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ORIGINAL ARTICLE

Overexpression of cytokeratin 17 protein in oral squamous cell carcinoma *in vitro* and *in vivo*

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OBJECTIVE: To determine the cytokeratin 17 (CK17) expression in oral squamous cell carcinoma (OSCC) both *in vitro* and *in vivo*.

METHODS: Comparative proteomic analysis of an *in vitro* cellular carcinogenesis model of OSCC (including a line of human immortalized oral epithelia cells (HIOECs), a line of cancerous HB96 cells and another kind of cells (HB56 cells) at the early stage of carcinogenesis was performed to identify differentially expressed proteins. CK17 was further validated *in vitro* (cellular carcinogenesis model and other three OSCC lines) and *in vivo* (tissues from six healthy persons and 30 primary OSCC patients) by Western blotting and immunohistochemistry respectively.

RESULTS: Increased CK17 expression was identified by two-dimensional gel electrophoresis and liquid chromatography–tandem mass chromatography in the HB56 and HB96 cells over HIOECs. Western blotting confirmed the increased CK17 expression in the HB56, HB96 cells and other three OSCC lines. Immunohistochemistry confirmed the increased CK17 expression in the cancerous tissues from OSCC patients compared with the paired adjacent non-malignant epithelia.

CONCLUSION: Increased CK17 expression may play an important role in the carcinogenesis progression of OSCC; however, further studies on the molecular function of CK17 are encouraged to clear the precise mechanism of CK17 in OSCC.

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Keywords: oral squamous cell carcinoma; cytokeratin 17; cellular carcinogenesis model; proteomics

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Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common malignancies in the head and neck region, afflicting about 300 000 patients worldwide each year (Petersen, 2003; Kademani, 2007). For many years, the 5-year survival rate of OSCC patients has remained relatively low, only approximately 50–60% (Neville and Day, 2002; Parkin *et al*, 2005) and the rate is even lower in patients diagnosed at later stages. Although early diagnosis and efficient treatment are the keys to the prognosis of OSCC, up to date, specific biomarkers for early diagnosis and prognostic monitoring of OSCC are lacking.

In recent years, proteomics has emerged as a powerful technology to identify differential protein expressions associated with cancer development and progression. Its two most popular and reliable techniques, two-dimensional gel electrophoresis (2-DE) and mass spectrometry, have been widely used in many fields of scientific research, especially in life science (Jung *et al*, 2000). Furthermore, *in vitro* cellular model is an important object in understanding cellular events related to pathological or physiological conditions in humans. It is an indispensable study tool in researching for molecular mechanisms, because of its homogeneity of cell population, accessibility, reproducibility, controllable growth rate and hence enough amount of material for analysis (Lenaerts *et al*, 2007).

Previously, we established a stable line of human immortalized oral epithelial cells (HIOECs) by transfecting normal epithelial cells with HPV16 E6/E7 gene (Sdek *et al*, 2006) and then subsequently derived it into a cancerous cell line (HB96) by treating with benzo[a]pyerene for 6 months (Zhong *et al*, 2008). Together with another kind of cells (HB56 cells) at the early stage of carcinogenesis, these three kinds of cells constituted the three major components of the *in vitro* cellular carcinogenesis model of OSCC. On the basis of this cellular carcinogenesis model, we compared the protein profile by comparative proteomic analysis and found a panel of differentially expressed proteins; we further validated several significantly upregulated proteins both *in vitro*

and *in vivo*, for their potential usefulness as clinical markers of OSCC. cytokeratin 17 (CK17) was chosen for such validation.

Cytokeratin 17, being a component of cytoskeleton protein with a low-molecular weight of 48.1 kDa and 432 residues, is an acid-type I cytokeratin with an isoelectric pH = 4.7. The CK17 gene has seven introns and eight exons covering approximately 5.14 kbp on 17q12-q21 with the transcript length of 1299 bp. The main function of CK17 is involved in the formation and maintenance of various skin appendages, specifically in determining shape and orientation of hair. CK17 is also considered a marker of basal cell differentiation in complex epithelia and recognizes cervical stem cells (Martens *et al*, 2004). On the aspect of carcinogenesis or cancerization, the detailed molecular mechanism of CK is still unclear. It is mainly expressed by squamous, basal, transitional cell carcinomas and adenocarcinomas with squamous differentiation (Miettinen *et al*, 1997). The expression of CK17 protein has been found increased in cancerous tissues compared with normal tissues in cervical squamous cell carcinoma (Carrilho *et al*, 2004; Ikeda *et al*, 2008), laryngeal squamous cell carcinoma (Cohen-Kerem *et al*, 2002, 2004), oesophageal squamous cell carcinoma (Takahashi *et al*, 1995; Luo *et al*, 2004) and lung cancers (Wetzels *et al*, 1992). For oral cancer, originated from squamous cell epithelia, there are two reports describing the CK17 expression, one is a transcriptomic analysis of clinical samples from tongue squamous cell carcinoma, which reports some upregulated genes, including CK17 (Ye *et al*, 2008); another is a correlation analysis on CK17 expression and oral submucous fibrosis, which reports a positive correlation between CK17 expression and disease severity in 28 clinical tissue samples (Lalli *et al*, 2008). To our knowledge, there are few reports on the expression of CK17 protein encompassing OSCC in both of the *in vitro* cellular carcinogenesis model and *in vivo* clinical tissue samples at the same time. In this study, we first identified the increase in CK17 protein expression in the HB56 and HB96 cells over the HIOECs by comparative proteomic analysis and then further validated the CK17 protein expression *in vitro* and *in vivo*, as well as its potential clinical value for OSCC.

Materials and methods

Cell cultures

Several OSCC cell lines were used throughout in this study. HIOECs, HB56 and HB96 cells were previously described as three major components of our *in vitro* cellular carcinogenesis model of OSCC (Sdek *et al*, 2006; Zhong *et al*, 2008) and three OSCC cell lines of Tca8113, TSCC and CAL27. Tca8113 cell line was established in our laboratory; TSCC was established by Wuhan University, China and was received as gift; CAL27 was purchased from ATCC (Manassas, VA, USA). HIOECs were cultured in the defined keratinocyte-SFM (Gibco, Grand Island, NY, USA). HB96 and CAL27 cells were cultured in DMEM (Dulbecco's modified Eagle's medium; Gibco) supplemented with 10% fetal bovine serum, 1% glutamine and 1% penicillin-streptomycin. Tca8113 and TSCC cells were cultured in the RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum, 1% glutamine and 1% penicillin-streptomycin. All cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C.

Clinical tissue samples

From February 2007 to July 2007, thirty primary OSCC patients without prior radiotherapy or chemotherapy were enrolled into this study. They were 21 males and nine females, aged 31–84 years with a mean of 53.8 years. After signing the informed consent forms which was approved by Ethical Committee of Ninth People's Hospital, School of Medicine, Shanghai Jiao Tong University, they underwent radical surgery at the Department of Oral and Maxillofacial Surgery, Ninth People's Hospital, School of Medicine, Shanghai Jiao Tong University. Surgical tissue samples including cancerous tissue and adjacent non-malignant epithelium were collected by procedures previously described (Zhong *et al*, 2007a,c). The adjacent non-malignant epithelia were collected at sites at least 2 cm away from the edge of tumor mass, with best efforts to avoid contamination by the tumor cells. The sites of primary carcinoma were the tongue (n = 17), buccal mucosa (n = 4), retromolar region (n = 3), floor of mouth (n = 3), gingiva (n = 2) and palatoglossal arch (n = 1). The stage of disease was determined according to the tumor-node-metastasis staging system of the International Union Against Cancer (Sobin and Wittekind, 2002). The histological grade of the tumor was determined according to the degree of differentiation in the WHO histological criteria (Barnes *et al*, 2005). Normal oral mucosa tissues from six healthy persons (with informed consent forms) were used for immunohistochemical staining for CK17.

2-DE and liquid chromatography–tandem mass chromatography (LC-MS/MS)

Human immortalized oral epithelia cells and HB cells at 80% confluence were lysed in a 300 μl of ice-cold lysis buffer containing 8 M urea, 65 mM DTT, 4% w/v CHAPS, 40 mM Tris and freshly prepared 100 μg/ml of phenylmethanesulfonyl fluoride. They were sonicated for 10 times (15 s each) and centrifuged at 12 000 g for 90 min. The concentration of crude proteins in the supernatant was determined with Bradford protein assay reagent (Bio-Rad protein Dye assay reagent; Bio-Rad, Hercules, CA, USA). The 17 cm pH3-10 IPG strips (Bio-Rad Cat. No. 163–2009) were subjected to isoelectric focusing (IEF) following the manufacturer's instructions with modifications. Isoelectric focusing was run at 250 V for 30 min at linear mode, followed by 1000 V for 60 min at rapid mode, 10 000 V for 5 h at linear mode, 10 000 V for 6 h at rapid mode and 500 V at linear mode. After IEF, strips were equilibrated for 2×15 min at room temperature in equilibration buffers (6 M urea, 30% glycerin, 2% sodium dodecyl sulfate (SDS), 375 mM Tris–HCl pH 8.8), first with supplemented 2% DTT and then with 2.5% iodoacetamide to

replace DTT. Equilibrated IPG strips were transferred onto 12% uniform polyacrylamide gels and the second-dimensional SDS-polyacrylamide gel electrophoresis (PAGE) was performed with a Hofer SE 600 Ruby System (Amersham Biosciences, Uppsala, Sweden). The constant current was first 5 mA/gel and then 30 mA/gel until the bromophenol blue frontier reached the bottom of gels. The gels were stained by $AgNO_3$ for 20 min and scanned by the Bio-Rad GS710 scanner.

Following the automated protein localization, gel spots were excised by a Bio-Rad spot cutter controlled by BIO-RAD PDQUEST software version 7.3.0. Excised gels were triturated and washed with ammonium bicarbonate and acetonitrile, digested with trypsin. The peptide solutions were dried by vacuum centrifugation, desalted and cleaned using a C18 Ziptip (Millipore, Billerica, MA, USA). Peptide mixtures were separated and identified by a Finnigan LTQ mass spectrometercoupled with a Surveyor HPLC system (ThermoQuest, San Jose, CA, USA). Protein identification using MS/MS raw data was performed with SEQUEST program in the BIOWORKS 3.1 software suite (University of Washington, licensed to Thermo Finnigan) based on the IPI Human database version 3.15.1 (containing 58 099 entries). Trypsin was selected as protein cleavage specificity. Protein identification results were filtered with the Xcorr (≥ 1.9 for a 1+ tryptic peptide, ≥ 2.2 for a 2+ tryptic peptide, ≥ 3.75 for a 3+tryptic peptide) and ΔCn (≥ 0.1).

Western blot analysis

Cultured cells grown to 80% confluence were lysed in ice-cold 2× lysis buffer containing 125 mM Tris–HCl (pH 6.8), 5% w/v SDS and 24.75% glycerol and subjected to total protein extraction according to standard procedures. After concentration determination by the Bradford assay (BCA™; PIERCE, Rockford, IL, USA), protein samples (50 μg/lane) were separated by 12% SDS-PAGE and then electrophoretically transferred onto polyvinylidene difluoride membranes using a wet transfer system (Invitrogen, Carlsbad, CA, USA). The membranes were blocked with blocking buffer containing 5% dry milk in phosphate-buffered saline (PBS) with 0.1% Tween 20 for 2 h and incubated at 4°C overnight with anti-mouse CK17 monoclonal antibody (clone E3; Millipore) at 1:200 dilution. After washing, the blot was then incubated with fluorescent-conjugated anti-mouse IgG secondary antibody (Fermentas, Vilnius, Lithuania) at 1:1000 dilution for 1 h. Finally, the immunoreactive bands were scanned and analysed using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). β-actin was used as internal control protein.

Immunohistochemical analysis

Thirty pairs of tissue samples from each patient, including cancerous tissue and adjacent non-malignant epithelium, were detected using immunohistochemical staining for CK17. The procedure of immunohistochemistry was according to the method we previously described (Zhong *et al*, 2007a,b). Briefly, after deparaffinization and endogenous peroxidase blocked, the sections were heated by water bath at 98°C with 0.01 M citrate buffer solution (pH 6.0) for 20 min, then incubated with the mouse monoclonal antibody to CK17 (ABcam, Cambridge, UK) at 1:100 dilution overnight at 4°C, and visualized using 3,3′-diaminobenzidine (DAB) detection kit (Dako Cytomation, Glostrup, Denmark). The 1:100 dilution was the best dilution compared with 1:50 and 1:200. Negative control was prepared using PBS instead of antibody. Microscopic examination was performed by two pathologists, and all samples were blinded. The CK17 positive grade was determined based on the proportion of stained cells on a scale of negative to strong as follows: negative, 0% of stained cells with the grade of 0; weak, 1–25% of stained cells with grade 1; moderate, 26–50% of stained cells with grade 2; and strong, more than 50% of stained cells with grade 3.

Statistical analysis

All data were analysed by the statistical software SPSS 10.0 for Windows (SPSS Inc., Chicago, IL, USA). The statistical difference of the initial data was analysed by the nonparametric tests. When the P-value was < 0.05, the difference was regarded as statistically significant.

Results

2-DE and LC-MS/MS

Using 2-DE, we compared the protein expression profiles among HIOECs, HB56, and HB96 cells. There were 54 spots in the 2-DE gel representing proteins up- or downregulated among these cells. Using mass spectrometry, we identified 45 differentially expressed proteins. Of those, 26 different proteins were from HIOECs, three proteins from HB56 cells and 19 proteins from HB96 cells (detail in Table 1). Among them, one protein significantly upregulated in HB cells was marked by the circles in Figure 1 and later identified by LC-MS/MS as CK17 protein.

CK17 protein expressions in a panel of OSCC cell lines

Western blot analysis revealed that CK17 protein expression all increased in the HB56, HB96, Tca8113, TSCC and CAL27 cells compared with the level in HIOECs upon normalization against β-actin protein signal (Figure 2). For the *in vitro* cellular carcinogenesis model of OSCC, CK17 expression pattern by Western blotting was consistent with that obtained form the comparative proteomic analysis.

CK17 protein expressions in primary OSCC tissue samples

Immunohistochemistry showed the CK17 protein expression predominantly in the cellular cytoplasm. Cancerous tissues from OSCC patients showed positive reactivity to CK17 of grade 1 in 10.0% (3/30) cases, grade 2 in 40.0% (12/30) cases and grade 3 in 50.0% (15/30) cases. However, adjacent non-malignant epithelia showed negative reactivity in 20.0% (6/30) cases, positive reactivity of grade 1 in 46.7% (14/30) cases, grade 2 in 26.7% (8/30) and grade 3 in 6.7% (2/30)

Table 1 The differentially expressed proteins identified in the human immortalized oral epithelia cells (HIOECs), HB56 cells and HB96 cells

Name of cells	*Abbreviations of gene names according to the identified differentially expressed proteins*
HIOECs	ACP1, ANXA1, ECH1, ETHE1, GLO1, GRB2, HCC1, ISG15, CK7, NDUFV2, PCBP2, PFN2, PRDX1, PSMB3,Q8N849, RANBP1, RPS12, SOD2, S100A6, S100A8, TPD52L2, TPI1, TXNL4A, VBP1, ZYX, 44 kDa protein-ENSP00000319797
HB56 cells	ANXA2, CFL, CK17
HB96 cells	ANXA2, CAPZA1, CTSB, EEF2, ERH, GAPDH, GNB1, LASP1, LGALS1, M6PRBP1, PDHB, PFN2, RANBP1, RPP2, SERPINB5, STMN1, TUBB2C, TUFM, UCHL1

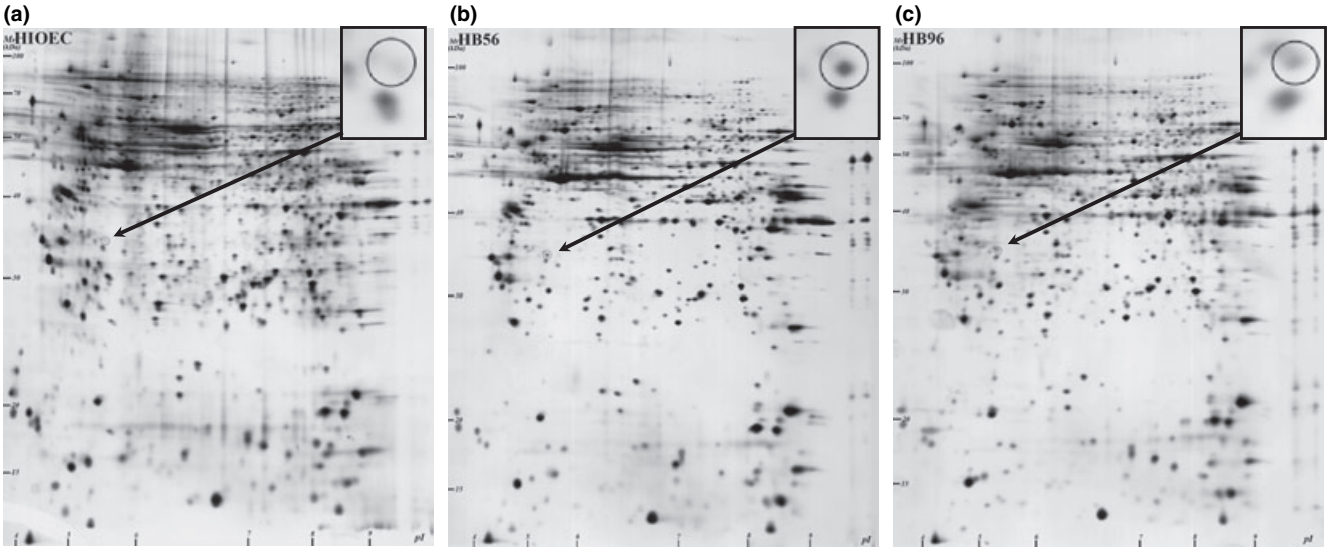

Figure 1 (**a**) Two-dimensional gel electrophoresis (2-DE) electrophotograms of the protein from human immortalized oral epithelia cells. (**b**) 2-DE electrophotograms of the protein from HB56 cells. (**c**) 2-DE electrophotograms of the protein from HB96 cells. The IEF was pH 3–10. The differentially expressed protein spots of cytokeratin 17 in the three kinds of cells were marked with ring

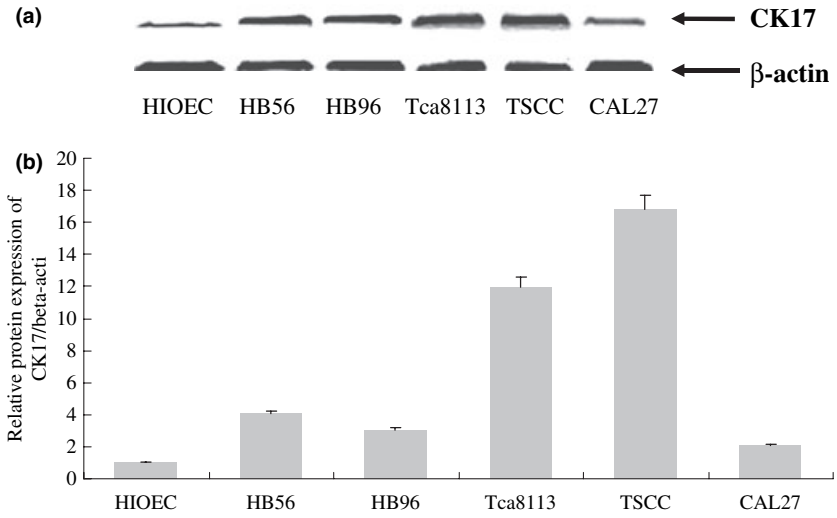

Figure 2 Western blot analysis of cytokeratin 17 (CK17) protein expression in the human immortalized oral epithelia cells, HB56 cells, HB96 cells and other three oral squamous cell carcinoma cell lines. The relative level of CK17 protein expression for each sample was normalized against β-actin. Increased CK17 protein expression was found in the HB56 cells, HB96 cells, Tca8113 cells, TSCC cells and CAL27 cells

(Table 2). The positive rate of CK17 protein in the cancerous tissues was significantly higher than that in the adjacent non-malignant epithelia ($\chi^2 = 23.859$, $P < 0.001$). The immunoreactions of CK17 protein expression in the cancerous tissues were consistently stronger than those in the paired adjacent non-malignant epithelia (Wilcoxon Signed Ranks Test, $Z = -3.892$, $P < 0.001$) (Figure 3). In the normal oral

Table 2 The immunohistochemical cytokeratin 17 (CK17) positive grade in the different types of tissues from oral squamous cell carcinoma patients

Type of tissue	*Total cases*	*CK17 positive grade*			
		0	*1*	*2*	*3*
Adjacent non-malignant epithelia	30	6	14	8	2
Cancerous tissue	30	0	3	12	15

Chi-square test, the positive rate of CK17 protein in the cancerous tissues was significantly higher than that in the adjacent non-malignant epithelia, $\chi^2 = 23.859$, $P < 0.001$.

mucosa tissues from healthy persons only the supra-basal layer of mucosa might be positive for CK17 staining (Figure 3e). The difference of CK17 protein positive score in cancerous tissues was analysed by classification of different clinicopathological characteristics of OSCC patients. Unfortunately, no significant difference was found between the CK17 protein expression and the T stage ($P = 0.576$), N stage ($P = 0.403$), clinical stage ($P = 0.554$), pathological differentiation grade ($P = 0.107$), smoking ($P = 0.486$), and drinking ($P = 0.359$) (Table 3).

Discussion

The carcinogenesis mechanism of OSCC is very complex and remains poorly understood. Based on our *in vitro* cellular carcinogenesis model of OSCC including HIOECs, HB56 and HB96 cells (Sdek *et al*, 2006; Zhong *et al*, 2008), 45 differentially expressed proteins representing different stages of OSCC are identified by comparative proteomic analysis. Of the identified proteins, CK17 protein shows significant upregulation in cancerous HB cells, both HB56 and HB96 cells. Western blot analysis confirms its increased expression of CK17 protein *in vitro* in the cellular carcinogenesis model of OSCC similar to the results obtained by comparative proteomic analysis. Increased expression of CK17 protein is also confirmed by Western blotting in other three OSCC cell lines. With clinical tissue samples from primary OSCC patients, the cancerous tissues also show a significant increase in CK17 protein expression *in vivo*, compared with the adjacent non-malignant epithelia. This is also consistent with the result of the comparative proteomic analysis. To our knowledge, this is the first report of increased expression of CK17 protein in OSCC both *in vitro* and *in vivo*.

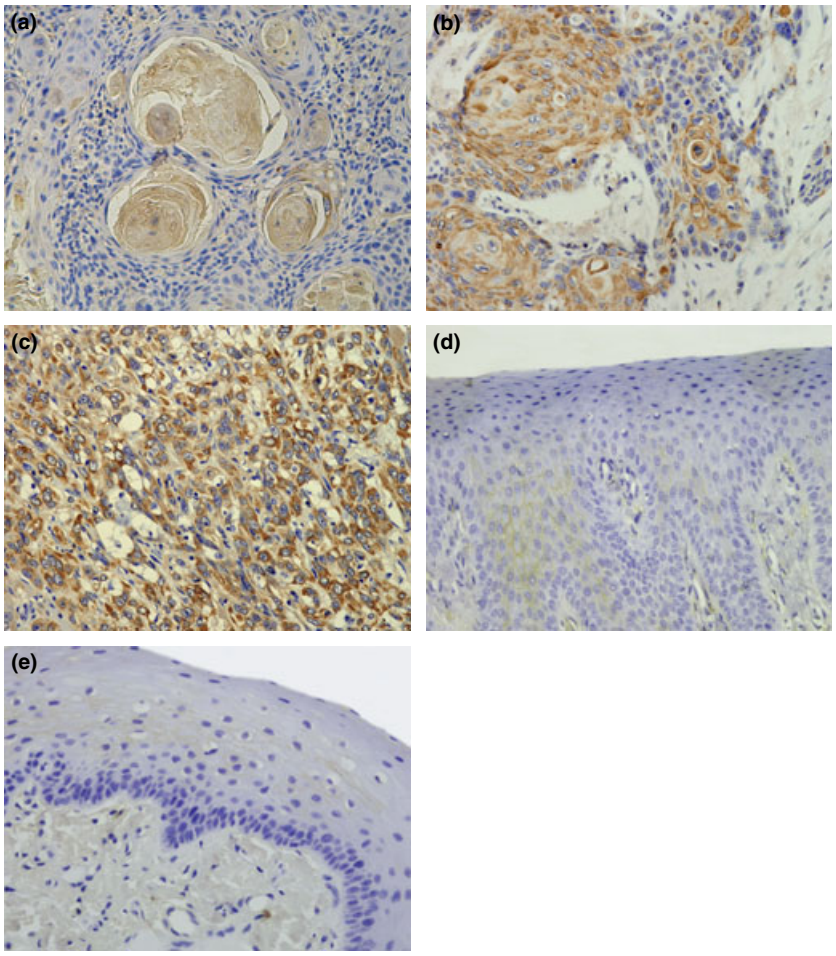

Figure 3 Immunoreactions of cytokeratin 17 (CK17) protein expression in the cancerous tissues and non-malignant epithelia from oral squamous cell carcinoma patients and the normal oral mucosa from healthy persons. (**a**) Well differentiated, (**b**) moderately differentiated, (**c**) poorly differentiated cancerous tissues, (**d**) non-malignant epithelia, (**e**) normal oral mucosa (×200). The positive staining of CK17 protein was in the supra-basal layer of non-malignant epithelia and normal oral mucosa. The positive grade of CK17 expression was obvious higher in the cancerous tissues than non-malignant epithelia. The location of the CK17 protein immunoreaction was cellular cytoplasm

Table 3 The correlation between the positive grade of cytokeratin 17 (CK17) protein expression and the clinicopathological characteristics of oral squamous cell carcinoma patients

Classification	*Case no.*	*Cancerous protein positive grade*	*Nonparametric tests value*	P-*value*
Smoking				
Yes	13	2.46 ± 0.78	$Z = -0.697$	0.486
No	17	2.35 ± 0.61		
Drinking				
Yes	12	2.25 ± 0.75	$Z = -0.917$	0.359
No	18	2.50 ± 0.62		
T stage				
T1	6	2.00 ± 1.41	$\chi^2 = 1.982$, d.f. = 3	0.576
T2	13	2.57 ± 0.65		
T3	4	2.20 ± 0.84		
T4	7	2.33 ± 0.50		
N stage				
N0	16	2.47 ± 0.72	$Z = -0.837$	0.403
N1–2	14	2.31 ± 0.63		
Clinical stage				
I	5	0.50 ± 0.71	$\chi^2 = 2.091$, d.f. = 3	0.554
II	8	0.67 ± 0.71		
III	2	0.67 ± 1.15		
IV	15	1.00 ± 0.63		
I + II	13	2.55 ± 0.69	$Z = -1.004$	0.316
III + IV	17	2.32 ± 0.67		
Pathological differentiation grade				
Well	12	2.50 ± 0.67	$\chi^2 = 4.475$, d.f. = 2	0.107
Moderate	15	2.20 ± 0.68		
Poor	3	3.00 ± 0.00		

In some squamous cell carcinomas, such as cervical, laryngeal, oesophageal and lung squamous cell carcinoma (Wetzels *et al*, 1992; Takahashi *et al*, 1995; Cohen-Kerem *et al*, 2002, 2004; Carrilho *et al*, 2004; Luo *et al*, 2004; Ikeda *et al*, 2008), increased CK17 expression has been reported with potential clinical value and pathological value. However, CK17 expression has not been well studied in OSCC; there are only two reports on describing the CK17 expression in clinical tissue samples associated with OSCC (Lalli *et al*, 2008; Ye *et al*, 2008). In one report, increased CK17 mRNA level in the cancerous tissues compared with the paracancerous tissues from tongue squamous cell carcinoma patients was found by genomic analysis. Unfortunately, no further protein validation has been reported (Ye *et al*, 2008). In another report focusing on oral submucous fibrosis and potential risk of malignant transformation, increased CK17 protein expression was found in the suprabasal layers of oral mucosa and the increased CK17 protein expression was found to correlate with disease severity of oral submucous fibrosis and to be at most risk to undergo malignant transformation (Lalli *et al*, 2008). In this study, we first identified the increased CK17 protein expression accompanied by the cancerization of the HIOECs and then validated the increased CK17 expression both *in vitro* and *in vivo*. In this study, CK17 positive expression pattern in the normal oral mucosa from healthy persons and the adjacent non-malignant epithelium from OSCC patient are in the supra-basal layer of mucosa and that in the cancerous tissues of OSCC is in the whole layer of mucosa. The CK17 positive grade is lower in the adjacent non-malignant epithelium than the cancerous tissue from OSCC patient. Therefore, the increased expression of CK17 may play an important role in the carcinogenesis of OSCC, while the detailed mechanism of CK17 on carcinogenesis of OSCC is not clear. The possible mechanism is the increased CK17 expression and alternated CK17 expression pattern in the oral mucosa. Further molecular research studies on the carcinogenesis progression of OSCC are suggested to clear the precise mechanism of CK17 on OSCC; large sample size studies with long-term follow-up studies are also encouraged to clear the clinical value of CK17 as a potential biomarker for OSCC.

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Author contributions

Dr K-J Wei contributed in immunohistochemistry and prepared manuscript; Dr L Zhang contributed in western blotting; Dr X Yang contributed in cell culture; Dr L-P Zhong designed the study, wrote the manuscript and made contributions to statistical analysis; Dr J Li contributed in immunohistochemical tissue preparation; Dr W-T Chen and Z-Y Zhang contributed in clinical tissue preparation.

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