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

BHARATH KUMAR VELMURUGAN1*, CHUN-WEN CHIU2*, YUEH-MIN LIN3,4,5, MAHALAKSHMI BHARATH6, CHUNG-MIN YEH3,7, YU-EN CHEN3, CHIA-MIN CHUNG8,9 and SHU-HUI LIN3,5

1Department of Biotechnology, Asia University, Taichung, Taiwan, R.O.C.; 2Department of Emergency Medicine, Changhua Christian Hospital, Changhua, Taiwan, R.O.C.; 3Department of Surgical Pathology, Changhua Christian Hospital, Changhua, Taiwan, R.O.C.; 4School of Medicine, Chung Shan Medical University, Taichung, Taiwan, R.O.C.; 5Department of Medical Laboratory Science and Biotechnology, Central Taiwan University of Science and Technology, Taichung, Taiwan, R.O.C.; 6Institute of Research and Development, Duy Tan University, Da Nang, Vietnam; 7Department of Medical Technology, Jen-Teh Junior College of Medicine, Nursing and Management, Miaoli, Taiwan, R.O.C.; 8Graduate Institute of BioMedical Sciences, China Medical University, Taichung, Taiwan, R.O.C.; 9Environment-Omics-Diseases Research Center, China Medical University Hospital, Taichung, Taiwan, R.O.C.

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-GSK3β (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.

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.
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 with p-GSK3β (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...
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.

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 of p-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.

| Factors          | Numbers | %     | p-GSK3β  |
|------------------|---------|-------|----------|
| Gender           | Female  | 9     | 6        |
| Flexible         | Male    | 143   | 94       |
| Age, year        | ≥49     | 41    | 27       |
| Flexible         | 50-59   | 59    | 38.8     |
| Flexible         | 60-69   | 35    | 23       |
| Flexible         | ≥70     | 17    | 11.2     |
| T (tumor size)   | I       | 37    | 24.3     |
| Flexible         | II      | 50    | 32.9     |
| Flexible         | III     | 16    | 10.5     |
| Flexible         | IV      | 49    | 32.3     |
| N (lymph node)   | N0      | 95    | 62.5     |
| Flexible         | N1      | 18    | 11.8     |
| Flexible         | N2      | 39    | 25.7     |
| Flexible         | N3      | 0     | 0        |
| M (metastasis)   | No      | 152   | 100      |
| Flexible         | Yes     | 0     | 0        |
| AJCC cancer stage| I       | 28    | 18.4     |
| Flexible         | II      | 34    | 22.4     |
| Flexible         | III     | 21    | 13.8     |
| Flexible         | IV      | 69    | 45.4     |
| Histological grade| Well   | 20    | 13.1     |
| Flexible         | Moderate | 129  | 84.9     |
| Flexible         | Poor    | 3     | 2        |
| Clinical therapy | Radiotherapy | No | 46 | 31.1 |
| Flexible         | Yes     | 102   | 68.9     |
| Chemotherapy     | No      | 108   | 73       |
| Flexible         | Yes     | 40    | 27       |

Velmurugan et al: p-GSK3β Predicts Poor Survival in OSCC

Table I. Characteristics of patients.

| Factors          | Numbers | %     |
|------------------|---------|-------|
| Gender           | Female  | 9     |
| Flexible         | Male    | 143   |
| Age, year        | ≥49     |
| Flexible         | 50-59   |
| Flexible         | 60-69   |
| Flexible         | ≥70     |
| T (tumor size)   | I       |
| Flexible         | II      |
| Flexible         | III     |
| Flexible         | IV      |
| N (lymph node)   | N0      |
| Flexible         | N1      |
| Flexible         | N2      |
| Flexible         | N3      |
| M (metastasis)   | No      |
| Flexible         | Yes     |
| AJCC cancer stage| I       |
| Flexible         | II      |
| Flexible         | III     |
| Flexible         | IV      |
| Histological grade| Well   |
| Flexible         | Moderate |
| Flexible         | Poor    |
| Clinical therapy | Radiotherapy | No | 46 | 31.1 |
| Flexible         | Yes     | 102   |
| Chemotherapy     | No      | 108   |
| Flexible         | Yes     | 40    |

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

**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 |               |                         |              |                       |                 |           |
| 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.
We further stratified cancer patients based on the levels of p-GSK3β, 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 research 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.

Acknowledgements

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

1. Abdulla R, Adyanthaya S, Kini P, Mohanty V, D’Souza N and Subbannayya Y: Clinicopathological analysis of oral squamous cell carcinoma among the younger age group in coastal karnataka, india: A retrospective study. J Oral Maxillofac Pathol 2(2): 180-187, 2018. PMID: 30158769. DOI: 10.4103/jomfp.JOMFP_16_18
2. Chen TW, Lee CC, Liu H, Wu CS, Pickering CR, Huang PJ, Wang J, Chang IY, Yeh YM, Chen CD, Li HP, Luo JD, Tan BC and Chan TEH: Apcob3a is an oral cancer prognostic biomarker in taiwanese carriers of an apobec deletion polymorphism. Nat Commun 8(1): 465, 2017. PMID: 28878238. DOI: 10.1038/s41467-017-00493-9
3. Bagan JV and Scully C: Recent advances in oral oncology 2008: squamous cell carcinoma aetiopathogenesis and experimental studies. Oral Oncol 45(7): e45-48, 2009. PMID: 19196543. DOI: 10.1016/j.joraloncology.2008.12.012
4. Kademani D: Oral cancer. Mayo Clinic Proc 82(7): 878-887, 2007. PMID: 17605971. DOI: 10.4065/82.7.878
5. Mishra R: Glycogen synthase kinase 3 beta: Can it be a target for oral cancer. Mol Cancer 9(144), 2010. PMID: 20537194. DOI: 10.1186/1476-4598-9-144
6. Cohen P and Frame S: The renaissance of gsk3. Nat Rev Mol Cell Biol 2(10): 769-776, 2001. PMID: 11584304. DOI: 10.1038/35096075
7. Miyashita K, Nakada M, Shakoori A, Ishigaki Y, Shimasaki T, Motoo Y, Kawakami K and Minamoto T: An emerging strategy for cancer treatment targeting aberrant glycogen synthase kinase 3 beta. Anticancer Agents Med Chem 9(10): 1114-1122, 2009. PMID: 19925395. DOI: 10.2174/187152009789734982
8. Harwood AJ: Regulation of gsk-3: A cellular multiprocessor. Cell 105(7): 821-824, 2001. PMID: 11439177. DOI: 10.1016/s0092-8674(01)00412-3
9. Doble BW and Woodgett JR: Gsk-3: Tricks of the trade for a multi-tasking kinase. J Cell Sci 116(pt 7): 1175-1186, 2003. PMID: 12615961. DOI: 10.1242/jcs.00384
10. Meares GP and Jope RS: Resolution of the nuclear localization mechanism of glycogen synthase kinase-3: Functional effects in apoptosis. J Biol Chem 282(23): 16989-17001, 2007. PMID: 17438332. DOI: 10.1074/jbc.M700610200
11. Rom S, Fan S, Reichenbach N, Dykstra H, Ramirez SH and Persidsky Y: Glycogen synthase kinase 3β inhibition prevents monocyte migration across brain endothelial cells via rac1-gtapase suppression and down-regulation of active integrin conformation. Am J Pathol 181(4): 1414-1425, 2012. PMID: 22636953. DOI: 10.1016/j.ajpath.2012.06.018
12. Velmurugan BK, Chang WH, Chung CM, Yeh CM, Lee CH, Yeh KT and Lin SH: Ddx2 overexpression in oral squamous cell carcinoma is associated to lymph node metastasis. Cancer Biomark 22(4): 747-753, 2018. PMID: 29945346. DOI: 10.3233/cbm-181302
13. Velmurugan BK, Lee CH, Chiang SL, Hua CH, Chen MC, Lin SH, Yeh KT and Ko YC: Pp2a deactivation is a common event in oral cancer and reactivation by fty720 shows promising therapeutic potential. J Cell Physiol 233(2): 1300-1311, 2018. PMID: 28516459. DOI: 10.1002/jcp.26001
14. Georgy SR, Gangkrama M, Srivastava S, Partridge D, Auden A, Dworkin S, McLean CA, Jane SM and Darido C: Identification of a novel proto-oncogenic network in head and neck squamous cell carcinoma. J Natl Cancer Inst 107(9), 2015. PMID: 26063791. DOI: 10.1093/jnci/djv152
15. Ma C, Wang J, Gao Y, Gao TW, Chen G, Bower KA, Odetallah M, Ding M, Ke Z and Luo J: The role of glycogen synthase kinase 3beta in the transformation of epidermal cells. Cancer Res 67(16): 7756-7764, 2007. PMID: 17699780. DOI: 10.1158/0008-5472.CAN-06-4665
16. Grassilli I, Ianzano L, Bonomo S, Missaglia C, Cerrito MG, Giovannoni R, Masiero L and Lavitrano M: Gsk3a is redundant with gsk3b in modulating drug resistance and chemotherapy-induced necroptosis. PLoS One 9(7): e100947, 2014. PMID: 24984063. DOI: 10.1371/journal.pone.0100947
17. Darrington RS, Campbell GM, Bongoa-Vergniory N, Gorrono-Extebarria I, Uysal-Onganer P, Kawanow Y, Wamnax J and Kypita RM: Distinct expression and activity of gsk-3alpha and gsk-3beta in prostate cancer. Int J Cancer 131(6): E872-883, 2012. PMID: 22539113. DOI: 10.1002/ijc.27620

Received March 21, 2020
Revised April 12, 2020
Accepted April 13, 2020