Overexpression of insulin-like growth factor binding protein 3 in oral squamous cell carcinoma

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Abstract. Previously, we established an *in vitro* cellular carcinogenesis model of oral squamous cell carcinoma (OSCC), including a human immortalized oral epithelial cell (HIOEC) line and its derived cancerous HB96 cell line. Further cDNA microarray analysis showed a significant up-regulated gene, insulin-like growth factor binding protein 3 (IGFBP3), accompanying with *in vitro* cancerization from HIOEC to HB96. In order to investigate IGFBP3 up-regulation and its potential usefulness as a molecular marker in OSCC, we detected the IGFBP3 expression with a panel of OSCC lines, and clinical samples of cancerous tissues and paired adjacent non-malignant epithelia from primary OSCC patients. Western blotting and real-time PCR showed increased IGFBP3 mRNA level and protein expression in OSCC cell lines compared with HIOEC *in vitro*; immunohistochemistry and real-time PCR also showed increased IGFBP3 mRNA level and protein expression in cancerous tissues compared with adjacent non-malignant epithelia from OSCC patients. Positive correlations were found between the IGFBP3 protein-positive grade in cancerous tissue and the tumor size as well as lymph node metastasis, a larger tumor size and positive lymph node metastasis indicating a higher level of IGFBP3 protein-positive grade. Based on these results, IGFBP3 may be used as a positive biomarker for OSCC development and progression.

Introduction

Oral squamous cell carcinoma (OSCC), being one of the most common malignancies in the head and neck region, afflicts about 300,000 patients worldwide each year (1,2). The 5-year survival rate of OSCC patients is ~50-60% (3,4). Understanding the molecular mechanisms of OSCC oncogenesis will provide valuable information on diagnosis and prognosis, as well as developing novel therapy. Previously, we established a stable cell line, human immortalized oral epithelial cell (HIOEC), by transfection with HPV16 E6/E7 gene into normal epithelial cells (5), and then subsequently derived it into a cancerous cell line (HB96) by treatment with benzo[a]pyerene for 6 months (6). HB96 cells developed moderately differentiated squamous cell carcinoma when implanted in nude mice (6). Based on these two cell lines, we compared the global gene expression profile by cDNA microarray of Affymetrix U133 plus 2.0 and found significant increase of insulin-like growth factor binding protein 3 (IGFBP3) mRNA level in HB96 cells compared with HIOECs.

IGFBP-3 is a member of a protein family that can bind IGF-I and thereby regulate the mitogenic activity of IGF-I (7). The IGFBP-3 gene is transcriptionally activated by the tumor suppressor p53 (8), and it is assumed that increased expression of IGFBP-3 contributes to p53-dependent apoptosis (9). IGFBP-3 can block the proliferation of various cell types *in vitro* by at least two distinct ways (7). As mentioned above, IGFBP-3 binds IGF-I and thereby regulates IGF-I dependent signaling. Second, there is evidence that mutants of IGFBP-3 that fail to interact with IGF-I are still able to induce apoptosis in PC-3 cells (9). Abnormal expression or malfunction of IGFBP3 is associated with tumor development and progress. Reduced IGFBP3 expression has been reported in several cancers such as lung cancer (10-12), hepatocellular carcinoma (13,14), ovarian cancer (15) and prostate cancer (16-18). However, increased IGFBP3 expression has also been reported in some other cancers such as renal cell carcinoma (19-21), esophageal carcinoma (22), breast cancer (23-25), colon cancer (26) and cervical neoplastic progression (27). In head and neck cancer, there are still controversial reports on the IGFBP3 expression, increased (28) and reduced (29) expression. To our knowledge, there are few reports analyzing the IGFBP3 expression in OSCC *in vitro* and *in vivo*. In this study, we found the overexpression of IGFBP3 mRNA level and protein expression in a panel of OSCC cell lines and clinical tissue samples from OSCC patients. The correlations between the IGFBP3 expression and the clinicopathological characteristics of OSCC patients were also investigated.

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Materials and methods

Cell cultures. Six cell lines were used in this study, including two cell lines of HIOEC and HB96 from our previously established *in vitro* cellular carcinogenesis of OSCC (5,6), four OSCC cell lines of Tca8113, TSCC, CAL27 and OSC, and normal oral mucosal epithelia from a healthy person with signed informed consent forms was also studied. Tca8113 cell line was established in our laboratory; TSCC was established by Wuhan University, P.R. China and was given as a gift; CAL27 was purchased from ATCC (Manassas, VA); OSC was presents from Kochi Medical School, Japan. HIOECs were cultured in the defined keratinocyte-SFM (Gibco, USA). HB96 and CAL27 cells were cultured in DMEM (Dulbecco's modified Eagle's medium, Gibco, USA) supplemented with 10% fetal bovine serum, 1% glutamine and 1% penicillin-streptomycin. Tca8113, TSCC and OSC cells were cultured in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum, 1% glutamine and 1% penicillin-streptomycin. Normal oral mucosa was obtained from patients undergoing surgery for cleft palate or lip reconstruction. Specimens were washed immediately in cold, sterile phosphate-buffered saline (PBS). After removing connective tissue, the healthy specimens were cut into small pieces and incubated overnight in Dispase II (Boehringer Mannheim, USA) at 4˚C, then the normal oral mucosal epithelial cells were prepared as previously described (5). All cells were cultured in a humidified atmosphere of 5% CO_2 at 37˚C.

Clinical tissue samples. From February 2007 to July 2007, 30 primary OSCC patients without prior radiotherapy or chemotherapy were enrolled into the study. They were 21 males and 9 females, aged from 31 to 84 years with a mean of 53.8. After signing the informed consent forms, they underwent radical surgery at our Department of Oral and Maxillofacial Surgery. Surgical tissue samples including cancerous tissues and adjacent non-malignant epithelia were collected by procedures previously described (30,31). The adjacent non-malignant epithelia were collected at sites at least 2 cm away from the edge of tumor masses, with best efforts of avoiding contamination by the tumor cells. The sites of primary carcinoma were tongue (n=17), buccal mucosa (n=4), retromolar region (n=3), floor of the mouth (n=3), gingiva (n=2) and palatoglossal arch (n=1). The stage of disease was determined according to the tumor-node-metastasis staging (TNM) system of the International Union Against Cancer (32). The histological grade of the tumor was determined according to the degree of differentiation of the WHO histological criteria (33).

Western blotting. Cultured cells grown to 80% confluence were lysed in ice-cold 2X 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, USA), protein samples (50 μg/lane) were separated by 12% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel), and then electrophoretically transferred onto polyvinylidene difluoride membranes using a wet transfer system (Invitrogen, USA). The membranes were blocked with blocking buffer containing 5% dry milk in PBS with 0.1% Tween-20 for 2 h and incubated at 4˚C overnight with mouse anti-IGFBP3 monoclonal antibody (Clone 84728.111, GeneTex, USA) at 1:500 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 analyzed using the Odyssey Infrared Imaging System (LI-COR Biosciences, USA).

Real-time RT-PCR. Total RNAs were isolated from liquid nitrogen-pulverized tissue samples, or cultured cells at 80% confluence, with TRIzol reagent (Invitrogen) according to the manufacturer's instruction. DNA contamination was digested by RNAse-free DNase I. RNA concentration and purity were assessed at 260 nm using a spectrophotometer (DU 530, Beckman-Coulter Inc., USA). Total RNA (2 μg) was reverse transcribed into first strand cDNA with M-MLV reverse transcriptase (Promega, USA) and random primers (Amersham Biosciences, USA). Real-time PCR was performed with a Takara PCR thermal cycler dice detection system and SYBR-Green dye (Takara, Japan). The primers for real-time PCR were designed by primer premier 5.0 (Premier Biosoft International, CA). The primers of IGFBP3 (NM_000589) were: forward, 5'-AGAGCACAGATACCCAGAACT-3'; reverse, 5'-TGAGGAACTTCAGGTGATTCAGT-3' and the length of PCR product was 105 bp. The primers of ß-actin were: forward, 5'-TGGATCAGCAAGCAGGAGTA-3'; reverse, 5'-GGTTTTGTCAAGAAAGGG-3' and the length of PCR product was 100 bp. The conditions for PCR reactions were: 95˚C for 10 sec followed by 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. The relative quantification of IGFBP3 mRNA level compared with ß-actin was calculated according to the $2^{-\Delta\Delta Ct}$ method (34):

$$-\Delta\Delta Ct = [Ct_{(\text{cancerous tissues ß-actin})} - Ct_{(\text{cancerous tissues IGFBP3})}] / [Ct_{(\text{adjacent non-malignant epithelia ß-actin})} - Ct_{(\text{adjacent non-malignant epithelia IGFBP3})}].$$

All samples were run in duplicates and the relative quantification of each target gene transcription was performed twice.

Immunohistochemistry. Thirty pairs of tissue samples from each patient, including cancerous tissues and adjacent non-malignant epithelia, were detected using immunohistochemical staining for IGFBP3. The procedure of immunohistochemistry was according to the method as we previously described (30,35). 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 IGFBP3 (Clone 84728.111, GeneTex) at 1:100 dilution overnight at 4˚C, and visualized using 3,3'-diaminobenzidine (DAB) detection kit (Dako Cytomation, 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 IGFBP3 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

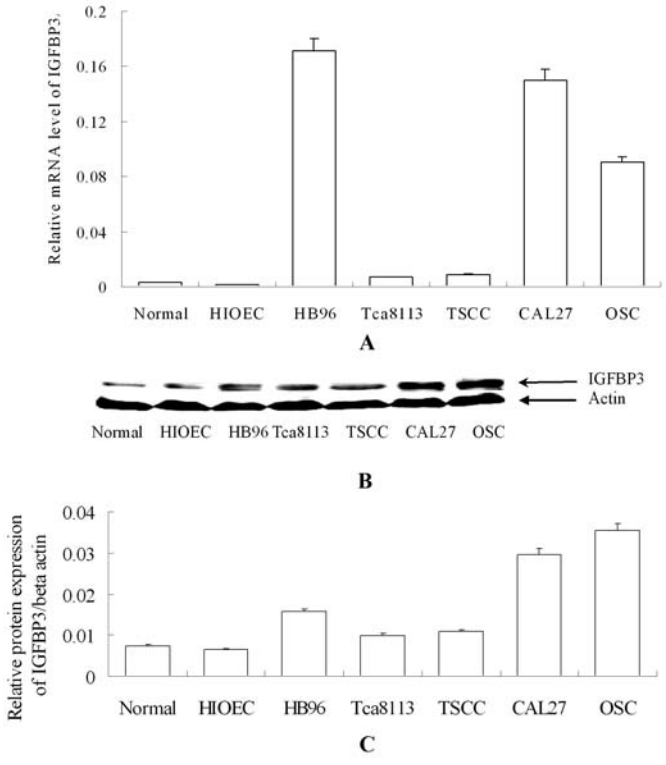

Figure 1. The expression of IGFBP3 in OSCC cell lines by real-time PCR and Western blotting. (A) Real-time PCR showed that IGFBP3 mRNA level in HB96, Tca8113, TSCC, CAL27 and OSC cell lines was higher than that in HIOEC. (B and C) Western blotting also showed that IGFBP3 protein level in HB96, Tca8113, TSCC, CAL27 and OSC cell lines was higher than that in HIOEC. IGFBP3 mRNA and protein levels in normal oral mucosal epithelia were almost the same as those in HIOECs.

Table I. The immunohistochemical IGFBP3 positive grade in the different types of tissues from OSCC patients.

		IGFBP3 positive grade			
Type of tissue	Case no.	0	1	2	3
Adjacent non-malignant epithelia	30	17	10	3	0
Cancerous tissues	30	7	15	5	3

Figure 2. The relative IGFBP3 mRNA level in clinical tissue samples from OSCC patients by real-time PCR. IGFBP3 mRNA level in the cancerous tissues was 3.515-fold higher than that in the adjacent non-malignant epithelia (t=3.594, P=0.001).

grade 1; moderate, 26-50% of stained cells with grade 2 and strong, >50% of stained cells with grade 3.

Statistical analysis. The data were analyzed by the statistical software package of SPSS10.0 for Windows (SPSS Inc., USA). The statistical difference of initial data was analyzed by non-parametric tests. At P-value <0.05, the difference was regarded as statistically significant.

Results

IGFBP3 expression at mRNA and protein levels in a panel of OSCC cell lines. Real-time PCR showed the increased IGFBP3 mRNA levels in the HB96, Tca8113, TSCC, CAL27 and OSC cells compared with the HIOEC normalized against ß-actin (Fig. 1A). Western blotting showed that IGFBP3 protein expression increased in all OSCC cell lines compared with the HIOEC normalized against ß-actin protein signal (Fig. 1B and C). IGFBP3 mRNA and protein levels in the normal oral mucosal epithelial cells were almost the same as those in the HIOECs.

IGFBP3 expression at mRNA and protein levels in primary OSCC tissue samples. Real-time PCR showed that IGFBP3 mRNA levels in the cancerous tissues were 3.515-fold higher than that in the adjacent non-malignant epithelia (t=3.594, P=0.001) (Fig. 2), with standard error of 0.700, standard deviation of 3.834 and 95% of confidence interval for mean from 2.084 to 4.974. Immunohistochemistry showed the IGFBP3 protein expression predominantly in the cellular cytoplasm. Cancerous tissues from OSCC patients showed positive reactivity to IGFBP3 of grade 0 in 23.3% (7/30) cases (Fig. 3A), grade 1 in 50.0% (15/30) (Fig. 3B), grade 2 in 16.7% (5/30) (Fig. 3C) and grade 3 in 10.0% (3/30) (Fig. 3D). However, adjacent non-malignant epithelia showed negative reactivity in 56.7% (17/30) cases (Fig. 3E), positive reactivity of grade 1 in 33.3% (10/30) cases (Fig. 3F), and grade 2 in 10.0% (3/30) (Table I). The positive rate of IGFBP3 protein in the cancerous tissues (76.7%, 23/30) was significantly higher than that in the adjacent non-malignant epithelia (43.3%, 13/30) (χ^2=6.944, P=0.008). The immunoreactions of IGFBP3 protein-positive grade in the cancerous tissues were consistently higher than those in the paired adjacent non-malignant epithelia (Wilcoxon's signed-ranks test, Z=-3.522, P<0.001).

The correlations between the IGFBP3 expression levels (mRNA and protein) and the clinicopathological characteristics of OSCC patients such as TNM stage, clinical stage, patho-

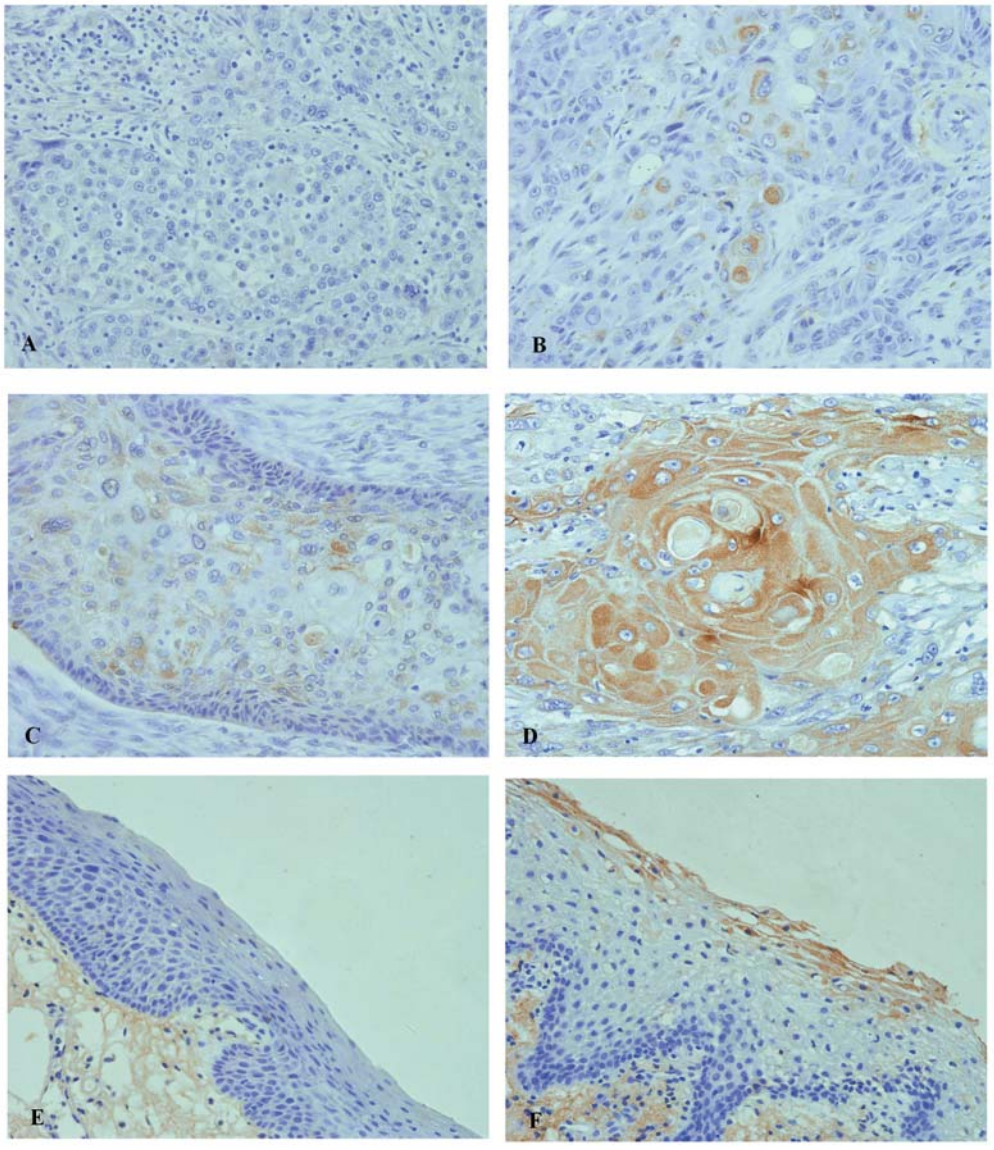

Figure 3. The IGFBP3 protein expression level in clinical tissue samples from OSCC patients by immunohistochemistry (x400). IGFBP3 protein-positive grades in the cancerous tissues and adjacent non-malignant epithelia: (A) grade 0 in the cancerous tissue; (B) grade 1 in the cancerous tissue; (C) grade 2 in the cancerous tissue; (D) grade 3 in the cancerous tissue; (E) grade 0 in the adjacent non-malignant epithelia and (F) grade 1 in the adjacent non-malignant epithelia.

logical differentiation grade, smoking and drinking, were analyzed. No significant correlations were found between the IGFBP3 mRNA level with T stage, N stage, clinical stage, pathological differentiation grade, smoking or drinking (Table II). However, significant difference of IGFBP3 protein expression was found by classification of N stage and clinical stage (Table II). The IGFBP3 protein-positive grade of cancerous tissues from OSCC patients with positive lymph node metastasis was significantly higher than that from OSCC patients without lymph node metastasis; the IGFBP3 protein positive grade of cancerous tissues from OSCC patients at late clinical stage was significantly higher than that from OSCC patients at early clinical stage. Positive correlation was also found between the IGFBP3 protein-positive grade and N stage (Spearman's rho correlation coefficient = 0.417, P=0.022), as well as clinical stage (Spearman's rho correlation coefficient = 0.407, P=0.026). Furthermore, positive correlation was also found between the IGFBP3 protein-positive grade and T stage (Spearman's rho correlation coefficient = 0.425, P=0.019), a lager size of tumor indicated a higher IGFBP3 protein-positive grade. No significant correlation was found between the IGFBP3 protein-positive grade and pathological differentiation grade of cancerous tissues.

Disscussion

In vitro cellular model is important in understanding cellular events related to pathological or physiological conditions in humans. It is an indispensable study tool in investigating for molecular mechanisms, because it has many advantages such as homogeneity of cell population, accessibility, reproducibility, controllable growth rate, and hence enough amount of material for analysis (36). *In vitro* cellular carcinogenesis model is very important, especially for

Table II. The correlation between the status of IGFBP3 expression and the clinicopathological characteristics of OSCC patients.

Classification	Case no.	Relative mRNA level	Non-parametric test values	P-value	Cancerous protein positive grade	Non-parametric test values	P-value
Smoking							
Yes	13	4.834±4.857			1.38±0.96		
			Z=-1.611	0.107		Z=-1.333	0.182
No	17	2.518±2.551			0.94±0.83		
Drinking							
Yes	12	4.375±5.123			1.25±0.87		
			Z=-0.466	0.641		Z=-0.091	0.465
No	18	2.942±2.687			1.06±0.94		
T stage							
T1	6	3.414±3.805			0.50±0.55		
T2	13	3.627±4.570	χ^2=0.024		1.15±1.07	χ^2=5.497	
				0.999			0.139
T3	4	2.569±1.442	df=3		1.25±0.50	df=3	
T4	7	3.934±3.914			1.57±0.79		
N stage							
N0	16	4.418±4.813			0.81±0.83		
			Z=-0.873	0.383		Z=-2.244	0.025
N1-2	14	2.725±2.628			1.50±0.85		
Clinical stage							
I	5	3.707±4.178			0.40±0.55		
II	8	2.672±1.714	χ^2=0.250		1.00±1.07	χ^2=8.865	
				0.969			0.031
III	2	2.098±0.236	df=3		0.50±0.71	df=3	
IV	15	4.090±4.788			1.53±0.74		
I+II	13	3.070±2.794			0.77±0.93		
			Z=-0.105	0.917		Z=-2.192	0.028
III+IV	17	3.856±4.527			1.41±0.80		
Pathological differentiation grade							
Well	12	3.642±4.858			1.42±0.90		
			χ^2=2.221			χ^2=2.121	
Moderately	15	3.827±3.828		0.329	0.93±0.70		0.346
			df=2			df=2	
Poorly	3	1.451±0.819			1.00±1.73		

cancer research, not only on the aspect of molecular mechanisms, but also on the aspect of molecular biomarkers. Based on our *in vitro* cellular carcinogenesis model of OSCC (5,6), we found significant increase of IGFBP3 mRNA level in HB96 cells compared with that in HIOECs by cDNA microarray technology. Then, we confirmed the cDNA microarray result in the two OSCC cell lines and clinical tissue samples from OSCC patients. *In vitro*, real-time PCR and Western blotting confirm the increased IGFBP3 mRNA level and protein expression in OSCC cell lines compared with HIOECs. *In vivo*, real-time PCR and immunohistochemistry also confirm the increased IGFBP3 mRNA level and protein expression in cancerous tissues from OSCC patients compared with paired adjacent non-malignant epithelia. Furthermore, we found positive correlation between the IGFBP3 protein expression and clinical stages, the patients at a later clinical stage with larger size of tumor and positive lymph node metastasis have a higher IGFBP3 protein-positive grade in the cancerous tissues. Thus, IGFBP3 expression correlates with the tumor progress of OSCC.

The above is contradictory to most of previous studies, because increased IGFBP3 expression is considered to inhibit cell proliferation and induce cell apoptosis as a protective factor (7-9). However, the precise mechanism of IGFBP3 has

not been clearly understood. IGFBP-3 has been shown to stimulate cell proliferation under various experimental conditions through IGF-dependent and independent mechanisms (37,38). In some cases, IGFBP-3 has a positive effect on cell growth. For example, IGFBP-3 has been shown to augment IGF-1 mitogenic responses in bovine mammary epithelial cells and in a human breast cancer cell line (39,40) and to enhance the activity of IGF-1 on cultured bovine fibroblasts (41). IGFBP-3 can enhance IGF activity by presenting and slowly releasing IGF-I for receptor interactions while protecting the receptor from down-regulation by high IGF-I exposure, and IGFBP3 serves as a risk factor (42-44). In this study, clinically, positive correlation between the IGFBP3 protein expression and clinical stages also supports the IGFBP3 as a risk factor. However, deeper molecular studies are encouraged to be performed to clear the detail mechanism of IGFBP3 on the carcinogenesis of OSCC.

This study revealed the significant increase of IGFBP3 mRNA and protein levels in OSCC *in vitro* and *in vivo*; it also revealed the positive correlation between IGFBP3 protein expression and clinical stage (tumor size and lymph node metastasis), an OSCC with larger tumor size or positive lymph node metastasis has a higher level of IGFBP3 protein-positive grade. Thus, IGFBP3 may be used as a positive biomarker for OSCC development and progression. However, the use of such marker should be with caution. For OSCC patients, prognostic evaluation will still be essential. At this point, further studies with larger sample size and longer term of follow-up are required. Since IGFBP3 is largely increased in OSCC tumors and cells, further studies are needed to support the direct roles of IGFBP3 on oncogenesis at molecular and cellular levels.

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