Increased Expression of *EIF5A2*, Via Hypoxia or Gene Amplification, Contributes to Metastasis and Angiogenesis of Esophageal Squamous Cell Carcinoma

Yan Li,[1] Li Fu,[1,2] Jian-Biao Li,[1] Yanru Qin,[3] Ting-ting Zeng,[1] Jie Zhou,[1] Zhao-Lei Zeng,[1] Jinna Chen,[1] Ting-Ting Cao,[2] Xiaojiao Ban,[1] Chaonan Qian,[1] Zongwei Cai,[4] Dan Xie,[1] Peng Huang,[1] and Xin-Yuan Guan[1,2]

[1]*Sun Yat-Sen University Cancer Center, State Key Laboratory of Oncology in South China, Collaborative Innovation Center for Cancer Medicine, Guangzhou, China;* [2]*Department of Clinical Oncology, The University of Hong Kong, Hong Kong, China;* [3]*Department of Clinical Oncology, The First Affiliated Hospital, Zhengzhou University, Zhengzhou, China; and* [4]*Hong Kong Baptist University, Hong Kong, China*

BACKGROUND & AIMS: Solid tumors often become hypoxic, leading to activation of hypoxia-response genes. We investigated the effects of overexpression of the hypoxia response genes *eIF5A2* in esophageal squamous cell carcinoma (ESCC). **METHODS:** We used quantitative real-time polymerase chain reaction and immunohistochemistry analyses to compare expression of *eIF5A2* between paired ESCC samples and nontumor esophageal tissues, and fluorescence in situ hybridization to detect gene copy-number alterations. Luciferase reporter and chromatin immunoprecipitation assays were used to study interactions between *eIF5A2* and hypoxia-inducible factor-1α (*HIF1*α). We determined the effects of *eIF5A2* overexpression and knockdown in ESCC cell lines and growth of ESCC xenograft tumors in nude mice. **RESULTS:** Levels of *eIF5A2* messenger RNA and protein were increased in >40% of ESCC samples compared with matched nontumor tissues, along with levels of HIF1α and vascular endothelial growth factor. Increased levels of EIF5A2 were significantly associated with ESCC metastasis to lymph nodes ($P < .001$) and tissue invasion ($P = .037$), and shorter survival times of patients ($P < .001$). Amplification of *eIF5A2* was detected in 35.14% of ESCC samples that overexpressed eIF5A2. Hypoxia increased expression of *eIF5A2* 4- to 8-fold in ESCC cell lines; we observed bidirectional regulation between *eIF5A2* and *HIF1*α. Transient transfection of ESCC cell lines with *eIF5A2* increased their migratory and invasive abilities and markers of the epithelial to mesenchymal transition, and *eIF5A2* knockdown or HIFα inhibition reduced these. In mice, xenograft tumors grown from ESCC cells that expressed eIF5A2 formed tumors more rapidly than cells that expressed only vector (controls); they also expressed higher levels of HIF1α and vascular endothelial growth factor, and formed more microvessels than controls. Knockdown of *eIF5A2* in ESCC cells with interfering RNAs reduced their growth as xenograft tumors in mice, particularly when mice were given docetaxel or cisplatin. **CONCLUSIONS:** *eIF5A2* is overexpressed by gene amplification or hypoxia in ESCCs, and associated with up-regulation of HIF1α, metastasis, and shorter survival times of patients. Increased expression of eIF5A2 increases metastasis and angiogenesis in ESCC via the *HIF1*α-mediated signaling pathway.

Keywords: Oxygen; Gene Regulation; EMT; Oncogene.

Esophageal squamous cell carcinoma (ESCC) is the seventh most common cancer and the fifth most common cause of cancer-related death in the world.[1] Esophagectomy is the potentially curative treatment in patients with early-stage ESCC.[2] However, most patients at presentation have locally advanced disease, and 20% to 30% has distant metastasis.[3] Tumor metastasis is the major cause of ESCC-related death, and identifying its mechanisms remains important and urgent. Like other solid tumors, the pathogenesis of ESCC is a long-term process involving multiple genetic and epigenetic alterations. Amplification of 3q26 is one of the most frequent genetic alterations in many malignancies, including nasopharyngeal carcinoma[4] and ESCC.[5] In our previous study, we isolated an oncogene, named *eukaryotic initiation factor 5A2* (*eIF5A2*), from chromosome 3q26.2.[6] Interestingly, overexpression of *eIF5A2* has been associated with tumor metastasis in various solid tumors, including colorectal[7] and liver cancers.[8] In hepatocellular carcinoma, overexpression of EIF5A2, which is mainly detected in tumor cells invading to surrounding tissue, can promote tumor invasion and metastasis by inducing epithelial-mesenchymal transition (EMT).[8] Although *eIF5A2* can promote cell motility in hepatocellular carcinoma and colorectal cancer, little progress has been made regarding the precise mechanism underlying the involvement of eIF5A2 in tumor metastasis.

One previous study found that yeast TIF51A and TIF51B, homologs of human EIF5A and EIF5A2, are regulated reciprocally by oxygen.[9] TIF51A is expressed under aerobic conditions, and TIF51B is expressed under anaerobic conditions. Accordingly, we propose here that besides being

Abbreviations used in this paper: **ChIP, chromatin immunoprecipitation; eIF5A2, eukaryotic initiation factor 5A2; EMT, epithelial-to-mesenchymal transition; ESCC, esophageal squamous cell carcinoma; FISH, fluorescence in situ hybridization; HIF-1, hypoxia-inducible factor-1; IF, immunofluorescence; IHC, immunohistochemistry; qRT-PCR, quantitative real-time polymerase chain reaction; sh, short hairpin; VEGF, vascular endothelial growth factor; WB, Western blotting.**

0016-5085/$36.00
http://dx.doi.org/10.1053/j.gastro.2014.02.029

regulated by gene amplification, EIF5A2 expression may also be regulated by hypoxic conditions in cancer. Hypoxia is a powerful driving force to breakdown normal tissue homeostasis and rearrangement of tumor–stroma interactions in tumor progression.[10] The best characterized hypoxia response pathway is mediated by hypoxia-inducible factor-1 (HIF-1).[11] By interacting with the co-activator CBP/p300, HIF-1 activates, transcription of target genes associated with angiogenesis, tumor cell survival, invasion, and metastasis.[11] To test whether *eIF5A2* can be induced by hypoxia and its role in tumor invasion and metastasis, we investigated the expression pattern of *eIF5A2* under hypoxic stress and its effect on tumor invasion and metastasis in ESCC. Our data indicated that *eIF5A2* expression could be induced by both gene amplification and hypoxia. Bidirectional regulation between *eIF5A2* and *HIF1α* plays an important role in tumor invasion, metastasis, and angiogenesis.

Materials and Methods

Cell Lines

ESCC cell lines KYSE140, KYSE180, KYSE410, KYSE510, and KYSE520 were obtained from DSMZ, the German Resource Center for Biological Material.[12] ESCC cell lines EC18, EC109, HKESC1, and immortalized esophageal epithelial cell line NE1 were provided by Professors G. Srivastava and GS Tsao (The University of Hong Kong).

Primary Tumor Tissues, Tissue Microarray, and Immunohistochemistry

Patients with ESCC were selected consecutively from the surgical pathology archives of the Linzhou Cancer Hospital (Henan, China). No patient in the study had received preoperative radiation or chemotherapy. Tissue microarray was constructed as reported previously.[6] Studies using human tissues were reviewed and approved by the Committees for Ethical Review of Research involving Human Subjects of Sun Yat-sen University. Immunohistochemistry (IHC) was performed using the standard streptavidin-biotin-peroxidase complex method.

Fluorescence In Situ Hybridization

A bacterial artificial chromosome clone containing *eIF5A2* and the centromere probe of chromosome 3 were labeled with Spectrum-orange-deoxyuridine triphosphate and Spectrum-green-deoxyuridine triphosphate by nick translation (Vysis; Abbott Laboratories, Abbott Park, IL), respectively. Fluorescence in situ hybridization (FISH) was performed as described previously.[6]

Interactions Between Eukaryotic Initiation Factor 5A2 and Hypoxia-Inducible Factor-1α

Luciferase assay, chromatin immunoprecipitation (ChIP), and electrophoretic mobility shift assay were used to study the mechanism of EIF5A2 and HIF1α interaction (Supplementary Materials).

Functional Assays

Both in vitro and in vivo assays were applied to investigate tumor growth, cell motility, and metastasis (Supplementary Materials).

Statistical Analysis

Data were expressed as mean ± SEM. The correlation between EIF5A2 expression and clinicopathologic features of ESCC patients was analyzed by the Pearson χ^2 test. Survival curves were generated according to the Kaplan-Meier method, and statistical analysis was performed using the log-rank test. The Cox proportional hazards regression model was used to identify independent prognostic factors. All statistical analyses were performed using statistical software (SPSS 16.0 for Windows; SPSS, Inc., Chicago, IL).

Results

Eukaryotic Initiation Factor 5A2 Is Frequently Overexpressed in Esophageal Squamous Cell Carcinoma

Expression of *eIF5A2* was compared by quantitative real-time polymerase chain reaction (qRT-PCR) between tumor and paired nontumor tissues in 71 ESCC cases. Compared with corresponding nontumor tissues, overexpression of *eIF5A2* (defined as a >2.5-fold increase) was detected in 31 of 71 (43.66%) of ESCCs ($P = .05$, Figure 1*A*). Compared with the immortalized esophageal epithelial cell line NE1, Western blot analysis (WB) showed that expression of EIF5A2 was increased in most ESCC cell lines except in KYSE510 (Figure 1*B*). Expression of EIF5A2 was also investigated by IHC with a monoclonal EIF5A2 antibody using an ESCC tissue microarray containing 300 pairs (tumor vs corresponding nontumor tissues) of ESCCs. Informative IHC data were obtained from 232 tumor tissues and 215 nontumor tissues. The frequency of positive staining of EIF5A2 was significantly higher in tumors (95 of 232 [40.95%]) than that in nontumor tissues (36 of 215 [16.74%]) ($P < .001$; Figure 1*C*). Similar to our previous report,[8] the expression level of EIF5A2 was often higher in tumor cells at the edge of the tumor tissue (Figure 1*C*).

Clinical Significance of Eukaryotic Initiation Factor 5A2 Overexpression in Esophageal Squamous Cell Carcinoma

The correlation of EIF5A2 overexpression with ESCC clinicopathologic features was analyzed statistically using IHC data from 232 informative ESCCs. The results found that EIF5A2 expression was positively correlated with lymph node metastasis ($P < .001$), depth of tumor invasion ($P = .037$), and advanced stages ($P = .001$; Table 1). Kaplan-Meier analysis showed that the overall survival rate of ESCC patients with EIF5A2 overexpression was poorer ($P < .001$; Figure 1*D*). Multivariate analysis also demonstrated that EIF5A2 is an independent prognostic factor (Supplementary Table 1). The correlation between *eIF5A2* overexpression and clinicopathologic features in the qRT-PCR cohort (n = 71) was summarized in Supplementary Table 2. These