grading. For laboratory analysis, each sample was deparaffinized by Xylene and then transferred to 96% Ethanol. The Ethics Committee of MUMS approved all experimental procedures prior to the beginning of this study.

RNA extraction and cDNA synthesis

To extract mRNA from each sample and evaluate the expression of *CK7* and *CK20*, we performed quantitative reverse transcription polymerase chain reaction (qRT-PCR) according to the manufacturer's instructions (High Pure RNA Paraffin Kit, FFPET RNA Tissue; Roche, Germany). A NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, Thermo, USA) was used to evaluate the purity of extracted mRNA using an absorbance ratio of 260 nm/280nm.

Complementary DNA (cDNA) was synthesized using the Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). The cDNA synthesis protocol included a 20 μ L reaction containing 5x Reaction Buffer (4 μ L), Ribolock RNase inhibitor (1 μ L), 10 mMol dNTP Mix (2 μ L) and Reverse Transcriptase (1 μ L) under ABI thermocycler (One Step, USA). Finally, the quality and concentration of cDNA for each sample was checked using a NanoDrop 2000 spectrophotometer.

qRT-PCR Analysis

We performed qRT-PCR, to evaluate *CK7* and *CK20* expression, using the SYBR Green master mix kit (Thermos Scientific, Germany) on an

ABI thermocycler (One Step, USA) with appropriate primers (Table 1). All reactions were run in duplicate in separate wells and contained 20 µL mixture, where each reaction contained 0.5 µM (10 Pico molar) of forward primer, 0.5 μM (10 Pico molar) of reverse primer, 10 μM SYBR Green master mix, 7 µM Diethyl pyrocarbonate water, and 2 µL of DNA extract (concentration of 4 ng/ml). The PCR experiment began with one cycle at 94 °C for ten seconds (holding process), followed by 40 amplification cycles at 94 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds. The final amplification occurred at 72 °C for 30 seconds. We conducted the $\Delta\Delta$ CT method for differential gene expression of CK7 and CK20 and used the Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) housekeeping gene as a reference (Figs. 1 and 2) (13).

Table 1. Primers sequence genes

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Gene		Sequence 5'-3' (position)		
CV7	Forward	5'- GTTCCATTTGCAAAGGCTGT- 3'		
CK7	Reverse	5'- CAGGTGGTTACCCGAAAGA -3'		
CV20	Forward	5'- GGAAGTCGATGGCCTACACAA -3'		
CK20	Reverse	5'- GGCCTGGAGCAGCATCAA -3'		
GAPDH	Forward	5'-CCC ATC ACC ATC TTC CAG G-3'		
	Reverse	5'-CAT CAC GCC ACA GTT TCC C-3'		



Fig. 1. Amplification plot for Ck7, Ck20 and GAPDH expression.

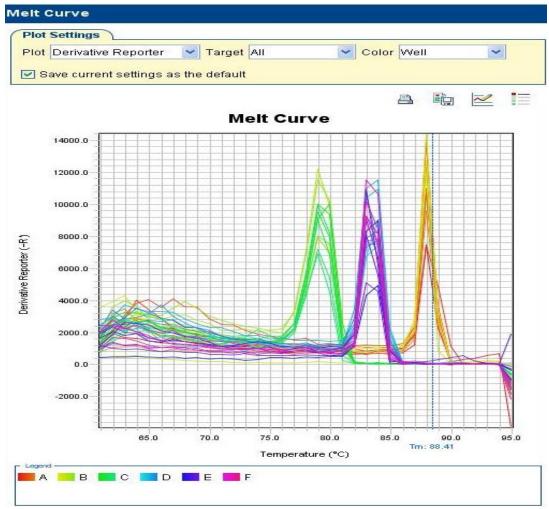


Fig. 2. Melt curve for Ck7, Ck20, GAPDH expression.

Statistical analysis

We performed statistical analysis using SPSS software version 16 (SPSS Inc., Chicago, IL, USA). Descriptive data including age, sex and mRNA expression were presented as a mean with the standard error of the mean. The normality of data was checked using a one-Sample Kolmogorov-Smirnov test. mRNA expressions of the CK7 and CK20 genes were compared to tumoral and dysplastic tissues using an independent samples t-test. Evaluation of CK7 and CK20 expression in tumor and dysplastic tissues and its relationship clinicopathological parameters including tumor stage and histological grade were assessed using a one-way ANOVA test. A Pearson's correlation coefficient test was used to investigate the correlation between CK7 and CK20 expression levels in tumors

dysplastic tissues and demographic information including age and gender. A p-value of less than 0.05 was considered statistically significant.

Results

Studied Patients characteristics

Our experiments included a total of 110 samples, specifically 38 dysplastic samples and 24 samples each of all three OSCC grades (I, II, and III). There were 59 female and 51 male patients with a mean age of 53.48±15.18 years. The patient's demographic features that were used to reveal any differences between the OSCC and dysplastic samples were depicted in Table 2. The differences, including age, gender, and consumption of smoke, alcohol, and drugs, were not significant (p> 0.05).

Table 2. Demographic features of studied participants.

Variables		Dysplastic group Number (%)	OSCC group Number (%)	p-value
Age	<60 years >60 years	18 (16.33) 20 (18.18)	42(38.18) 30 (27.27)	0.14
Gender	Female Male	20 (18.18) 18 (16.33)	39(35.45) 33 (30)	0.55
Smoking consumption	No Yes	24 (21.18) 14 (12.72)	42 (38.18) 30 (27.27)	0.17
Alcohol consumption	No Yes	23 (20.90) 15(13.63)	49 (44.54) 23 (20.90)	0.36
Drug consumption	No Yes	18 (16.33) 20 (18.18)	40 (36.36) 32 (29.09)	0.27

Squamous cell carcinoma of oral cavity (OSCC); p-value was calculated based on chi-square test.

Evaluation of CK7 and CK20 Expression in tumoral and dysplastic mucosa specimens

Table 3 illustrates the differential expression of *CK7* and *CK20* between dysplastic and OSCC sampled tissues. The results showed that *CK7* expression was significantly higher in OSCC

tumor tissues than dysplastic mucosa sampled tissues (p= 0.001, Table 3). Further, there was a significant difference in *CK20* expression between tumor tissues compared to the dysplastic tissues which had a significantly higher expression of CK20 (p= 0.001, Table 3).

Table 3. Expression level of *CK7* and *CK20* studied genes between two studied groups based on QRT analysis.

Variables	Dysplastic group Mean±SE	OSCC group Mean±SE	p-value
CK7 Expression	0.33±0.25	3.75±1.73	0.001*
CK20 Expression	0.47±0.26	5.12±2.48	0.001*

SE; standard error of mean, Squamous cell carcinoma of oral cavity (OSCC); p-value was calculated based on Mann-Whitney test and*less than 0.05 considered as significant level.

Association of studied cytokine expression (CK7, CK20) with demographic and pathological features

We first examined the correlation between *CK7* expression and demographic and histopathological characteristics, including grade and stage of tumors, in tumoral and dysplastic samples (Table 4 and 5). Between both groups, there was no significant difference between *CK7* expression and age (p= 0.14) or gender (p= 0.55). Next, we found that patients with higher OSCC development (grade III) experienced higher *CK7* expression than in grade I and grade II patients respectively (Table 5). However, these correlations were not significant between grade I (Spearman's correlation test, p= 0.18,

r= 0.36) and grade II patients (Spearman's correlation test, p= 0.24, r= 0.32). The correlation between CK7 expression and the stage of studied tumors, however, was found to be significant (p= 0.001). Patients in the advanced stage of disease had a significantly higher expression of CK7 compared to OSCC patients in the early stages or patients with dysplastic tissue (p= 0.001).

In addition to CK7, we examined the expression of CK20 and its correlation to demographic and pathological characteristics in OSCC and dysplastic tissues (Table 4). Between both groups, we found that there was no significant CK20difference between expression and age (p> 0.05) or gender (p> 0.05). However, we found that CK20

expression differed significantly among the three OSCC grades (p= 0.001). Patients in grade III experienced a higher *CK20* expression than in grade I and grade II patients (Table 5). However, this finding was not significant between grade I and grade II patients (p> 0.05). Our results were significant

regarding CK20 expression and the stage of studied tumors (p= 0.001). Lastly, our results showed that patients in the advanced stages of OSCC present significantly higher expression of CK20 compared to OSCC patients in the early stages and patients with dysplastic tissue (p= 0.001).

Table 4. Association of expression of *CK7* and *CK20* genes with age and gender.

Gene	Variables		Dysplastic group		OSCC group Mean±SE		
			Mean±SE	Grade I	Grade II	Grade III	
СК7	Age, p-value correlation)	e (Pearson	0.66 (0.12)	0.93 (0.02)	0.15 (0.39)	0.96 (0.01)	
	Gender	Female Male	0.23±0.15 0.45±0.29	2.68±0.43 2.62±0.38	2.99±0.63 2.69±0.52	5.32±1.32 6.14±1.72	
	p-value		0.09	0.75	0.33	0.33	
	Age, p-value correlation)	e (Pearson	0.60 (0.14)	0.46 (0.20)	0.08 (0.46)	0.92 (0.02)	
СК20	Gender	Female Male	0.44±0.19 0.50±0.33	4.22±1.18 3.41±0.57	4.22±1.47 3.55±0.88	7.07±3.06 8.16±2.05	
	p-value		0.67	0.10	0.30	0.42	

^{*}p-value less than 0.05 was considered as significant level. SE; standard error of mean, Squamous cell carcinoma of oral cavity (OSCC).

Table 5. Association of expression of *CK7* and *CK20* genes between two studied groups with pathological features including grade and stage of disease.

Gene	Variable	s	Gene expression Mean±SE	on p-value	
		Dysplastic tissue	0.33±0.25		
CK7	Grade	Grade I	2.65±0.39	0.001*	
		Grade II	2.85 ± 0.59		
		Grade III	5.76 ± 0.55		
		Dysplastic tissue	0.33±0.25	0.001*	
	Stage	Early stage	2.79±0.10		
		Advance stage	4.52±0.39		
СК20		Dysplastic tissue	0.47±0.26		
	Grade	Grade I	3.79±0.97	0.01*	
		Grade II	3.90 ± 1.24		
		Grade III	7.65±2.54		
	G	Dysplastic tissue	0.33±0.25	0.00*	
	Stage	Early stage	4.14±0.24	0.001*	
		Advance stage	5.88±0.59		

^{*}p-value less than 0.05 was considered as significant level. SE; standard error of mean, p-value calculated based on one-way ANOYA test.

Discussion

The overexpression of CK, a group of epithelium filaments, was reported amongst different cancers (7, 8). Among 20 specified types of CK, the individual expression of *CK7* and CK20 or their co-expression was also reported (10). However, their co-expression in salivary glands and OSCC tumor tissue and how it relates to pathologic markers is much less understood (11, 14, 15). Therefore, further investigation is required to elucidate the diagnostic potential of *CK7* and *CK20* co-expression in OSCC tumors.

According to previous investigations, salivary gland tumors were assessed for their CK7/20 profile. They found that all salivary gland neoplasms had a CK7+/CK20- immune profile while squamous cell carcinomas (SCC) samples showed negative CK7/20 expression. The authors conclude that these profiles cannot discriminate between the different types of salivary gland neoplasms or the differences between benign and malignant salivary gland tumors (16).In rare cases spindle cell squamous cell carcinoma of the tongue, immunohistochemical analysis revealed that tumor cells were positive for some pancytokeratin (17). In the case of a lower left gingival tumor, CK7 expression was found positive in a mixture of SCC and tubular formation. However, CK7 was found to be negatively expressed in their margin tissues (18). There were also reports about the negative expression CK7 CK20 of or basaloid squamous cell carcinoma (19) and Adenoid squamous cell carcinoma (20, 21). Recently, however, SCC tissues were identified by the positive expression of CK7 in dysplastic and invasive lesions of luminal and basal tissues (6). Chue et al. found that CK7 expression was positive in most carcinoma cases except colon, prostate, kidney and thymus carcinomas, lung carcinoid tumors, gastrointestinal tract and Merkle cell carcinoma (6). CK7 expression was positive in all salivary glands, however, CK20 expression was negative (6). This study aligns with our results with regards to CK7 expression in the salivary gland but differed for CK20 expression. These results could be attributed to

a difference in protocol between the studies and the lack of SCC samples in the assay conducted by Chue et al (6).

Abdul-Maksoud et al. evaluated CK20 expression in 80 samples with bladder cancer using qRT-PCR and compared the results to healthy samples (7). They investigated 54 samples of transitional cell tumoral tissue as the invasive group and SCC tissue as the noninvasive group (20). Similar to our study, they found that increased CK20 expression was associated with disease stage and grade. They concluded that the detection of CK20 had a predictive prognostic value differentiating between invasive and noninvasive carcinoma. Though their study results were like our findings, we both investigated different CK7 and CK20 genes in OSCC tissues. Lastly, the expression of CK19 and CK20 was investigated to improve bladder cancer screening and diagnosis (22). Morsi et al. found that CK19 and CK20 were the best candidates as they provided the highest sensitivity and specificity (22).

In this current study, we first examined the correlation between the coordinate expression of CK7 and CK20 on the RNA level to different histological grades and stages. We then investigated the presence of non-OSCC specimens in dysplastic specimens and OSCC samples with different histopathological grades. While our study used a large sample size, there were a few limitations including the absence of healthy individuals to measure CK7 and CK20 expression levels, or their expression in adjacent tumors tissues. The association between prognosis, tumorigenicity and survival rate of OSCC patients to the expression of CK7 and CK20 may be of interest for further investigations.

We concluded that the expression of *CK7* and *CK20* was significantly higher in OSCC tumor samples compared to dysplastic samples. This finding could be due to the amplification role of these two markers during carcinogenic processes of oral mucosa cancers. This significant difference was also found to be related to histopathological grade, where

patients with higher development in grade III experienced a higher expression level of both *CK7* and *CK20* in comparison to grade I and grade II patients.

Lastly, mRNA expressions of *CK7* and *CK20* were significantly higher in advanced stages compared to earlier stages of OSCC tissues. Therefore, measuring the coordinate expression levels of *CK7* and *CK20* could serve as a potential prognostic biomarker and

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improve the diagnosis of higher grades of OSCC tumor tissue.

Acknowledgements

The authors appreciate the Research Council of Mashhad University of Medical Sciences, Faculty of dentistry for their financial support under thesis number 970702.

The authors declare that there are no conflicts of interest.

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