DNMT1–MicroRNA126 Epigenetic Circuit Contributes to Esophageal Squamous Cell Carcinoma Growth via ADAM9–EGFR–AKT Signaling

Ronghua Liu1,2, Jie Gu3, Pei Jiang1,2, Yijie Zheng1,2, Xiaoming Liu1,2, Xuechao Jiang1,2, Enyu Huang1,2, Shudao Xiong1,4, Fengkai Xu3, Guangwei Liu1,2, Di Ge3, and Yiwei Chu1,2

Abstract

Purpose: MicroRNAs (miRNA) are involved in and are controlled by epigenetic regulation, and thereby form a reciprocal regulatory circuit. Using next-generation sequencing (NGS)–based miRNA profiling, this study aimed to discover esophageal squamous cell carcinoma (ESCC)–specific miRNAs and miRNA-related epigenetic modulations.

Experimental Design: NGS-based miRNA profiles were generated for four pairs of ESCC tissues and adjacent normal tissues. In situ hybridization was used to assess miRNA expression and its correlation with prognosis. miRNA-related DNA methylations were identified using bisulfite genomic sequencing, and the role of DNA methyltransferase 1 (DNMT1) was investigated using RNA interference. miRNA targets were screened by mRNA sequencing, and functional validation was performed in vitro and in vivo.

Results: NGS-based miRNA profiling identified 78 differentially expressed miRNAs in ESCC. Among them, microRNA126-3p (miR-126) was significantly downregulated, and its downregulation correlated with poor ESCC prognosis. Downregulation of miR-126 was due to promoter hypermethylation of its host gene, Egfl7. DNMT1 was aberrantly upregulated in ESCC and responsible for the hypermethylation of Egfl7. Intriguingly, DNMT1 was suppressed by overexpression of miR-126, indicating the existence of a regulatory feedback circuit. ADAM9 was identified as a key target of miR-126. Ectopic expression of miR-126 or silencing of ADAM9 reduced ESCC cell proliferation and migration by inhibiting epidermal growth factor receptor–AKT signaling.

Conclusions: Our results indicate that miR-126 is a potential prognostic indicator for ESCC and suggest that a novel “DNMT1–miR-126 epigenetic circuit” is involved in ESCC progression. Consequently, miR-126–based epigenetic modulations may provide a basic rationale for new approaches to antitumor therapeutics.Clinical Cancer Res; 1–10. © 2014 AACR.

Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive malignancies of the gastrointestinal tract (1). Despite improvements in diagnostic approaches and the introduction of adjuvant chemotherapy, the 5-year survival rate in patients with esophageal cancer remains less than 30% (2). Previous studies have described complicated genetic and epigenetic alterations involved in ESCC progression (3, 4), but the underlying mechanisms have not been fully elucidated. Therefore, to develop better approaches for ESCC diagnosis and therapy, a comprehensive understanding of the molecular variables that affect ESCC pathways is needed.

MicroRNAs (miRNA) have emerged as key players in human tumors; their dysregulation affects cell proliferation (5), differentiation (6), invasion (7), and the epithelial–mesenchymal transition (EMT; ref. 8). miRNA expression profiles have been developed for use as prognostic/predictive biomarkers and been used to identify the molecular signatures and underlying functional mechanisms of various cancers. Previous miRNA profiling studies were conducted using miRNA expression microarray platforms (9, 10). However, in recent decades, the emergence of next-generation sequencing (NGS) platforms has revolutionized genomic studies. In comparison with microarray, NGS-based sequencing has advantages for the analysis of widely expressed miRNAs, the identification of miRNA sequence variations, and the discovery of novel miRNAs (11, 12). Although, microarray-based miRNA profiles of ESCC have been described (13), to our knowledge, no studies have used an NGS-based platform to identify ESCC-specific miRNAs. Furthermore, the pathologic mechanisms underlying their dysregulation remain incompletely understood.
Translational Relevance
Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive malignancies of the gastrointestinal tract. Previous studies have described complicated genetic and epigenetic alterations involved in ESCC progression, but the underlying mechanisms have not been fully elucidated. Recent evidence shows that microRNAs (miRNA) are involved in and are controlled by epigenetic regulation, and thereby form a reciprocal regulatory circuit in many systems. In this study, using NGS-based miRNA profiling, we found the downregulation of miR-126 in ESCC and proposed a “DNMT1–miR-126 epigenetic circuit” involved in ESCC progression. Moreover, miR-126 acted as a novel tumor suppressor that effectively suppresses ESCC cell proliferation and migration by repressing ADAM9–EGFR–AKT signaling transduction and as an independent prognostic indicator for patients with ESCC. Taken together, our funding of “DNMT1–miR-126 epigenetic circuit” suggests another insight into pathologic mechanisms underlying ESCC initiation and progression, implying its clinical significance in developing targets for ESCC prediction and therapy.

Materials and Methods
Samples and cells
ESCC specimens embedded in paraffin were obtained from 185 patients who underwent esophagectomy at Zhongshan Hospital (Shanghai, China) in 2007 with informed consent, and clinicopathologic characteristics of patients were listed in Supplementary Table S1. Charlson comorbidity index (CCI) was used for the analysis of comorbidities (17). Each of the paraffin blocks was well formalin-fixed, paraffin-embedded, and without necrosis and hemorrhage. Follow-up was conducted every 6 months, and ended in December 2012, with a median follow-up of 32 months. The clinical endpoint was the survival state of patient at the last follow-up. Another 30 pairs of frozen human primary human ESCC tissues (EC) and matched adjacent noncancerous tissues (NM) were obtained from 30 patients at Zhongshan Hospital in 2012. Tumor stage was determined according to seventh edition of the Union for International Cancer Control-American Joint Committee on Cancer tumor, node, metastasis staging system (18). Approval for the study was obtained from the Research Ethics Committee of Zhongshan Hospital.

ESCC cell line ECa-109 was obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Science (TCHu 69, Shanghai, China), and ESCC cell line KYSE-510 (ICRBI436, National Institute for Biomedical Innovation, Japan) and normal human esophageal epithelial cell line HEEpiC (HEEC; #2720; ScienCell Research Laboratories) were supplied by Dr. Lu (Department of Thoracic Surgery, Zhongshan Hospital, Shanghai, China) (19), which were authenticated using short tandem repeat (STR) profiling and used in the sixth month after receipt. All the cells were cultured in RPMI-1640 supplemented with 10% FBS, 2 μmol/L glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin sulfates.

NGS-based miRNA profiling
Total RNA from four pairs of ECs and matched NMs were isolated using a mirVana miRNA Isolation Kit (Ambion) according to the manufacturer’s instructions. After PAGE purification, small RNA molecules were amplified and sequenced on an Illumina platform according to the manufacturer’s instructions (20).

Tissue microarray and in situ hybridization
Tissue microarrays were constructed as described in a previous study (21). Digoxin (DIG)-labeled oligonucleotide probes complementary to hsa-miR-126 were designed as previously described and purchased from ExonBio (22). The probe binding sequence was 5’-UCGILIACCGI1GAGUAA1LI1GCGG-3’. The slides were hybridized with DIG-conjugated hsa-miR-126 probes overnight at 42°C and stained using an Enhanced Sensitive in situ hybridization (ISH) Detection Kit II (AP) with phosphatase-conjugated anti-DIG antibody (Boster) according to the manufacturer’s instructions.

Statistical analysis
Data are presented as the mean ± SEM of replicate experiments (n ≥ 3). Results were analyzed using the SPSS 16.0 software (SPSS) and PRISM 5.0 (GraphPad Software Inc.). The Student unpaired t test or unpaired t test with Welch’s correction was used to analyze intergroup differences for two groups, ANOVA was used to analyze more than two groups, and Pearson’s correlation coefficient was used to analyze the correlation between groups. The cumulative survival time was calculated using the Kaplan–Meier method and analyzed using the log-rank test. Univariate and multivariate analyses were based on the Cox proportional hazards regression model. P values < 0.05 were considered statistically significant.

Results
NGS-based discovery of miR-126 and validation of its downregulation in ESCC
To identify ESCC-specific miRNA profiles, NGS-based miRNA profiling was performed on four pairs of frozen ECs and adjacent NMs. When ECs and NMs were compared, 78 miRNAs were...
differentially expressed, of which 44 were downregulated and 34 were upregulated in ECs ($P < 0.05$ with fold-change > 3; Fig. 1A; Supplementary Table S2). Among the differentially regulated miRNAs, the downregulation of miR-126-3p in ECs exhibited the strongest statistical significance. For validation, qPCR was used to assess the expression of miR-126 in 30 pairs of frozen ECs and matched NMs, as well as in ESCC cell lines (ECa-109 and KYSE-510) and HEECs, normal human esophageal epithelial cells. The results showed that miR-126 expression was also downregulated in ESCC tissues ($P < 0.001$; Fig. 1B) and cell lines ($P < 0.001$; Fig. 1C), when compared with expression in NMs and HEECs, respectively.

Downregulation of miR-126 correlates with poor prognosis in ESCC patients

To address the clinical significance of miR-126 in ESCC, ISH was performed in 185 ESCC specimens. Patients with negative miR-126 expression exhibited lower 5-year disease-specific survival rate than that with positive miR-126 expression ($P = 0.014$, Fig. 1D). In multivariate analysis, CCI, tumor stage, and miR-126 expression were identified as independent prognostic factors in patients’ disease-specific survival (Supplementary Table S3). These results suggest that downregulation of miR-126 is an independent indicator of prognosis in ESCC.

Hypermethylation of the *Egfl7* promoter induces miR-126 downregulation in EC

Given that the miR-126 gene is located in an intron of *Egfl7* and its expression is reportedly upregulated in cancer cells treated with inhibitors of DNA methylation (23), we analyzed the sequence of *Egfl7* and found that a large CpG island was present in the promoter region (Fig. 2A). To understand the correlation between methylation of the *Egfl7* promoter and the downregulation of miR-126, we analyzed CpG island methylation in ECs and
matched NMs. The results showed that Egfl7 promoter methylation was higher in ECs than in NMs (Fig. 2B). Samples with higher methylation exhibited lower miR-126 expression (Fig. 2C). To determine the effect of methylation of the Egfl7 promoter on the expression of miR-126, genomic DNA was extracted from ECa-109 and KYSE-510 cells treated with 5-aza-dC or vehicle. After sodium-bisulfite modification, methylation-specific PCR (MSP) was carried out using the specific primers for the unmethylated (U) or methylated (M) Egfl7 promoter sequence. As a result, compared with vehicle-treated cells in which the Egfl7 promoter was hypermethylated, cells treated with the demethylating agent 5-aza-dC to induce demethylation of the Egfl7 promoter showed more unmethylated sequences and an increase in miR-126 (Fig. 2D). These results indicate that hypermethylation of the Egfl7 promoter directly downregulates miR-126 in ESCC.

**DNMT1–miR-126 regulatory circuit contributes to Egfl7 promoter hypermethylation**

DNA methyltransferases (DNMT) are critical for gene-specific methylation and the consequent transcriptional silencing of cancer-related genes (24). In this study, we found that DNMT1 expression was dramatically increased in ESCC tumor tissues and cell lines (Fig. 3A; Supplementary Fig. S1A). Furthermore, correlation analysis showed that the expression of DNMT1 inversely correlated with that of endogenous miR-126 (Fig. 3A). To investigate the role of DNMT1 in the regulation of miR-126 expression, we silenced DNMT1 expression in cells using siRNA. DNMT1 knockdown reduced the hypermethylation of the miR-126 host gene (Fig. 3B), leading to miR-126 upregulation (Fig. 3C). Interestingly, overexpression of miR-126 suppressed DNMT1 expression in both KYSE-510 and ECa-109 cells (Fig. 3D). Collectively, overexpression of DNMT1 resulted in the hypermethylation and downregulation of miR-126, whereas overexpression of miR-126 suppressed DNMT1 expression. Our findings indicate the involvement of a novel DNMT1–miR-126 regulatory loop in ESCC.

**Overexpression of miR-126 suppresses ESCC cell proliferation and migration**

Given the low expression of miR-126 in ESCC, we further evaluated the biologic role played by miR-126 in ESCC cell lines. miR-126 mimics were transfected into ECa-109 and KYSE-510 cells to upregulate miR-126 expression (Supplementary Fig. S2A). Overexpression of miR-126 markedly suppressed cell proliferation (Fig. 4A) and the rate of colony formation (Fig. 4B) in ECa-109 and KYSE-510 cells when compared with the negative control. Further analysis showed that miR-126 mainly promoted cell apoptosis, but without a marked effect on the cell cycle (Supplementary Fig. S2B and C). In addition, overexpression of miR-126 inhibited ESCC cell migration in vitro (Fig. 4C; Supplementary Fig. S3A).

To confirm the suppressive role of miR-126 in ESCC, we next investigated the effect of miR-126 on ESCC tumor growth in vivo. ECa-109 and KYSE-510 cells transfected with either miR-126 or miR-NC (control group) were implanted subcutaneously into the bilateral posterior flank of nude mice. Compared with tumor growth in the control group, tumor growth in the...
miR-126–transfected group was markedly suppressed, as reflected by the smaller tumor sizes (Fig. 4D, Supplementary Fig. S3B). Collectively, these results indicate that miR-126 functions as a tumor suppressor by inhibiting ESCC cell growth and migration.

The ADAM9–EGFR–AKT signaling directly mediates the suppressive role of miR-126

To investigate the targets and downstream signaling pathway of miR-126, we compared mRNA profiles of ECa-109 cells transfected with miR-126 (126-EC cells) or miR-NC (NC-EC cells) mimics using NGS-based mRNA sequencing. In 126-EC cells, 31 genes were significantly downregulated relative to the levels in NC-EC cells (Supplementary Table S4), including ADAM9. Consistently, in bioinformatics analysis, ADAM9, a putative target of miR-126, was identified with four prediction algorithms (PicTar, TargetScan, miRanda, and miRDB; Supplementary Table S4). Therefore, ADAM9 was selected for further validation and functional analyses. Results from qPCR, Western blot analysis, and immunohistochemistry (IHC) showed that ADAM9 was overexpressed in ECs and ESCC cell lines and that its mRNA levels inversely correlated with miR-126 levels in ECs (Fig. 5A–B; Supplementary Fig. S4A). This inverse correlation strongly suggested the involvement of ADAM9 in the miR-126–related network. Bioinformatics analysis showed that the binding sites in the 3′-UTR of ADAM9 and miR-126 are highly conserved (Fig. 5C). We constructed the ADAM9-3′-UTR reporter plasmid and performed luciferase reporter assays in cells transfected with the 3′-UTR plasmid. As expected, miR-126 repressed luciferase activity in cells expressing ADAM9-3′-UTR, indicating a direct interaction between miR-126 and ADAM9 in ESCC cells (Fig. 5C). Furthermore, a decrease in ADAM9 was detected in miR-126–overexpressing ESCC cells (P < 0.05; Fig. 5D). To address the direct involvement of ADAM9 in miR-126–regulated tumor suppression, ECa-109, KYSE-510, and KYSE-510 cells were transfected with miR-126 and miR-NC mimics was determined by Western blotting.

ADAM9 activates EGFR by cleaving the transmembrane pro-heparin–binding epidermal growth factor-like growth factor (pro-HB-EGF) precursor to yield HB-EGF, a soluble EGFR ligand. HB-EGF binds and activates intracellular signaling cascades downstream of EGFR, such as the AKT pathway, and thereby promotes the growth of normal and neoplastic cells (25, 26). In our study, we found that the EGFR–AKT pathway was involved in the growth of normal and neoplastic cells (25, 26). In our study, we found that the EGFR–AKT pathway was involved in the suppressive action of miR-126/ADAM9 in ESCC. Both overexpression of miR-126 and blockage of ADAM9 reduced levels of p-EGFR and p-AKT (Fig. 6B), cell proliferation, and cell migration (Fig. 6C). These results indicate that ADAM9 is the target of miR-126 and is regulated by the “DNMT1–miR-126 circuit” in ESCC.

Discussion

The “epigenetic–miRNA loop,” wherein miRNAs regulate or are regulated by epigenetic factors, was proposed by Pagano and colleagues (27). They suggested that epigenetic–miRNA loops contributed to hematopoiesis by orchestrating gene expression in hematopoietic cells. In this study, we identified
the DNMT1–miR-126 epigenetic loop in ESCC and investigated its contribution to ESCC development via modulation of the ADAM9–EGFR–AKT pathway. Dysregulation of miRNAs in diseases is frequently due to epigenetic changes in the miRNA genome (28, 29). Previous studies of miRNA genomic sequences have indicated that promoter hypermethylation of miRNAs occurs frequently as a result of aberrant expression of DNMTs, histone deacetylases, and methyl-CpG-binding proteins (29–31). In the present study, we found that miR-126 was downregulated in ECs and that its downregulation was induced by promoter hypermethylation of its host gene. Dysregulation of miR-126 has also been observed in other tumors, such as colon cancer (32) and lung cancer (33), but little is known about its function in ESCC. This is the first report to show that miR-126 is downregulated and acts as a new tumor suppressor in ESCC. Moreover, we found that downregulation of miR-126 is a potential prognostic indicator for ESCC.

Figure 4.
Overexpression of miR-126 suppresses ESCC cell proliferation and migration. A, ECa-109 and KYSE-510 cells were transfected with miR-126 (100 nmol/L) or miR-NC (100 nmol/L) mimics for 24 hours, and cell viability and proliferation were assessed with the CCK-8 assay at 24, 48, 72, and 96 hours. B, cell colonies were counted after transfection for 12 days. The colony formation ratio was calculated as follows: (visible colony number/original number of cells) × 100. C, the total number of migrated cells was calculated 48 hours after transfection. D, ECa-109 or KYSE-510 cells transfected with miR-126 or miR-NC mimics were injected subcutaneously into either side of the posterior flank of the same nude mouse, respectively (n = 4). Photographs show mice and tumors 4 weeks after tumor cell implantation. Tumor sizes are shown as the mean ± SD. Values represent the mean ± SD (**, P < 0.01; ***, P < 0.001).

Although evidence from Zhang and colleagues indicated that the downregulation of miR-126 in breast cancer positively correlated with hypermethylation of the Egfl7 T2 promoter (35), the underlying molecular mechanism was not elucidated. Notably, our results demonstrate that DNMT1 is overexpressed in primary ECs and cell lines and that DNMT1 is responsible for the hypermethylation of the Egfl7 promoter, leading to miR-126 downregulation.

DNMT1, a member of the DNMT family (DNMT1, DNMT3A, and DNMT3B), is overexpressed in tumors and is essential for maintaining promoter hypermethylation of tumor-suppressive genes, leading to their silencing (36). In breast cancer, overexpressed DNMT1 induces miR-148a/152 silencing by maintaining the hypermethylation of their promoters and contributes to tumor transformation and angiogenesis (37). In ESCC, we demonstrate that overexpressed DNMT1 was responsible for the promoter hypermethylation of the miR-126 host gene, thereby downregulating miR-126 and inhibiting ESCC growth and cell migration. Interestingly, in a feedback mechanism, DNMT1 was suppressed by miR-126 overexpression in ESCC cells, indicating the presence of a novel "DNMT1–miR-126 epigenetic circuit." We also showed that DNMT1 was the direct target of miR-126 (Supplementary Fig. S5). Regulation of DNMT1 by miR-126 has also been described in CD4+ T cells from patients with systemic lupus erythematosus (37). Similar to the "DNMT1–miR-126 epigenetic circuit" in ESCC, a negative feedback regulatory loop between miR-148/152 and DNMT1 has been reported in breast cancer (38). Thus, epigenetic–miRNA loops are widely present in various tumors. Given that both miRNA and epigenetic modifications strongly regulate
the expression of various disease-associated genes (14), controlling the dynamic balance of epigenetic–miRNA loops might be of great clinical significance.

The expression of ADAM9 has been reported to correlate with cell adherence and migration (25, 33, 39, 40). This study found that ADAM9 functioned as a direct target of miR-126 and contributed to miR-126 repressing cell migration in ESCC. Consistently, the targeting of miR-126 to ADAM9 and the role of ADAM9 in cell migration was also reported in a previous study by Hamada and colleagues (39). A similar approach with our study using restoration of miR-126 and silencing of ADAM9 showed inhibition of the invasive growth. To systematically investigate the target of miR-126 in ESCC, RNA sequencing was applied in the present study to examine the differential gene expression in miR-126–overexpressed ESCC EC-109 cells versus control cells. ADAM9 was showed to be a functional target of miR-126, whereas other potential targets are under exploration. Therefore, apart from pancreatic cancer in Hamada's study (39), our study also identified the interaction between miR-126 and ADAM9 in ESCC. In addition, we investigated the downstream pathway and found that ADAM9 was critical for promoting cell proliferation in ESCC by targeting EGFR–AKT signaling. ADAM9 cleaved the pro-HB-EGF precursor to yield soluble HB-EGF, which binds to EGFR, thereby activating downstream signal transduction (26, 41). The EGFR–AKT pathway is important for cell growth and migration (42, 43). Our results showed that blocking ADAM9 inhibits EGFR–AKT activation by suppressing phosphorylation, but has no effect on ERK phosphorylation. We also found that the expression of phosphoinositide-3-kinase, regulatory subunit 2 (beta) was downregulated upon miR-126 overexpression (Supplementary Table S4 and Supplementary Fig. S6A). However, its expression in ECs and in NMds did not differ (P > 0.05), and its inverse correlation with miR-126 expression was poor (R = –0.1791; P = 0.5255; Supplementary Fig. S6B and S6C). Other growth-related targets of miR-126 identified in other tumors, such as Crk (44) and IRS (45), were not downregulated in miR-126–overexpressing ECa-109 cells (Supplementary Fig. S6A), implying they are not key targets of miR-126 in ESCC growth.

With accumulating evidence demonstrating the importance of miRNAs in cancer biology, miRNA-based cancer therapy emerges as a hot issue and also a big challenge in the current miRNA research. To be excited, MRX34, as the first microRNA-based therapy for cancer, is entering the phase I trial (46). In the present study, our primary data showed that the exogenous miR-126 mimics suppressed ESCC cell growth, which implies its potential therapeutic significance in ESCC using a miRNAs' "replacement" strategy. However, miR-126–based therapy for ESCC in practice still needs to be further explored, considering some challenges, especially technical limitations, such as how to improve its accumulation in the target tissues and balance the safety and efficiency. In general, there is a long way ahead to realize the translation of fundamental research of miR-126 to clinical applications in ESCC.

Figure 5.
ADAM9 is the target of miR-126 in ESCC. A, ADAM9 RNA levels in 30 matched esophageal cancer tissues and their adjacent normal tissues were determined using qPCR and normalized to GAPDH expression. Data shown are the mean ± SD values (**, P < 0.001). ADAM9 RNA levels in cancer tissues negatively correlated with endogenous miR-126 levels (P = 0.0019, R = –0.5446, n = 30). B, ADAM9 protein levels were measured in six matched ESCC samples and three cell lines by Western blotting. IHC staining for ADAM9 was performed in ECs and matched NMds. Magnification, ×200; ×400. C, alignment of miR-126 with the ADAM9 3'–UTR. MiR-126 (100 nmol/L) or miR-NC (100 nmol/L) mimics were cotransfected with the ADAM9–3–UTR reporter plasmid, along with a control Renilla luciferase pRL-TK vector, into Ecaries-109 or KYSE-510 cells. The relative luciferase activity in each group was analyzed 48 hours later and expressed relative to the activity in the miR-NC–transfected group. Data are representative of three independent experiments. Values represent the mean ± SD (**, P < 0.001). D, ADAM9 expression in ECa-109 and KYSE-510 cells transfected with miR-126 mimics or siRNA against DNMT1 (si-D) was determined by Western blotting.
In summary, this study proposes a "DNMT1–miR-126 epigenetic circuit" in ESCC and sheds light on its importance for ESCC progression via regulation of ADAM9–EGFR–AKT signaling. Altering critical gene expression and signal transduction by modulating miR-126-related epigenetic dynamics might be of clinical significance in ESCC prediction and therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: R. Liu, P. Jiang, Y. Zheng, D. Ge, Y. Chu
Development of methodology: R. Liu, J. Gu, P. Jiang, X. Liu, X. Jiang, E. Huang, E. Xu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Liu, J. Gu, X. Liu, E. Huang, Y. Chu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Liu, J. Gu, P. Jiang, X. Jiang, F. Xu, G. Liu, Y. Chu
Writing, review, and/or revision of the manuscript: R. Liu, J. Gu, Y. Zheng, X. Liu, S. Xiong, G. Liu, Y. Chu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Liu, J. Gu, P. Jiang, Y. Zheng, Y. Chu
Study supervision: Y. Zheng, D. Ge, Y. Chu

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