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Original Article

The prognosis outcome of oral squamous cell carcinoma using HIF-2α

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Abstract

Background: Hypoxia-induced factors (HIF) has a role in angiogenesis and regulate tumorigenesis of cancer cell. The HIF is the best-identified mechanism that shows imbalance between consumption and oxygen supply in progressing tumor. This study of HIF-2α expression in oral squamous cell carcinoma (OSCC) aimed to investigate the relationship of HIF-2α and pathology characteristics related to its clinical correlation.
Methods: Fifty-eight samples of OSCC and adjacent tissues were fixed in paraffin for microarray preparation. The tissue array then was stained using primary antibody HIF-2α (NB100-122) and autoprobe II ABC universal staining kit. Each tissue sample was captured using camera microscope, and images were analyzed with Photoshop 6.0 using the CMYK method. A statistical analysis was performed with the two-tailed *t*-test, Kaplan−Meier and log-rank test using Prism for Windows version 5.0.
Results: The samples of the non-cancerous matched tissues (NCMTs) paired with their OSCC samples showed HIF-2α overexpression with significance difference $p < 0.0001$. Although no significant difference was found between HIF-2α expression and overall survival rate, cancer-specific survival rate, and disease-free survival rate, the HIF-2α expression showed statistical significance for overall cancer stages with $p = 0.013$. In addition, patients with high HIF-2α expression tended to develop recurrence within 2 years compared to the low expression group.
Conclusion: HIF-2 expression has complicated roles in different cancer types, including OSCC. Our study indicated that HIF-2α overexpression can serve as a good biomarker for cancer status for all tumor stages and may predict an early recurrence within two years.
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Keywords: HIF-2α; IHC; OSCC; Predictor

1. Introduction

Oral squamous cell carcinoma (OSCC) is a common cancer, and its treatment can cause impairment of vital functions and result in cosmetic deformity. Like all solid tumors, OSCC is thought to be initiated and progress through a series of genetic alternations.[1] Its survival rate less than 50% within 5 years.

Tumor hypoxia causes a frequent failure of radiotherapy in solid tumors, and in some clinical and experimental reports, the intra-tumoral oxygen levels may affect the malignant potential of a neoplasm and influence biologic parameters.[2−7] Several factors that cause tumor hypoxia are due to reduced ability of blood to carry oxygen, an inability of cells to use oxygen, generalized or local reduced tissue perfusion, and deterioration of the diffusion geometry.[8]

Tumors can survive and even progress when the oxygen partial pressure falls below a critical value by some molecular mechanism. The mediator of hypoxia in tumor hypoxia, called hypoxia-inducible factors (HIFs), regulates tumorigenesis, including in tumor formation, progression, and response or resistance to certain therapy clearly seen in tumor hypoxia.[9]

Three HIFs (HIF-1, -2 and -3) have been found to regulate transcriptional programs in low oxygen condition. In many

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cellular processes, these HIFs facilitate both oxygen delivery and adaptation during oxygen deprivation including angiogenesis, glucose uptake and metabolism, apoptosis and cell proliferation by regulating some expression genes.[10] Although the two HIFs-(1a and 2a) seem have distinct roles, cooperate and overlap, HIF-1a has been shown predominantly regulate transcriptional targets in the cancer setting.[7–9]

The HIF-2 has been characterized and shares approximately 48% of amino-acid sequence homology with HIF-1, and its expressions are restricted to specific cell types, including fibroblasts of the kidney, type II pneumocytes, endothelial cells, interstitial cells of the duodenum and pancreas.[11] Moreover, HIF-2α expression is correlated with poor patient outcome in colorectal carcinoma, hepatocellular, melanoma, non-small cell lung cancers and ovarian cancer.[12]

Given HIF-2α has an important role in tumorigenesis, regulates some expression genes, and is less studied that its counterpart HIF-1a, we hypothesized that HIF-2α would display overexpression in tumor progression and would result in lower survival rate. To explore this notion, we focused on Taiwanese population and compared their clinical findings.

2. Methods

2.1. Ethics statement

The clinical study was reviewed by the Institutional Review Board (IRB) (approval number 2016-02-005BC) at the Taipei Veterans General Hospital, and informed consent from patients was signed.

2.2. Tissue samples

Tissue samples of OSCC were collected during surgical treatment at the Oral and Maxillofacial Department in Taipei Veteran General Hospital from 2002 to 2007. All patients' clinical data were observed from TNM staging, pathology criteria[13] and survival status, with no age or gender limitation. The data and tissue samples were excluded when there was a lack of pathology information (differentiation status, perineural invasion, lymphovascular permeation, tumor necrosis) or tissue sample loss of more than 50% during the IHC procedure. Only fifty-eight patients met the requirements.

2.3. Tissue microarray (TMA) preparation

All the OSCC tissues and non-cancerous matched tissues (NCMT) were fixed in 10% neutral-buffered formalin and then embedded in paraffin to produce tissue blocks. The sample area was marked after the pathological characteristics were verified by hematoxylin and eosin stain. Each block had 2 pieces of 2.0-mm core tissue harvested by array machine for tissue microarray. This arrayed tissue was placed on 4-mm positively charged slides then heated to 40° for 30 min. A total of 116 tissue samples for each OSCC and NCMT were arranged on 3 slides. After leveling paraffin and cores, the array was cooled to 4° for 15 min. The tissue microarray then was checked and, 48 of NCMTs were excluded due to lack of oral epithelium.

2.4. Immunohistochemistry (IHC)

IHC was performed using the AutoProbe II ABC universal staining kit (Bionexus, Oakland, CA, USA) under the manufacturer's suggested protocols. Section slides with tissue microarray were dewaxed by Xylene, soaked in gradient alcohol (dilution from 100%, 95%, 70%, 50%–30%) and finally soaked in sterilized water. After pressure-boiler treatment in antigen retrieval buffer (10 mM citric acid, pH 6.0) for 50 min, the samples were incubated in 5% hydrogen peroxide for 10 min to inactivate endogenous peroxidase activity. Sections were then incubated with primary antibody against HIF-2α (NB100-122 Novus Biologicals, A-B Littletown, CO, USA) at under 4° overnight. The group with antiserum served as the negative control. Peroxidase staining was then revealed with aminoethyl carbazole (AEC) (10-0047 Genemed Biotechnologies, Inc., South San Francisco, CA, USA). Mayer's hematoxylin (ScyTek Laboratories, Inc., Logan, UT, USA) was used as the counterstain.

2.5. Image analysis

Each tissue core was observed and captured using a camera. The image was analyzed by Photoshop 6.0, and 5 spots were selected randomly from each field. We used the yellow-CMYK method, and the yellow intensity of specimens had a direct relationship with traditional visual scoring. This highly applicable and easy technique can be used with different chromogens including AEC, DAB and NovaRed, and the method also has a high tolerance of hematoxylin, which is usually used as a counterstain in IHC. This indicated that the greater the expression of AEC staining, the higher the percentage of the yellow channel in the CMYK method[14] (Supplementary Fig. 1).

2.6. Statistical analysis

Correlation of clinicopathologic characteristics was evaluated with the two-tailed *t*-test. Overall survival (time range from first treatment to death/last follow-up) was calculated by the Kaplan–Meier method and log-rank test for differences between survival curves. All statistical analyses were performed with Prism for Windows version 5.0. (GraphPad Software, Inc., 2236 Avenida de la Playa, La Jolla, CA, USA)

3. Results

In total of 56 intensity scores of HIF-2α ranging from 10.4% to 60.2% were collected from OSCC tissues. According to the intensities of HIF-2α on the yellow-CMYK scale, they were sorted into three groups, namely high (23/56, 41%), moderate (25/56, 45%) and low/no expression (8/56, 14%) groups (see Fig. 1). Interestingly, as a hypoxia-responsive transcription factor, the majority of HIF-2α was expressed in

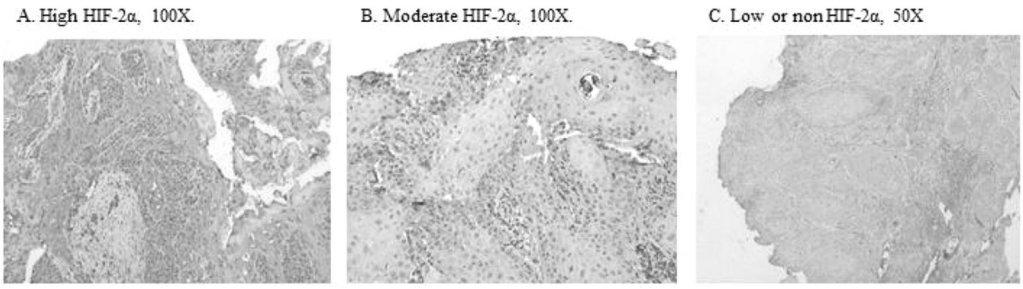

Fig. 1. HIF-2α showed high (A), moderate (B) and low expression (C) in tumor tissue.

the cytosol throughout the whole tumor region, and only some nuclear staining was noted.

Compared with NCMTs, OSCC showed significantly higher expression of HIF-2α ($p < 0.0001$) (Fig. 2). This result suggested that elevation of HIF-2α expression was highly related to the cancer status.

To explore the clinical significance of HIF-2α overexpression, we correlated the HIF-2α expression levels with various clinical parameters, including tumor size, lymph node involvement, differentiation status, perineural invasion, lymphovascular permeation, tumor necrosis, and recurrence. However, we observed no statistically significant difference with $p > 0.05$. However, HIF-2α expression showed statistical significance, with $p = 0.013$ for overall cancer staging (Fig. 3). It meant that HIF-2α may contribute to the progression of OSCC. In our study, we found that level of HIF-2α expression only can be used as a rough prognosis in patients with OSCC.

To investigate the relation of HIF-2α and survival, we applied Kaplan–Meier survival analysis to evaluate the 5-year survival rate of our cohort of oral cancerous patients. The result showed there was no significant difference between HIF-2α expression and overall survival rate, cancer-specific survival rate, and disease-free survival rate. Only slightly better survival was observed in low HIF-2α expression groups. Because oral cancer easily develops recurrence in the first two years after initial treatment, we then re-examined the survival for within 2 years. It showed a lower rate of poor survival for the patients with higher HIF-2α (Fig. 4a). We further analyzed the data of those patients in this cohort who developed recurrent disease after initial treatment. Patients in the high HIF-2α group tended to develop recurrence within 2 years sooner than the low expression group. No patients with high HIF-2α expression developed recurrence after 2 years (Fig. 4b). The results suggested that for OSCC patients with high HIF-2α expression, the first two years is a critical follow-up period for developing recurrent oral cancer, which may serve as a potential early recurrence marker for OSCC.

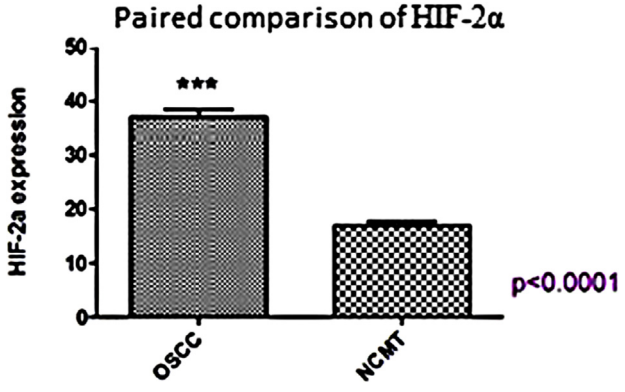

Fig. 2. HIF-2α expression between OSCC and NCMT.

4. Discussion

Hypoxia-inducible factor (HIF)-1 and HIF-2 are heterodimeric transcription factors. The HIF-1α composed of HIF-1α and HIF-1β which identical to aryl hydrocarbon nuclear translocator (ARNT). The alternative dimerization partner which keeping its functional homology with HIF-1α also like endothelial PAS domain protein 1 (EPAS-1) called as HIF-2α, subsequently bind to hypoxia response elements in the promoters of target genes.[15,16]

In contrast to HIF-1α, HIF-2α may have different roles and sometimes plays a good prognostic role in different type of cancers.[17,18] For example, Florczyk et al. showed HIF-1α and HIF-2α have opposite regulatory roles on IL8 expression in endothelial cells.[19] They also have different patterns of activation, tissue expression and favorable oxygen condition in breast cancer.[20] In human lung adenocarcinoma, HIF-2α could sustain prolonged hypoxia, while the expression of HIF-1α would be turned off by HAF and increased natural antisense HIF-1α (aHIIF).[18,21]

Many studies have addressed the roles of HIF-1α and HIF-2α in oral cancers. Despite their differences in expression patterns, regulated genes and clinic-pathological parameters, they usually had similar tumor-promoting effects of angiogenesis and growth.[22]

Our study also suggested higher expression of HIF-2α in OSCC had lower survival rate, though without statistical significance. This weak correlation may result because the expression of HIF-2α in our OSCC specimen was primarily identified in the cytoplasm and only a small amount of nuclear staining was noted. By contrast, in the study of head and neck by Beasley et al., HIF-1α and HIF-2α expression were localized to tumor nuclei and HIF-2α expression was also seen in tumor-associated macrophages.[23]

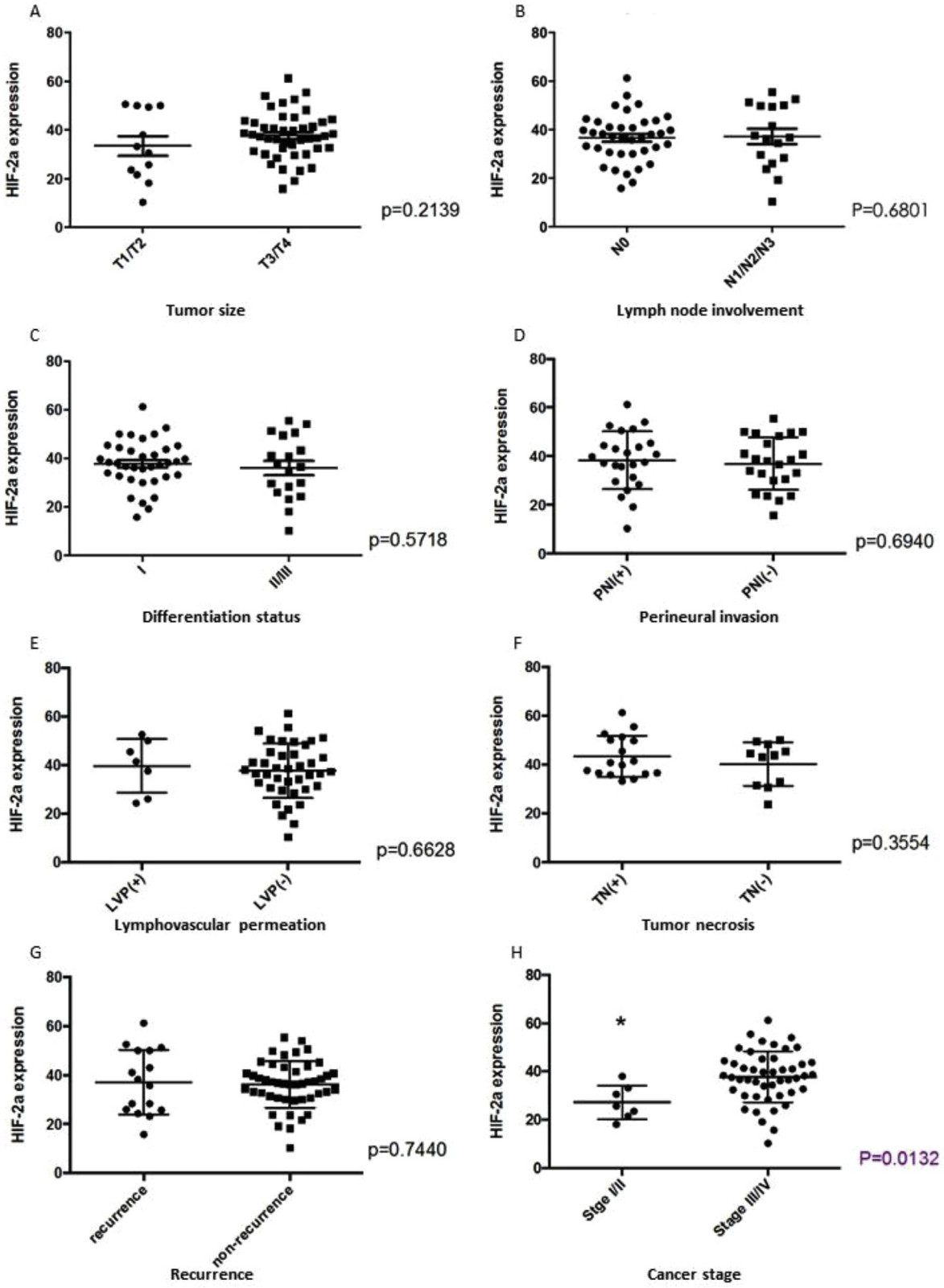

Fig. 3. HIF-2α expression in clinical parameters. (A) Tumor size, (B) Lymph node involvement, (C) Differentiation status, (D) Perineural invasion, (E) Lymphovascular permeation, (F) Tumor necrosis, (G) Recurrence, and (H) Cancer stage.

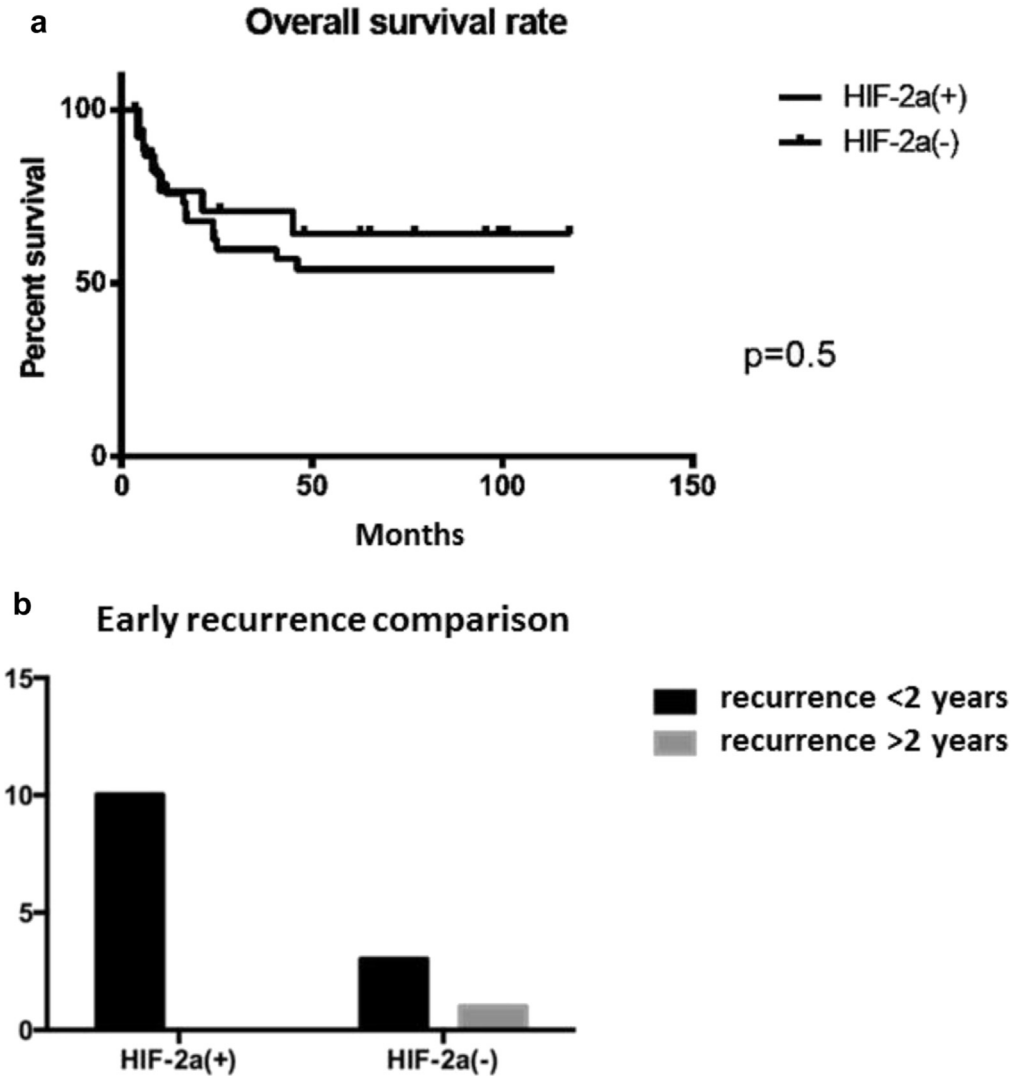

Fig. 4. a. HIF-2α expression and overall survival rate within 2 years. b. HIF-2α expression and early recurrence within 2 years.

When compared to NCMTs, the elevation of HIF-2α expression observed was significant in cancer tissues ($p < 0.05$) in all cancer stages, which was more obvious in stage II or IV patients. We assumed it was related to tumor progression. However, higher HIF-2α was not seen to be different with statistical significance in tumor size, lymph node involvement, differentiation status, and recurrence in our study. That may be due to small sample size. Our findings were consistent with those of other studies on some cancer types. For example, HIF-1α has been shown to have no clinical significance with clinical and pathological parameters in tongue carcinoma.[24] Moreover, both HIF-1α and HIF-2α expression levels showed no difference related to recurrence and primary tumor, including, T grade, T size and T stage in bladder cancer.[25]

For diagnostic purpose in OSCC, HIF-2α was correlated well with cancer status regardless of overall cancer staging, as in Eckart's study.[22]

For survival contribution, our results revealed that HIF-2α expression level had no significant roles in overall survival, disease-specific survival and disease-free survival rate. Liang et al., found a similar result in tongue squamous cell carcinoma, that HIF-2α showed no correlation with overall survival or disease-free survival, but shorter disease-free survival in HIF-1α overexpressed groups.[26] However, a study by Beasley et al. in HNSCC patients showed better overall survival and disease-free survival for HIF-1α highly expressed patients but not for HIF-2α.[23] On the contrary, some studies showed that negative or low HIF-1α expression had better disease-free survival rate in tumors of the floor mouth[27], OSCC[28,29] and osteosarcoma.[30] Therefore, the exact roles of HIF in OSCC are still controversial.

Interestingly, when we looked into survival during shorter period for patients developing recurrent diseases after initial treatment, patients with high HIF-2α expression tended to develop recurrence within 2 years compared to the low expression group. No patients with high HIF-2α expression developed recurrence after 2 years. This result suggested the first two years is a critical time for OSCC patients with high HIF-2α expression. A more frequent follow-up schedule is

necessary for this group of OSCC patients. The possible reasons may be related to failure of adjuvant chemoradiotherapy therapy, some recent studies have implied that HIF-2α had roles in chemo/radioresistance through various mechanisms.[31]

Generally, our study showed HIF-2α can serve as a good biomarker for cancer in all stages and is not a good independent prognostic factor for OSCC patients. A similar finding was noted for gastroesophageal carcinomas, that HIF-2α did not prove to be an independent prognostic marker, but the survival of patients with HIF-2α-negative tumors was higher than that of those with HIF-2α-positive tumors.[32] However, high expression of HIF-2α may contribute to the early recurrence within two years of OSCC patients, and be an indicator for intensive follow-up plan. A larger sample size of patients may be needed to validate the related clinical application.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jcma.2017.06.005.

References

1. Leemans CR, Braakhuis BJ, Brakenhoff RH. The molecular biology of head and neck cancer. *Nat Rev Cancer* 2011;**11**:9−22.
2. Vaupel P, Kelleher D. Tumor hypoxia: pathophysiology, clinical significance and therapeutic perspectives. *Exper Oncol* 2001;**23**:78−9.
3. Raleigh JA. Hypoxia and its clinical significance. *Semin Radiat Oncol* 1996;**6**:1−2.
4. Brown JM, Giaccia AJ. The unique physiology of solid tumors: opportunities (and problems) for cancer therapy. *Cancer Res* 1998;**58**:1408−16.
5. Semenza GL. Hypoxia, clonal selection, and the role of HIF-1 in tumor progression. *Crit Rev Biochem Mol Biol* 2000;**35**:71−103.
6. Semenza GL. HIF-1: mediator of physiological and pathophysiological responses to hypoxia. *J Appl Physiol* 2000;**88**:1474−80.
7. Sutherland RM. Tumor hypoxia and gene expression: implications for malignant progression and therapy. *Acta Oncol* 1998;**37**:567−74.
8. Hockel M, Vaupel P. Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. *J Natl Cancer Inst* 2001;**93**:266−76.
9. Lu X, Kang Y. Hypoxia and hypoxia-inducible factors: master regulators of metastasis. *Clin Cancer Res* 2010;**16**:5928−35.
10. Rankin EB, Giaccia AJ. The role of hypoxia-inducible factors in tumorigenesis. *Cell Death Differ* 2008;**15**:678−85.
11. Wiesener MS, Jurgensen JS, Rosenberger C, Scholze CK, Horstrup JH, Warnecke C, et al. Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs. *FASEB J* 2003;**17**:271−3.
12. Talks KL, Turley H, Gatter KC, Maxwell PH, Pugh CW, Ratcliffe PJ, et al. The expression and distribution of the hypoxia-inducible factors HIF-1alpha and HIF-2alpha in normal human tissues, cancers, and tumor-associated macrophages. *Am J Pathol* 2000;**157**:411−21.
13. *AJCC cancer staging manual*. Vol 6th ed. USA Springer; 2002.
14. Pham NA, Morrison A, Schwock J, Aviel-Ronen S, Lakovlev V, Tsao MS, et al. Quantitative image analysis of immunohistochemical stains using a CMYK color model. *Diagn Pathol* 2007;**2**:8.
15. Carroll VA, Ashcroft M. Targeting the molecular basis for tumour hypoxia. *Expert Rev Mol Med* 2005;**7**:1−16.
16. Kawanaka T, Kubo A, Ikushima H, Sano T, Takegawa Y, Nishitani H. Prognostic significance of HIF-2alpha expression on tumor infiltrating macrophages in patients with uterine cervical cancer undergoing radiotherapy. *J Med Invest* 2008;**55**:78−86.
17. Keith B, Johnson RS, Simon MC. HIF1alpha and HIF2alpha: sibling rivalry in hypoxic tumour growth and progression. *Nat Rev Cancer* 2012;**12**:9−22.
18. Loboda A, Jozkowicz A, Dulak J. HIF-1 versus HIF-2- Is one more important than the other? *Vasc Pharmacol* 2012;**56**:245−51.
19. Florczyk U, Czauderna S, Stachurska A, Tertil M, Nowak W, Kozakowska M, et al. Opposite effects of HIF-1alpha and HIF-2alpha on the regulation of IL-8 expression in endothelial cells. *Free Radic Biol Med* 2011;**51**:1882−92.
20. Camps C, Buffa FM, Colella S, Moore J, Sotiriou C, Sheldon H, et al. hsa-miR-210 is induced by hypoxia and is an independent prognostic factor in breast cancer. *Clin Cancer Res* 2008;**14**:1340−8.
21. Uchida T, Rossignol F, Matthay MA, Mounier R, Couette S, Clottes E, et al. Prolonged hypoxia differentially regulates hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha expression in lung epithelial cells: implication of natural antisense HIF-1alpha. *J Biol Chem* 2004;**279**:14871−8.
22. Eckert AW, Schutze A, Lautner MH, Taubert H, Schubert J, Bilkenroth U. HIF-1alpha is a prognostic marker in oral squamous cell carcinomas. *Int J Biol Markers* 2010;**25**:87−92.
23. Beasley NJ, Leek R, Alam M, Turley H, Cox GJ, Gatter K, et al. Hypoxia-inducible factors HIF-1alpha and HIF-2alpha in head and neck cancer: relationship to tumor biology and treatment outcome in surgically resected patients. *Cancer Res* 2002;**62**:2493−7.
24. Roh JL, Cho KJ, Kwon GY, Ryu CH, Chang HW, Choi SH, et al. The prognostic value of hypoxia markers in T2-staged oral tongue cancer. *Oral Oncol* 2009;**45**:63−8.
25. Ioachim E, Michael M, Salmas M, Michael MM, Stavropoulos NE, Malamou-Mitsi V. Hypoxia-inducible factors HIF-1alpha and HIF-2alpha expression in bladder cancer and their associations with other angiogenesis-related proteins. *Urol Int* 2006;**77**:255−63.
26. Liang X, Zheng M, Jiang J, Zhu G, Yang J, Tang Y. Hypoxia-inducible factor-1 alpha, in association with TWIST2 and SNIP1, is a critical prognostic factor in patients with tongue squamous cell carcinoma. *Oral Oncol* 2011;**47**:92−7.
27. Fillies T, Werkmeister R, van Diest PJ, Brandt B, Joos U, Buerger H. HIF1-alpha overexpression indicates a good prognosis in early stage squamous cell carcinomas of the oral floor. *BMC Cancer* 2005;**5**:1−9.
28. Tilakaratne WM, Nissanka-Jayasuriya EH. Value of HIF-1alpha as an independent prognostic indicator in oral squamous cell carcinoma. *Expert Rev Mol Diagn* 2011;**11**:145−7.
29. Eckert A, Lautner M, Schütze A, Taubert H, Schubert J, Bilkenroth U. Coexpression of hypoxia-inducible factor-1alpha and glucose transporter-1 is associated with poor prognosis in oral squamous cell carcinoma patients. *Histopathology* 2011;**58**:1136−47.
30. Yang Q, Zeng B, Dong Y, Shi Z, Jiang Z, Huang J. Overexpression of hypoxia-inducible factor-1alpha in human osteosarcoma: correlation with clinicopathological parameters and survival outcome. *Jpn J Clin Oncol* 2007;**37**:127−34.
31. Zhao JDF, Luo Y, Shen G, Zheng F, Xu B. The emerging role of hypoxia-inducible factor-2 involved in chemo/radioresistance in solid tumors. *Cancer Treat Rev* 2015;**41**:623−33.
32. Griffiths EAPS, McGrath SM, Valentine HR, Price PM, Welch IM, West CML. Hypoxia-associated markers in gastric carcinogenesis and HIF-2alpha in gastric and gastro-oesophageal cancer prognosis. *Br J Cancer* 2008;**98**:965−73.