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Original contribution

SMYD3 stimulates EZR and LOXL2 transcription to enhance proliferation, migration, and invasion in esophageal squamous cell carcinoma☆,☆☆

Ying Zhu MS[a,b], Meng-Xiao Zhu MS[a,b], Xiao-Dan Zhang PhD[a,b], Xiu-E Xu BSc[a,b], Zhi-Yong Wu MD[d], Lian-Di Liao BSc[a,b], Li-Yan Li PhD[a,b], Yang-Min Xie MS[a,e], Jian-Yi Wu BSc[a,c], Hai-Ying Zou PhD[a,c], Jian-Jun Xie PhD[a,c], En-Min Li PhD[a,c,*], Li-Yan Xu PhD[a,b,*]

[a]*The Key Laboratory of Molecular Biology for High Cancer Incidence Coastal Chaoshan Area, Shantou University Medical College, Shantou 515041, Guangdong, PR China*
[b]*Institute of Oncologic Pathology, Shantou University Medical College, Shantou 515041, Guangdong, PR China*
[c]*Department of Biochemistry and Molecular Biology, Shantou University Medical College, Shantou 515041, Guangdong, PR China*
[d]*Departments of Oncology Surgery, Shantou Central Hospital, Affiliated Shantou Hospital of Sun Yat-Sen University, Shantou 515041, Guangdong, PR China*
[e]*Experimental Animal Center, Shantou University Medical College, Shantou 515041, Guangdong, PR China*

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Summary Epigenetic alterations, including DNA methylation and histone modifications, are involved in the regulation of cancer initiation and progression. SET and MYND domain-containing protein 3 (SMYD3), a methyltransferase, plays an important role in transcriptional regulation during human cancer progression. However, SMYD3 expression and its function in esophageal squamous cell carcinoma (ESCC) remain unknown. In this study, SMYD3 expression was studied by immunohistochemistry in a tumor tissue microarray from 131 cases of ESCC patients. Statistical analysis showed that overall survival of patients with high SMYD3 expressing in primary tumors was significantly lower than that of patients with low SMYD3-expressing tumors ($P = .008$, log-rank test). Increased expression of SMYD3 was found to be associated with lymph node metastasis in ESCC ($P = .036$) and was an independent prognostic factor for poor overall survival ($P = .025$). RNAi-mediated knockdown of SMYD3 suppressed ESCC cell proliferation, migration, and invasion in vitro and inhibited local tumor invasion in vivo. SMYD3 regulated transcription of *EZR* and *LOXL2* by directly binding to the sequences of the promoter regions of these target genes, as demonstrated by a chromatin immunoprecipitation assay. Immunohistochemical staining of ESCC tissues also confirmed that protein levels of EZR and LOXL2 positively correlated with SMYD3 expression, and the Spearman correlation coefficients (r_s) were 0.78 (n = 81; $P < .01$) and 0.637 (n = 103; $P < .01$), respectively. These results indicate that SMYD3 enhances tumorigenicity in ESCC through enhancing transcription of genes involved in proliferation, migration, and invasion.

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* Corresponding authors at: The Key Laboratory of Molecular Biology for High Cancer Incidence Coastal Chaoshan Area, Shantou University Medical College, Shantou, Guangdong, PR China.
E-mail addresses: nmli@stu.edu.cn (E. -M. Li), lyxu@stu.edu.cn (L. -Y. Xu).

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1. Introduction

Esophageal cancer occurs with the highest rates in southern and eastern Africa and eastern Asia [1], with 90% of the cases being squamous cell carcinomas [2,3]. Most patients with esophageal squamous cell carcinoma (ESCC) are at an advanced stage with local invasion and metastasis at the time of diagnosis because there are few symptoms during the initial phase. Thus, it is important to explore the molecular mechanism(s) responsible for ESCC development.

Altered patterns of histone modifications are involved in many human diseases, including cancer [4]. Much is now known about the importance and the complexity of histone methylation. SET (Su) and MYND (myeloid-Nervy-DEAF-1) domain-containing protein 3 (SMYD3), a histone methyltransferase, dimethylates and trimethylates H3K4, which in turn activates transcription of its downstream target genes [5]. SMYD3 is highly overexpressed in most cancers [5-7]. Several trials have explored the effects of SMYD3 overexpression on cell viability, adhesion, and migration [8]. These studies suggest that SMYD3 may function as an oncogene in these cancers. However, the function and new target genes of SMYD3 in ESCC remain unclear.

In this study, we examined the expression of SMYD3 in ESCC. We found the expression of SMYD3 was up-regulated in human ESCC tissues. We further explored the function of SMYD3 in ESCC cell lines and nude mice. Furthermore, we identified EZR and LOXL2, 2 important ESCC functional genes, as new target genes of SMYD3 in ESCC cells.

2. Materials and methods

2.1. Cell lines and culture

Human ESCC cell lines SHEEC and KYSE150 were used. The SHEEC cell line was established by our laboratory in 2000 [9]. The KYSE150 cell line was previously established from a primary human ESCC in 1992 [10]. SHEEC cells were cultured in Dulbecco's modification of Eagle's medium Dulbecco (DMEM)/F12 medium (Thermo Fisher Scientific, Shanghai, China) with 10% newborn bovine serum. KYSE150 cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum. All cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

2.2. Patients and samples

For the retrospective study, 131 archival formalin-fixed, paraffin-embedded specimens were obtained from the Department of Pathology, Shantou Central Hospital, from 2000 to 2006. The samples were used for tissue microarray (TMA) construction before immunohistochemical staining. Information on sex, age, stage of disease, and histopathologic parameters retrieved from the medical records is shown in Supplementary Table S1. All tumors were confirmed to be ESCC by pathologists in the Clinical Pathology Department of the Hospital, and the cases were classified according to the seventh edition of the TNM classification of the American Joint Committee on Cancer [11]. Evaluation of tumor differentiation was based on histologic criteria of the guidelines of the World Health Organization Pathological Classification of Tumors [12]. This study was approved by the ethics committee of the Center Hospital of Shantou City and the local ethics committee, and only patients with written informed consent were included.

2.3. TMA construction and immunohistochemical staining

The tissue microarray construction and immunohistochemical staining of esophageal carcinoma tissues were described earlier [13,14]. TMA sections were sectioned (4 μm) and incubated with a rabbit polyclonal antibody against human SMYD3 antibody (1:50; Abgent, San Diego, CA). Detailed information about the primary antibodies is listed in Supplementary Table S2. Phosphate-buffered saline (PBS), instead primary antibody, was used for the negative controls. Then, immunostaining was carried out by a PV-9000 2-step plus Poly-HRP Anti-Rabbit IgG Detection System (ZSGB-BIO, Beijing, China) and a Liquid DAB Substrate Kit (Invitrogen, San Francisco, CA).

The immunohistochemical staining results were assigned a mean score taking into consideration both the intensity of staining and the proportion of tumor cells showing an unequivocal positive reaction. Each section was independently assessed by 2 histopathologists without prior knowledge of the patient data. For SMYD3, a staining index (values 0-12) was determined by multiplying the score for staining intensity with the score for positive area. The intensity of staining was scored as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. Tumor cell area was scored as follows: 1, positive staining in 1% to 25% of tumor cells; 2, positive staining in 26% to 50% of tumor cells; 3, positive staining in 51% to 75% of tumor cells; 4, positive staining in 76% to 100% of tumor cells. For statistical analyses, a *composite staining index* was defined as the product of the intensity and area scores. The X-tile software program (version 3.6.1), as previously described [15], was used to determine the best threshold value for classifying samples into high (scores of 5-12) and low (scores of 0-4) expression. For EZR (ezrin), immunohistochemical staining and standard for evaluation were fully performed as by Xie et al [14].

2.4. Western blotting analysis

Total proteins were extracted from cells, using Radio-Immunoprecipitation Assay (RIPA) buffer, and protein