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Increased Expression of p-GSK3β Predicts Poor Survival in T –III/IV Stage OSCC Patients

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Abstract. *Background/Aim: Glycogen synthase kinase 3 beta (GSK3-β) acts either as a tumor suppressor or an oncogene in various human cancers. The present study aimed to investigate the expression and activity of p-GSK3-β (Ser9) in oral cancer patients. Materials and Methods: We investigated the levels of p-GSK3β in 152 oral cancer tissues by immunohistochemistry, and explored their prognostic impact. Results: To investigate the role of p-GSK3β (Ser9) in OSCC progression, we first analyzed the expression levels of protein p-GSK3β in normal and oral cancer tissues using immunohistochemical staining. p-GSK3β immunostaining was detected in 32 of 152 (21.1%) oral cancer specimens. High p-GSK3β expression was significantly associated with T (III/IV) stage. Kaplan-Meier survival analysis revealed that high levels of p-GSK3β were correlated with poor survival (p=0.001) in T stage (III/IV) OSCC patients. Multivariate analyses indicated that TN stage, AJCC tumor stage, tumor differentiation status and clinical therapy, but not p-GSK3β levels, were independent prognostic factors. Significant mortality risk was found in T stage (III/IV) oral cancer patients with high levels of p-GSK3β (p=0.0006). Conclusion: GSK3β inactivation is a key event in oral cancer patients and targeting GSK3β might be valuable in treating oral cancer patients.*

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Key Words: pGSK3β, IHC, OSCC, survival, AJCC, T stage, prognostic factors.

Oral squamous cell carcinoma (OSCC) is the sixth most common cancer worldwide (1),and the fourth most common cancer affecting Taiwanese men (2). According to the American Cancer Society, men have twice the chance of developing oral cancer compared to women (3). The major risk factors for acquiring OSCC include smoking, chewing tobacco, consumption of alcohol, family history and infection with human papillomavirus (HPV). Oral cancer is asymptomatic and is usually diagnosed after migration and invasion in other organs (4). Surgery, chemotherapy and radiotherapy are the currently available treatments for oral cancer. In the last decade, improvement in clinical approaches with the use of highly targeted inhibitors and specific monoclonal antibodies, have led to an increase in patient survival rate. With the success rate of targeted therapy, scientific focus has shifted towards transcription factors, cell cycle regulators and metastasis-promoting factors, the deregulation of which causes uncontrolled cell division (5).

Glycogen synthase kinase 3 (GSK3), is a serine/threonine kinase that plays a critical role in cellular homeostasis and is ubiquitously expressed in all mammalian tissues. It regulates various physiological processes and is involved in the pathogenesis of a number of chronic and progressive diseases, like cancers, neurodegenerative diseases and diabetes mellitus

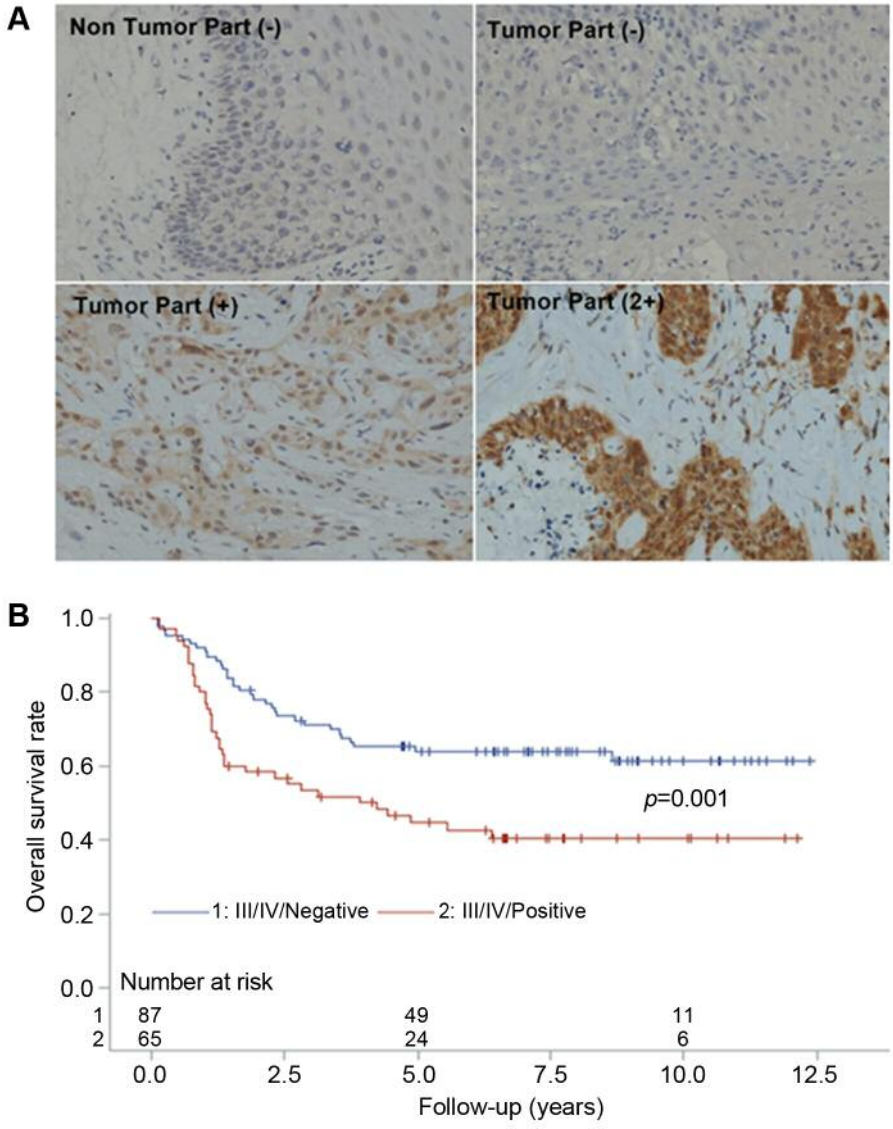

Figure 1. *p-GSK3β protein levels in OSCC samples. (A) Immunohistochemical analysis of p-GSK3β levels in non-tumor and tumor tissues. (B) Overall survival of T-III/IV stage oral cancer patients with high or low levels of p-GSK3β was analyzed using the Kaplan-Meier log-rank test.*

(6, 7). GSK3β plays a role in cell proliferation, cell cycle regulation, apoptosis, cell differentiation and migration (8, 9). Several new studies suggest an inconsistent role of GSK3 in different human cancers, acting either as a tumor suppressor or as an oncogene (10). Phosphorylation of GSK3β in the serine 9 (Ser9) residue leads to inhibition of its enzymatic activity (11). In contrast, GSK3β phosphorylation in the tyrosine216 (Tyr216) residue leads to increased enzymatic activity; however, the mechanisms regulating this modification remain elusive.

In the present study, we investigated the expression levels and the clinico-pathological characteristics associated withp-GSK3-β (Ser9) levels in 152 OSCC tissue samples.

Materials and Methods

Participants and clinical tissues. OSCC specimens from 152 patients were obtained from the Department of Pathology at Changhua Christian Hospital, Taiwan. Few patients had received chemotherapy (5-FU and cisplatin) or radiotherapy before surgery. This research was approved by the Ethics Committee of Changhua Christian Hospital (CCH IRB No. 170413). IRB agreed to use formalin-fixed, paraffin-embedded decoded tissue array samples without the need to obtain an informed consent from each patient.

Immunohistochemistry. Immunohistochemistry was performed according to standard protocols, as previously described (12). Two pathologists independently evaluated and scored the stained slides. The staining intensities were scored as no staining, -; low/moderate

Table I. *Characteristics of patients*.

Factors	Numbers	%
Gender		
Female	9	6
Male	143	94
Age, year		
≤49	41	27
50-59	59	38.8
60-69	35	23
≥70	17	11.2
T (tumor size)		
I	37	24.3
II	50	32.9
III	16	10.5
IV	49	32.3
N (lymph node)		
N0	95	62.5
N1	18	11.8
N2	39	25.7
N3	0	0
M (metastasis)		
No	152	100
Yes	0	0
AJCC cancer stage		
I	28	18.4
II	34	22.4
III	21	13.8
IV	69	45.4
Histological grade		
Well	20	13.1
Moderate	129	84.9
Poor	3	2
Clinical therapy		
Radiotherapy		
No	46	31.1
Yes	102	68.9
Chemotherapy		
No	108	73
Yes	40	27

staining, 1+; high staining, 2+. The primary p-GSK3β antibody (1:200, Cell signaling Technology, MA, USA) was used in the immunohistochemical analysis.

Statistical analysis. All data were analyzed by the SAS 9.4 Software (SAS Institute, Inc.; Cary, NC, USA). All statistical analyses were performed as described in our earlier studies (12, 13).

Results

Patient characteristics. Demographic and clinicopathological characteristics of the OSCC patients are summarized in Table I. Specimens from 152 patients were included in the current study (143 male and 9 female patients).Among the cancer patients, 43%, 25.7% and 59.2% were found to have stage III/IV disease, lymph node metastasis of N2/N3 and AJCC tumor stage (III/IV), respectively, and 87% were moderate or poor histological grade. A total of 102 patients (69%) and 40 patients (27%), were treated with radiotherapy and chemotherapy, respectively.

Table II. *Correlation between clinicopathologic features and expression of p-GSK3β in 152 oral cancer patients*.

Factors	p-GS3β			
	High No. (n=32)	Low No. (n=120)	aOR (95%CI)*	*p*-Value
Tumor size (SD)	2.80 (2.27)	3.07 (2.78)		0.39
T classification				
I/II	25	62	1	
III/IV	7	58	0.29 (0.12-0.74)	0.0094
N (lymph node)				
N0/N1	26	87	1	
N2/N3	6	33	0.60 (0.23-1.60)	0.31
M (metastasis)				
No	32	120	1	
Yes	0	0	ND	
AJCC cancer stage				
Early stage (I/II)	17	45	1	
Advance stage (III/IV)	15	75	0.52 (0.24-1.15)	0.11
Histological grade				
Well	6	14	1	
Moderate/Poor	26	106	0.57 (0.20-1.62)	0.29
Death				
No	17	66	1	
Yes	15	54	1.07 (0.49-2.35)	0.86

*Adjusted odds ratio (aOR) was controlled for gender and age.

p-GSK3β expression in OSCC is associated with tumor differentiation. Immunohistochemical results showed that 32 out of 152 OSCC patients (21.1%) had higher levels of p-GSK3β (Ser 9) and 120 had lower levels of p-GSK3β. As shown in Figure 1a, p-GSK3β was present both in the cytoplasm and nucleus of tumor cells, while in most of the cases high levels ofp-GSK3β were observed in the cytoplasm (Figure 1A).

The correlation between p-GSK3β levels and clinical features of OSCC is shown in Table II. p-GSK3β levels were positively correlated with T stage (p=0.0094; Table II). No significant correlation was found between p-GSK3β levels and other clinicopathologic parameters.

p-GSK3β levels and survival. The clinicopathologic factors and p-GSK3β levels related to the mortality of OSCC patients are shown in Table III. The mortality rate for patients with TN stage, AJCC tumor stage and tumor differentiation was 14.0, 26.3, 14.3 and 10.1 per 100 people-years, respectively. Independent mortality risk for OSCC patients with high p-GSK3β levels was not significant when compared to low p-GSK3β levels group.

Table III. *Correlation between clinicopathologic features and expression of p-GSK3β on mortality density.*

Factors	No. of patient	Follow-up (person-year)	No. of death	Mortality density[a]	aHR[b]	(95%CI)	Interaction *p*-Value
Overall mortality from primary malignancy to death							
T classification							
I/II	87	506.3	32	6.3	1		
III/IV	65	264.4	37	14.0	2.02	(1.40-2.92)	0.0002
N classification							
N0/N1	113	656.5	39	5.9	1		
N2/N3	39	114.2	30	26.3	3.06	(2.09-4.48)	<0.0001
AJCC tumor stage							
I/II	62	407.8	17	4.2	1		
III/IV	90	362.9	52	14.3	2.65	(1.75-4.02)	<0.0001
Tumor differentiation							
Well	20	119.7	3	2.5	1		
Moderate/Poor	132	651	66	10.1	2.73	(1.38-5.39)	0.0038[c]
Clinical therapy							
Surgery	49	306.4	14	4.6	1		
Chemotherapy/Radiotherapy	103	464.3	55	11.8	3.03	(1.95-4.69)	<0.0001
p-GSK3β expression							
Low	120	618.75	66	10.7	1		
High	32	151.9	17	11.2	1.1	(0.62-1.95)	0.7404

[a]Mortality density was displayed as per 100 people-years; [b]aHR (adjusted hazard ratio) for age and gender; [c]Significant multiplicative-scale interaction between T classification and p-GSK3β expression on mortality risk was p=0.0038.

Table IV. *T classification and the presence of p-GSK3β on mortality risk were identified.*

Factors	No. of patient	Follow-up (person-year)	No. of death	Mortality density[a]	aHR[b]	(95%CI)	*p*-Value
T classification/p-GSK3β							
I/II/Low	65	466.7	20	4.3	1		
I/II/High	51	272.1	28	10.3	2.127	(1.20-3.77)	**0.0101**
III/IV/Low	87	506.2	32	6.3	1.31	(0.75-2.29)	0.3444
III/IV/High	65	264.4	37	14.0	2.592	(1.51-4.48)	**0.0006**

[a]Mortality density was displayed as per 100 people-years; [b]aHR was adjusted for gender and age. Bold values show significance.

The above findings indicated that p-GSK3β levels significantly interacted with T stage (Table II), therefore, we further investigated the combined effect of p-GSK3β levels and T stage on OSCC patient's mortality. Cox proportional hazards models (Table IV) showed that, high p-GSK3β levels in T stage (III/IV) patients was associated with a high mortality rate (p=0.0006) compared to the low p-GSK3β levels group. Kaplan-Meier analysis showed that patients with high p-GSK3β levels have a shorter survival rate than patients with low levels of p-GSK3β (p=0.001; Figure 1b).

Discussion

Aberrant expression of GSK3β is involved in various types of human cancers as well as in other diseases. GSK3β can function either as a tumor suppressor or tumor promoter in various human cancers (5, 14). In the case of oral cancer, phosphorylation of Ser9 of GSK3β leads to its inactivation and, therefore, to activation of oncogenic signaling molecules (15, 16). Considering the role of GSK3 in extrinsic and intrinsic apoptotic pathways, and the no detrimental effect of active GSK3 on normal cells, targeting the GSK3β pathway is becoming a potential therapeutic target for oral cancer.

In this study, we compared p-GSK3β levels between OSCC tissues and adjacent normal tissues in relation to OSCC pathogenesis. IHC analysis showed that, p-GSK3β was markedly upregulated in OSCC tissues compared with the normal marginal tissues. p-GSK3β was localized in both the nucleus and cytoplasm, and high cytoplasmic levels of p-GSK3β were found in most samples. These results are consistent with previous findings (17) reporting that GSK3β is localized predominantly in the cytoplasm of oral cancer cells.

We further stratified cancer patients based on the levels of p-GSK-3β, and analyzed their relationship with other clinicopathologic parameters. Our findings showed that high p-GSK3β levels were significantly associated with T (III/IV) stage in OSCC patients. No significant association between p-GSK3β levels and other clinicopathological characteristics were found in our study. Cox proportional hazards model indicated that cytoplasmic p-GSK3β was not an independent prognostic factor in OSCC patients. Multivariate analysis was used to analyze the combined effect of clinicopathological factors, T (III/IV) and p-GSK3β levels on mortality risk. Significant correlation was observed between p-GSK3β expression and T-III/IV stage. Survival analysis showed that, T stage (III/IV) patients with high levels of p-GSK3β had a poorer survival than patients with low levels of p-GSK3β.

In summary, we report that GSK3β inactivation is a key event in oral cancer patients and targeting GSK3β might be valuable in treating oral cancer patients.

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study. This study was funded by Changhua Christian Hospital (108-CCH-IRP-044).

Authors' Contributions

VBK, CWC and SHL analyzed and drafted the article. YML, BM, CMY, YEC, CMC - assisted with data interpretation. VBK, SHL and BM reviewed and revised the article. All Authors read and approved the final article.

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