

Circulating lncRNA DLG1-AS1 in Plasma as a Novel Diagnostic Marker for Esophageal Squamous Cell Carcinoma (ESCC)

Xiuqin Wei

Beijing rehabilitation hospital

Qiang Gao

Beijing rehabilitation Hospital

Wei Jia

Beijing rehabilitation hospital

Chao Ma

Beijing rehabilitation Hospital

Mei Xue

Beijing rehabilitation Hospital

Yali Wang

Beijing rehabilitation Hospital

Mengqiao Zhang

Beijing rehabilitation Hospital

Lei Su (✉ leisu1968@163.com)

Xuanwu Hospital

Research

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Abstract

BACKGROUND: Esophageal squamous cell carcinoma (ESCC) in some cases can be diagnosed as esophageal varices (EV). DLG1-AS1 promotes cervical cancer, while its function in other malignancies remains unknown. Our aim for this study is to study the role of DLG1-AS1 in esophageal squamous cell carcinoma.

METHODS: Plasma levels of DLG1-AS1 in 66 early stage ESCC patients, 60 EV patients and 60 healthy controls were measured by RT-qPCR. Receiver operating characteristic (ROC) curve was applied to analyze the diagnostic value of DLG1-AS1 for early stage ESCC. Relationship between miR-145 and DLG1-AS1 was analyzed by overexpression experiments. Proliferation of cells was determined by CCK-8 assay.

RESULTS: DLG1-AS1 was upregulated in ESCC, but not in EV patients compared with healthy control. DLG1-AS1 overexpression distinguished ESCC patients from healthy controls and EV patients. Plasma miR-145 was inversely correlated with DLG1-AS1 in ESCC patients. Moreover, DLG1-AS1 overexpression resulted in the downregulation of miR-145, while miR-145 mimic transfection did not significantly alter DLG1-AS1. Overexpression of DLG1-AS1 mediated the promoted, while overexpression of miR-145 resulted in inhibited proliferation of ESCC cells. The role of DLG1-AS1 overexpression was inhibited by miR-145 mimic transfection.

CONCLUSION: Therefore, DLG1-AS1 may promote ESCC under the repression of miR-145.

Background

In spite of the low incidence rate, esophageal squamous cell carcinoma (ESCC) exhibits extreme aggressive nature and causes high mortality rate (1). Every year, ESCC causes more than 400000 deaths every year (1). The high mortality rate of ESCC is mainly caused by the low early diagnosis rate (2). Most patients with ESCC are diagnosed with inoperable conditions and the overall 5-year survival rate is below 15% (3). Several diagnostic biomarkers, such as carcinoembryonic antigen, have been developed to diagnose ESCC, while there are not sensitive enough at early cancer stages (4, 5).

In clinical practices, ESCC at early stages is sometimes misdiagnosed as esophageal varices (EV) due to their similar imaging features (6). Therefore, development novel biomarkers to distinguish ESCC from other esophageal lesions are urgently needed. Long (>200nt) non-coding RNAs (lncRNAs) are plays crucial roles in cancers (7). lncRNAs are usually spatially and temporally expressed (8), thereby providing potentials for disease diagnosis. Previous studies have identified a big number of lncRNAs with potential functions in ESCC(9, 10), while their functionality and clinical values for ESCC remains elusive. A recent study reported that DLG1-AS1 could promote cervical cancer (11). Interestingly, our preliminary deep sequencing data revealed its upregulation in ESCC but not in EV, and the inverse correlation between DLG1-AS1 and miR-145 (data not shown), which suppresses ESCC (12). We therefore performed more in-depth investigations in this study to analyze the role of DLG1-AS1 in ESCC and its interaction with miR-145.

Methods

Research subjects

ESCC group included 66 patients with ESCC (40 cases of stage I and 26 cases of II, 39 males and 27 females, 31 to 64 years, 45.4 ± 5.6 years). EV group included 60 patients with EV (35 males and 25 females, 32 to 64 years, 45.8 ± 5.1 years). Therefore were 60 healthy volunteers (35 males and 25 females, 31 to 64 years, 45.9 ± 5.3 years) in control group. Based on the tumor location, the 66 ESCC patients induced 19, 25 and 22 cases at upper, middle, and lower sites of esophagus, respectively. All these participants were enrolled in Xuanwu Hospital, Capital Medical University during the time period between July 2016 and August 2018. Patients with history of other malignancies or initiated therapy, with other clinical disorders, failed to cooperate with researchers, did not provide consent for potential publication of the present study were not included. Ethics Committee of the aforementioned hospital approved this study.

Plasma and cells

Before therapies, fasting blood (5 ml) was extracted and blood samples were centrifuged in EDTA tubes at 1200g for 10 min to collect plasma.

Human ESCC cell lines KYSE510 and Hs 285.T (ATCC, USA) were used. Cell culture medium was RPMI-1640 medium (10 % FBS). A 5 % CO₂ and 95% humidity incubator was used to cultivate cells at 37 °C.

RT-qPCR

Ribozol reagent (Sigma-Aldrich, USA) was used for RNA isolation. Following cDNA synthesis, qPCRs were performed to determine DLG1-AS1 expression with 18S rRNA as endogenous control. MiRNA RTs and qPCRs were performed using mirVana qRT-PCR miRNA Detection Kit (Invitrogen) to determine miR-145 expression with internal control of U6. Three technical replicates were included in each experiment, and $2^{-\Delta\Delta Ct}$ method was applied for normalizations.

Transient cell transfection

Negative control miRNA and miR-145 mimic, as well as DLG1-AS1 expression vector (pcDNA3.1) and empty vector were all from Sangon (Shanghai, China). Through Lipofectamine 3000 Reagent-mediated transient transfections, KYSE510 and Hs 285.T cells were transfected with either 8 nM vector or 36 nM miRNA. To perform NC experiments, empty vector or NC miRNA transfection was performed. To perform Control (C) experiments, untransfected cells were further cultivated for 54h. The duration of transfections was 6h. Cell culture was performed for further 48h after transfections before subsequent experiments.

Methylation-specific PCR (MSP)

KYSE510 and Hs 285.T cells were used to isolate genomic DNAs and the genomic DNA was converted by EZ DNA Methylation-Gold™ kit (ZYMO RESEARCH). After that, PCR reactions were performed using Taq DNA polymerase kit (NEB).

CCK-8 assay

KYSE510 and Hs 285.T cells with transfections were transferred to a 96-well plate with 3500 cells in 0.1 ml medium per well. Cells were cultivated under the aforementioned conditions. To determine cell proliferation, CCK-8 solution was added to 10% at 2h before the measurement of OD values (450 nm), which was performed at 24, 48, 72 and 96h later.

Statistical process

Gene expression levels were expressed as average values of three technical replicates and data were compared by unpaired t test. Mean \pm values were used to express data of triplicates of multiple transfection groups, and were compared by ANOVA Tukey's test. Correlations were analyzed by linear regression. ROC curve analysis was performed for diagnostic analysis. Differences were statistically significant at $p < 0.05$.

Results

Plasma DLG1-AS1 was specifically upregulated in ESCC patients

RT-qPCR was performed to determine DLG1-AS1 expression in plasma. Differences of DLG1-AS1 levels in plasma among ESCC group (n=66), EV group (n=60) and Control group (n=60) were explored. DLG1-AS1 in plasma in ESCC group was significantly upregulated in comparison to EV and Control groups (Fig.1, $p < 0.05$). Moreover, plasma levels of DLG1-AS1 were not statistically significant different between EV and Control groups (Fig.1).

Plasma DLG1-AS1 showed diagnostic value for ESCC

In ROC analysis EV patients were first used as true negative cases, with this analysis, AUC was 0.91 (standard error: 0.026; 95% confidence interval: 0.85-0.96; Fig.2A; $p < 0.0001$). After that, healthy controls were further used true negative cases, with this analysis, AUC was 0.91 (standard error: 0.025; 95% confidence interval: 0.86-0.96; Fig.2B; $p < 0.0001$).

DLG1-AS1 downregulated miR-145 in ESCC cells

Plasma miR-145 in ESCC patients was also determined. Correlation analysis revealed that miR-145 was inversely correlated with DLG1-AS1 in ESCC patients (Fig.3A). DLG1-AS1 expression vector or miR-145 mimic were transfected into KYSE510 and Hs 285.T cells. At 24 h after transfection, transfections were confirmed (Fig.3B, $p < 0.05$). DLG1-AS1 overexpression resulted in the downregulation of miR-145 (Fig.3C, $p < 0.05$), while miR-145 overexpression did not significantly alter DLG1-AS1 expression (Fig.3D).

DLG1-AS1 promoted ESCC cell proliferation through miR-145

Analysis of cell proliferation data showed that overexpression of DLG1-AS1 mediated the promoted, while overexpression of miR-145 resulted in inhibited proliferation of ESCC cells (Fig.4, $p < 0.05$). The role of DLG1-AS1 overexpression was significantly inhibited by miR-145 mimic transfection. Therefore, DLG1-AS1 could downregulate miR-145 to suppress ESCC (Fig.4, $p < 0.05$).

DLG1-AS1 promoted the methylation of miR-145 gene in ESCC cells

MSP was performed to analyze the methylation of miR-145 gene in ESCC cells with DLG1-AS1 overexpression. It was observed that, DLG1-AS1 expression vector transfection increased methylation of miR-145 in comparison to empty vector transfection (Fig.5).

Discussion

We analyzed the role of DLG1-AS1 in ESCC. We found DLG1-AS1 can be used as early diagnostic biomarker for ESCC. DLG1-AS1 may promote ESCC cell proliferation by downregulating miR-145, which is a tumor suppressive miRNA in ESCC (12).

Due to the lack of cures for metastatic cancers, early diagnosis is always critical for the survival of cancer patients (2, 13). However, many types of cancer do not have classic clinical symptom at early stages. Therefore, development of molecular biomarkers is needed to detect the development of cancer before the appearance of visible tumors (14). DLG1-AS1 is a recently identified lncRNA with known oncogenic functions only in cervical cancer (11). However, its clinical significance is unknown. In the present study we revealed the upregulation of DLG1-AS1 in ESCC but not in EV. To test its values for the early detection of ESCC, only patients at stage I and II, which are considered as early cancer stages, are included. It was found that altered expression of DLG1-AS1 distinguished ESCC from EV and healthy controls. Therefore, plasma circulating DLG1-AS1 may be used to assist early diagnosis of ESCC.

Besides the roles in regulating the protein-coding genes (15, 16), lncRNAs can also participate in cancer biology by regulating other non-coding RNAs, such as miRNA (17, 18). We found that DLG1-AS1 was the upstream inhibitor of miR-145 in regulating ESCC cell proliferation. However, no specific binding site of miR-145 was found on DLG1-AS1. We proved that DLG1-AS1 overexpression in ESCC cells could increase the methylation of miR-145 gene. Therefore, DLG1-AS1 may downregulate miR-145 through methylation. Our study suggested that DLG1-AS1 might be used as a maker for the early diagnosis of ESCC. However, our study is limited by the small size. In addition, we failed to explore the in vivo function of DLG1-AS1 in ESCC. Future studies are needed to include more ESCC patients to further analyze the diagnostic accuracy. In addition, animal model experiments are also needed to explore the functions of DLG1-AS1 in vivo.

Conclusion

In conclusion, DLG1-AS1 was upregulated in ESCC, and DLG1-AS1 overexpression has early diagnostic values for ESCC. In addition, DLG1-AS1 may promote ESCC cell proliferation by downregulating miR-145.

Abbreviations

esophageal squamous cell carcinoma (ESCC);

esophageal varices (EV);

Receiver operating characteristic (ROC)

negative control (NC);

control (C)

Declarations

Ethics approval and consent to participate

This study was approved by Ethics Committee of Xuanwu Hospital, Capital Medical University. And informed consent was obtained from all individual participants.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

XQW and QG: experimental work, manuscript writing, literature search and data analysis; WJ, CM, MX, YLW and MQZ: experimental work, data analysis and statistical analysis. LS: project management, manuscript review and research design. All authors read and approved the final manuscript.

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Figures

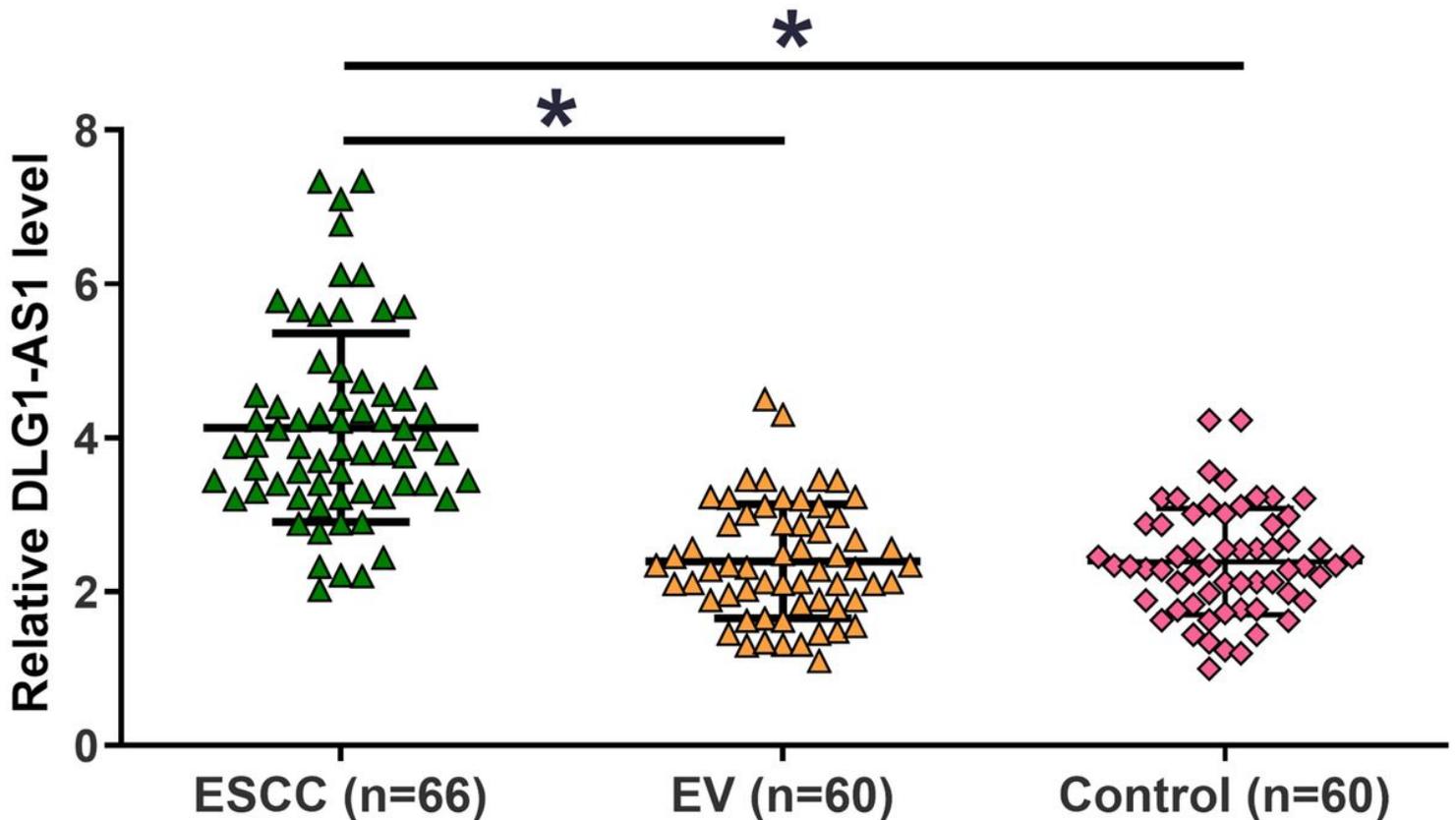


Figure 1

Plasma DLG1-AS1 was specifically upregulated in ESCC patients. Analysis of qPCR results showed that plasma levels of DLG1-AS1 were significantly higher in ESCC group than in EV and Control groups. Moreover, plasma levels of DLG1-AS1 were not statistically significant different between EV and Control groups (*, $p < 0.05$).

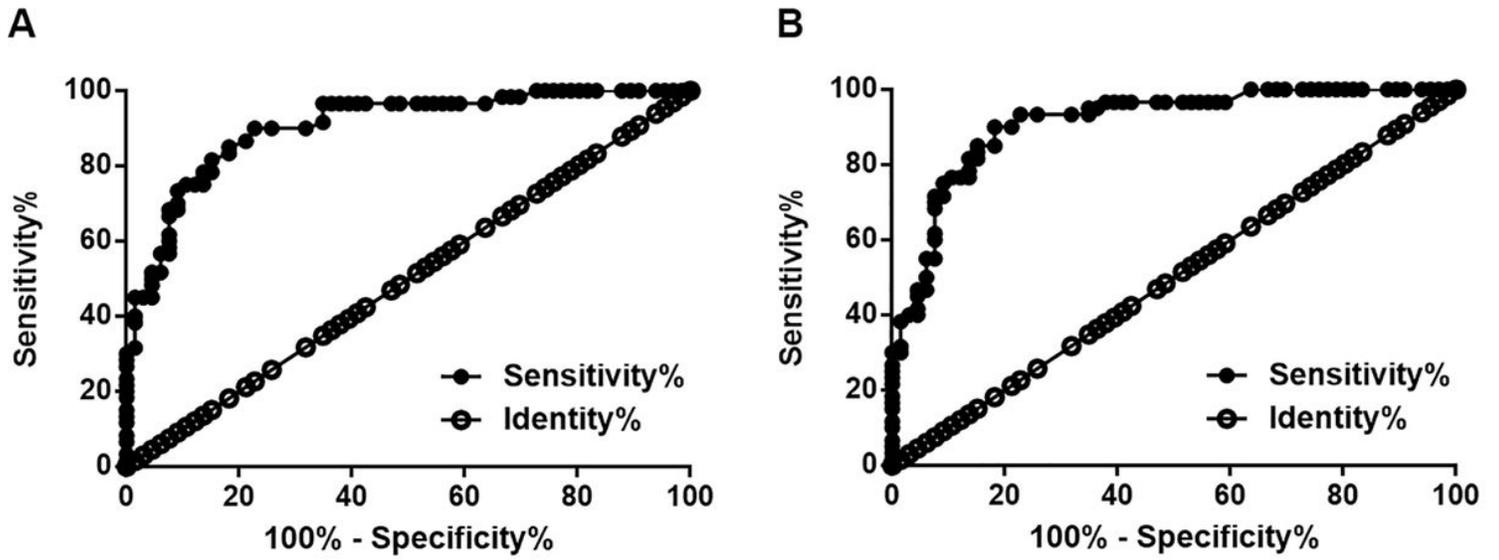


Figure 2

DLG1-AS1 may promote ESCC by downregulating miR-145. ROC curve analysis showed that DLG1-AS1 overexpression distinguished ESCC patients from EV patients (A) and healthy controls (B).

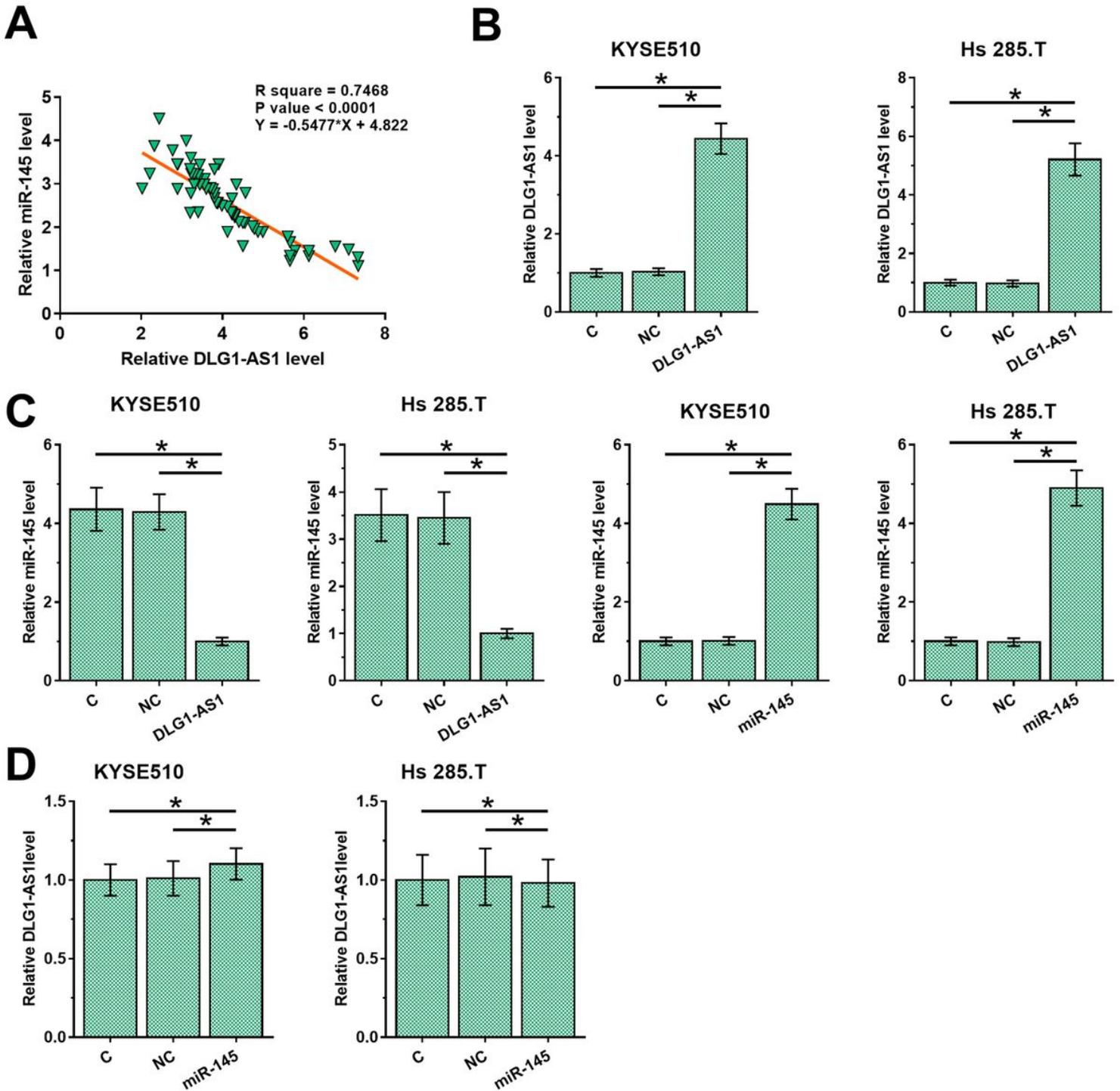


Figure 3

DLG1-AS1 downregulated miR-145 in ESCC cells. Linear regression revealed that plasma miR-145 was inversely correlated with DLG1-AS1 in ESCC patients (A). At 24 h after transfection, DLG1-AS1 and miR-145 were significantly upregulated comparing to two controls in KYSE510 and Hs 285.T cells (B). In addition, cells with DLG1-AS1 expression vector transfection exhibited downregulated miR-145 (C), while miR-145 overexpression failed to significantly affect DLG1-AS1 (D) (*, $p < 0.05$).

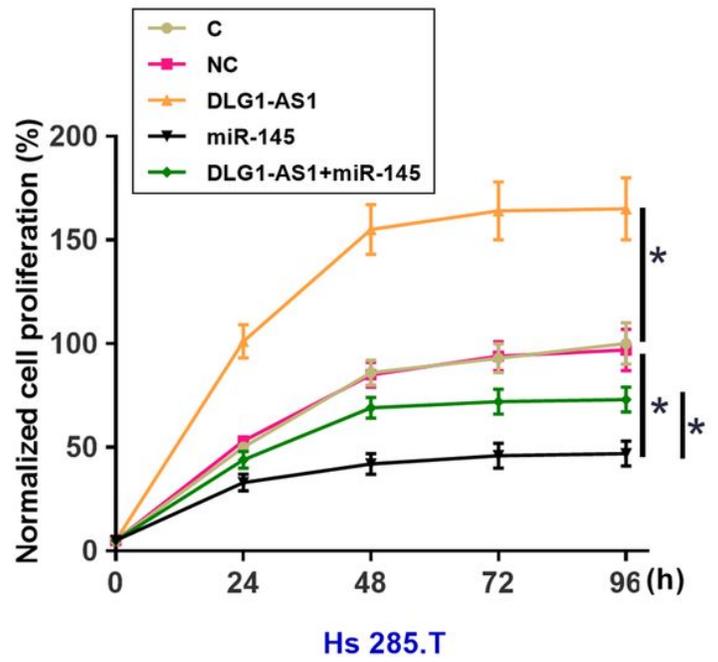
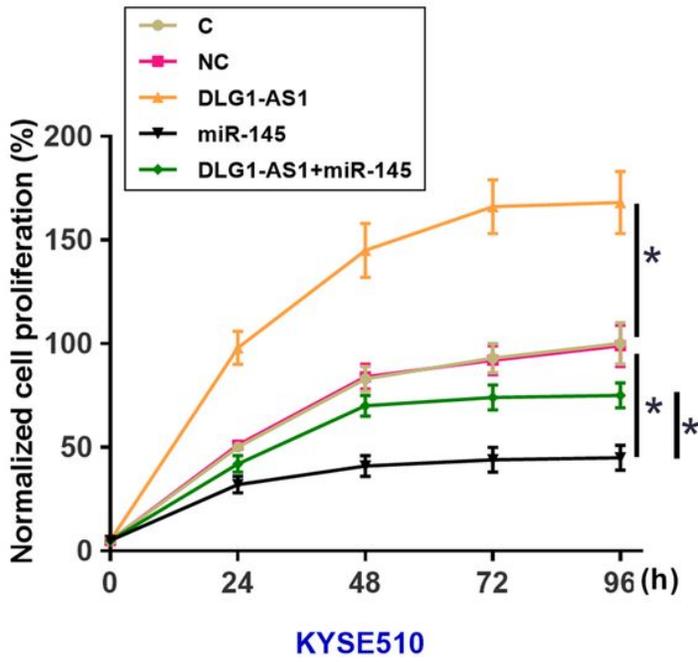


Figure 4

DLG1-AS1 promoted ESCC cell proliferation through miR-145. Overexpression of DLG1-AS1 mediated the promoted, while overexpression of miR-145 resulted in inhibited proliferation of ESCC cells. In addition, miR-145 overexpression attenuated the effects of DLG1-AS1 overexpression. Therefore, DLG1-AS1 may promote ESCC by downregulating miR-145 (*, $p < 0.05$).

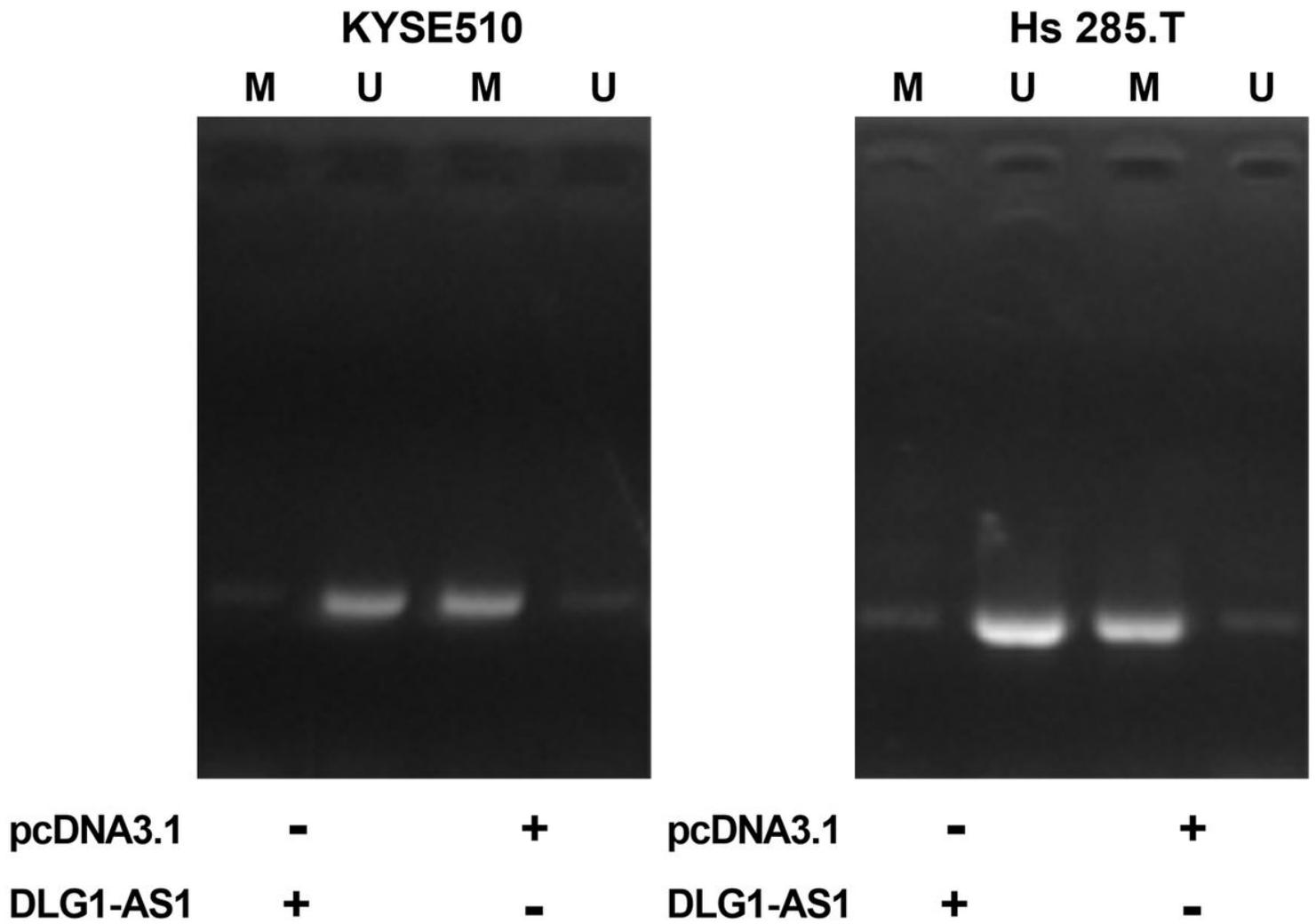


Figure 5

DLG1-AS1 promoted the methylation of miR-145 gene in ESCC cells. MSP was performed to analyze the methylation of miR-145 gene in ESCC cells with DLG1-AS1 overexpression. It was observed that DLG1-AS1 expression vector transfection significantly increased the methylation of miR-145 gene compared to empty vector transfection. M, methylation; U, unmethylation.