

# Paclitaxel-resistant related lncRNA DBH-AS1 promotes the proliferation and invasion of esophageal cancer

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**Abstract. – OBJECTIVE:** Chemoresistance is one of the main obstacles in the clinical treatment of cancer. However, secondary resistance to paclitaxel poses new challenges for cancer treatment. Long noncoding RNAs regulate cellular functions at different levels and mechanisms and play an important role in the biological behavior of tumors.

**MATERIALS AND METHODS:** LncRNA microarrays were used to detect lncRNAs in Paclitaxel-resistant cells and corresponding parental cells. Cell counting kit 8 and Transwell analysis were used to test the effect of lncRNA on function.

**RESULTS:** The expression of lncRNA DBH-AS1 in TE-4 TAX-R cells was significantly higher than that in TE-4 cells. Transwell analysis showed that the overexpression of lncRNA DBH-AS1 increased the invasion of Eca cells. Cell scratches and Transwell analysis showed that the overexpression of lncRNA DBH-AS1 in Eca cell culture supernatants promoted the migration and invasion of HUVEC. In addition, lncRNA DBH-AS1 relies on miR-21 to regulate the expression of YOD1.

**CONCLUSIONS:** Paclitaxel-resistant lncRNA DBH-AS1 appears to promote Eca cell proliferation and invasion by acting as a ceRNA and regulating miR-21-5p/YOD1 signaling pathway.

*Key Words:*

Paclitaxel, LncRNA, MiRNA, YOD1, Esophageal cancer.

## Introduction

Esophageal cancer is the eighth most common cancer worldwide and the sixth leading cause of cancer-related death<sup>1-3</sup>. The incidence and mortality of Eca are increased by obesity, smoking, and nutritional deficiencies. Surgery remains one of the most traditional methods for treating Eca in the early stage, with locally advanced patients requiring perioperative chemotherapy or radiotherapy and chemotherapy<sup>4-6</sup>.

In recent years, paclitaxel (TAX) has been considered as an effective drug for Eca treatment<sup>7</sup>. However, the development of TAX resistance has seriously affected its clinical efficacy. Therefore, understanding the underlying molecular mechanisms of Eca progression is important to propose new and improved Eca therapies as well as to inhibit tax resistance.

Long non-coding RNA (lncRNA) is a class of non-coding RNA molecules with a length greater than 200 NT<sup>8-10</sup>. LncRNAs regulate cellular function through chromatin modification, transcriptional regulation, microrRNA function regulation, and other levels and mechanisms, and play an important role in tumor biological behavior<sup>11-15</sup>. However, the role of lncRNA in disease progression after Eca paclitaxel resistance is rarely reported. In this study, we found that lncRNA DBH-AS1 induced paclitaxel resistance and promoted the expression of YOD1 through a competitive endogenous RNA (ceRNA) mechanism by regulating the expression of MiR-21-5p in Eca cells. In conclusion, lncRNA DBH-AS1 directly enhanced the malignant characteristics of Eca cells.

## Materials and Methods

### Tissue Specimens

A total of 121 pairs of Eca tumor and normal adjacent tissues were collected. The enrolled patients did not receive preoperative systemic or local antitumor therapy. After collection, pathological examination was performed to confirm the diagnosis of Eca and specimens were frozen at -80°C. All patients gave informed consent to participate, and all human studies were approved by the Ethics Committee of Fujian Provincial Hospital (No. K2020-03-032). All experiments were performed in accordance with relevant guidelines and regulations.

### Cell Culture, *LncRNA DBH-AS1* Overexpression and Knockdown

Human ECA cell lines TE-4 and TE-8 were purchased from ATCC. All Eca cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS) at 37°C and 5% CO<sub>2</sub>. According to previous study<sup>16</sup>, taxol resistant TE-4 cells (TE-4 tax-r) were established by exposure to increasing concentrations of taxol. *DBH-AS1* was cloned into PLVX vector through gene synthesis, and *dbh-as1* overexpression vector (plvx -*DBH-AS1*) was transferred into cells.

### Real-Time PCR Analysis

Total RNA was extracted and Real-time PCR analysis was conducted with the SYBR Green PCR kit (TaKaRa Bio, Otsu, Shiga, Japan) on a StepOne Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) according to previous study<sup>17</sup>. The relative gene expression was calculated by the 2<sup>-ΔΔCT</sup> method, and the mRNA and lncRNA expression was corrected by GAPDH expression.

### CCK-8 and Colony Formation Assays

The activity of cells was detected using Cell Counting Kit-8 at 24 h, 36 h, 48 h, and 96 h after inoculation with 10 μL of CCK-8 in a 37°C incubator for 2 h according to previous study<sup>17</sup>.

### RNA Pulls Down

RNA pulls down were performed by Western blot analysis as previously described<sup>17</sup>. The complex was eluted and detected by qPCR.

### Transwell and Wound Scratch Assay

Transwell and wound scratch assays were performed as previously described<sup>17</sup>.

### Lncrna Expression Analysis

The total RNA of the sample was quantified with nanodrop nd-2000 (Thermo Fisher Scientific, Waltham, MA, USA), and the RNA integrity was detected with Agilent Bioanalyzer 2100 (Agilent Technologies). The following tests were completed by Shanghai Bohao Biotechnology Co., Ltd., (Shanghai, China). Differential genes and differential lncrna were screened by *p*-value and fold change value of *t*-test. The screening criteria are up-regulated or down regulated refractive value ≥ 2.0 and *p*-value ≤ 0.05.

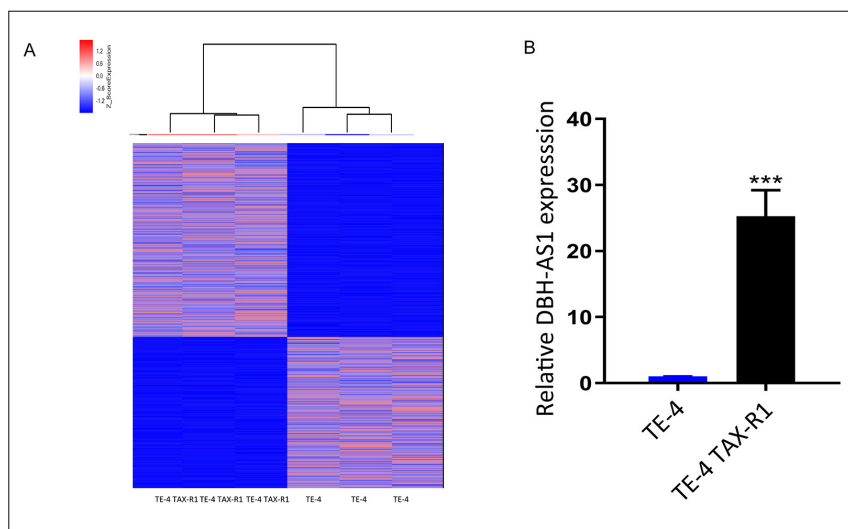
### Statistical Analysis

SPSS 22.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Results are expressed as mean ± standard deviation. A *p*-value < 0.05 was considered statistically significant.

## Results

### *LncRNA DBH-AS1* Is Up-Regulated in Taxol Resistant TE-4 Cells

We constructed a tax tolerant cell line TE-4 tax-r and analyzed the lncRNA expression difference between TE-4 tax-r and its parent cell TE-4 by lncRNA chip. As shown in Figure 1A, there are significant differences in the expression of many lncRNA between TE-4 tax-r cells and TE-4 cells. As shown in Figure 1B, the



**Figure 1.** *LncRNA DBH-AS1* was upregulated in paclitaxel-resistant TE-4 cells. **A**, Heat map of the difference in lncRNA expression between TE-4 cells and TE-4 tax-r cells. **B**, Real time PCR was used to detect the expression of lncRNA *DBH-AS1* in TE-4 and TE-4 tax-r cells. \**p*-value < 0.05.

expression of lncRNA DBH-AS1 in TE-4 tax-r cells was significantly higher than that in TE-4 cell.

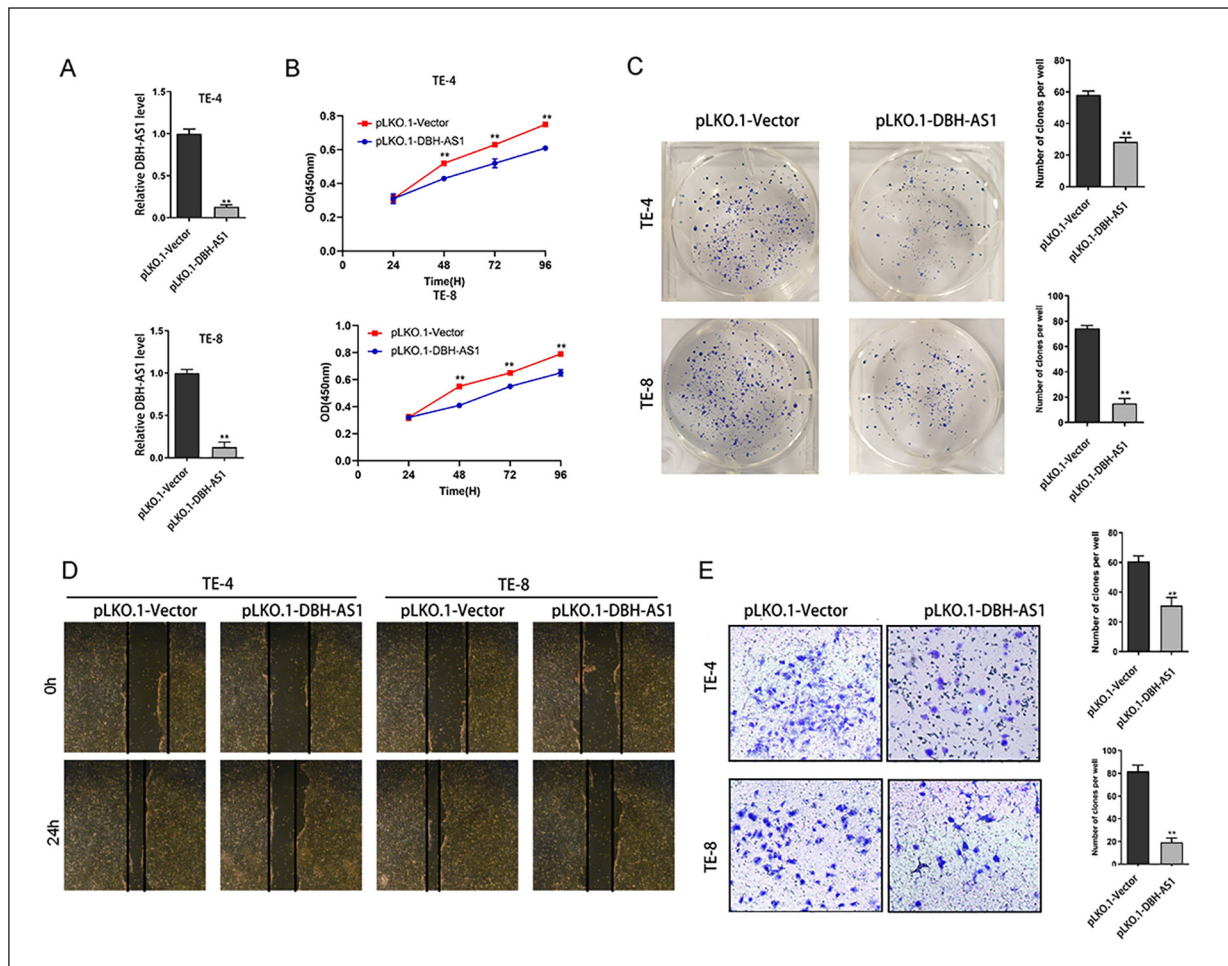
**LncRNA DBH-AS1 Knockdown Can Inhibit the Proliferation and Invasion of Eca**

Next, we constructed the specific target of harpin RNA (short Harpin RNA, shRNA) of lncRNA DBH-AS1 (Figure 2A). The viability of Eca cells in pLKO group was lower. lncRNA DBH-AS1 group was significantly stronger than pLKO group 1 vector group (Figure 2B). Clone formation experiments showed that lncRNA DBH-AS1 knockdown could reduce the number

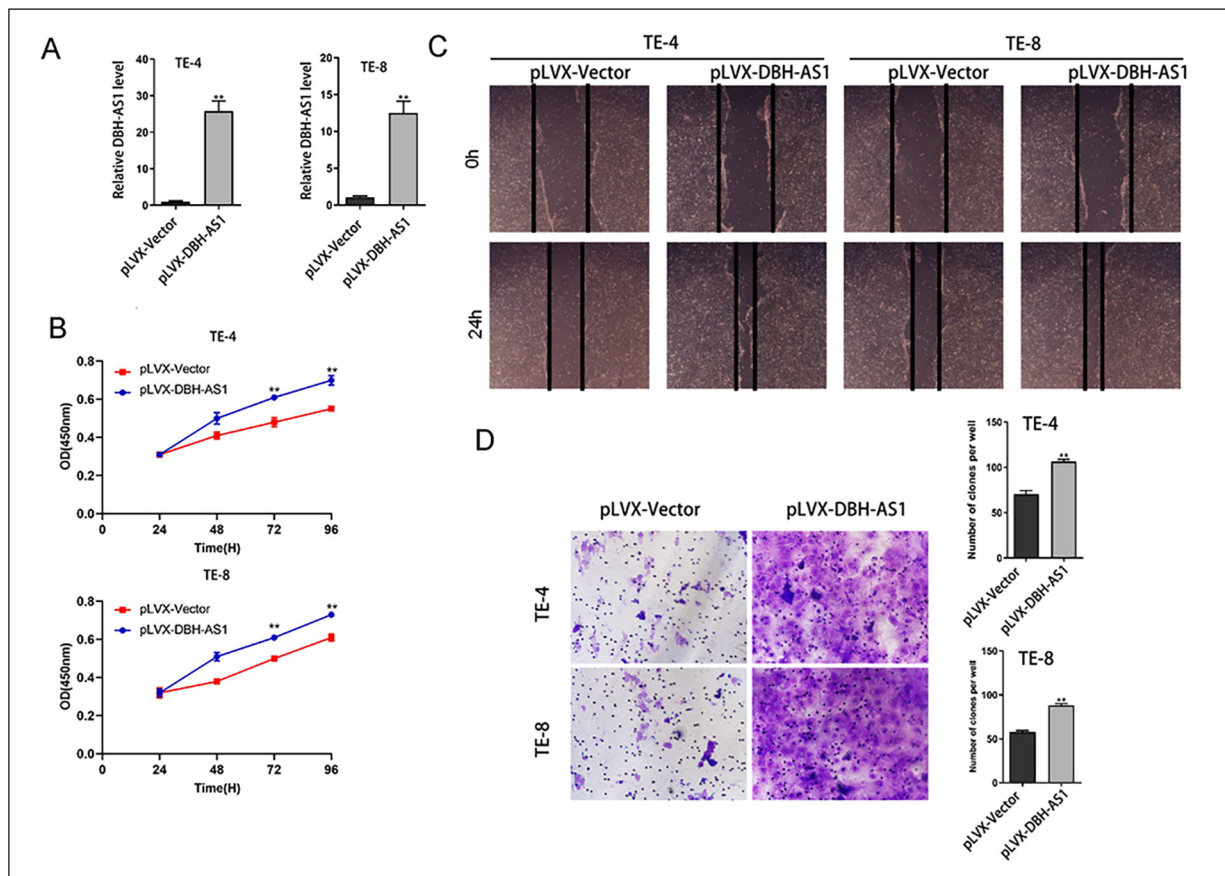
of clones in Eca cell (Figure 2C). Cell scratch test showed that lncRNA DBH-AS1 knockdown could reduce the migration ability of Eca cells (Figure 2D). Transwell experiment showed that lncRNA DBH-AS1 knockdown could reduce the invasive ability of Eca cell (Figure 2E).

**LncRNA DBH-AS1 Overexpression Promotes Eca Proliferation and Invasion**

Next, we constructed a lentiviral vector over-expressing lncRNA DBH-AS1 (Figure 3A). The viability of Eca cell in pLVX DBH-AS1 group was higher than that in pLVX vector group (Figure 3B). Cell scratch test showed that over-expression of lncRNA DBH-AS1 could increase the



**Figure 2.** LncRNA DBH-AS1 knockdown suppressed the Eca proliferation and invasion. **A**, Real time PCR was used to detect the expression of lncRNA DBH-AS1 in lncRNA DBH-AS1 knockdown Eca cells. **B**, CCK-8 was used to detect the proliferation of lncRNA DBH-AS1 knockdown Eca cells. **C**, Clone formation was used to detect the proliferation of lncRNA DBH-AS1 knockdown Eca cells. Magnification  $\times 50$ . **D**, The migration of lncRNA DBH-AS1 knockdown Eca cells was detected by cell scratch test. Scale: 100  $\mu\text{m}$ . **E**, Transwell assay was used to detect the invasion of lncRNA DBH-AS1 knockdown Eca cells. Scale: 100  $\mu\text{m}$ . \**p*-value < 0.05.



**Figure 3.** LncRNA DBH-AS1 overexpression promoted the Eca proliferation and invasion. **A**, Real time PCR was used to detect the expression of lncRNA DBH-AS1 in Eca cells overexpressing lncRNA DBH-AS1. **B**, CCK-8 was used to detect the proliferation of lncRNA DBH-AS1 overexpressing Eca cells. **C**, The migration of lncRNA DBH-AS1 overexpressed Eca cells was detected by cell scratch test. Scale: 100  $\mu$ m. **D**, Transwell assay was used to detect the invasive ability of lncRNA DBH-AS1 overexpression Eca cells. Scale: 100  $\mu$ m. \* $p$ -value < 0.05.

migration ability of ECA cells (Figure 3C). Transwell experiment showed that overexpression of lncRNA DBH-AS1 increased the invasive ability of Eca cell (Figure 3D).

#### ***LncRNA DBH-AS1 Regulates EMT Related Genes in Eca Cells***

As shown in Figure 4A-D, overexpression of lncRNA DBH-AS1 promoted the expression of ZEB1, snail and fibronectin, and inhibited the expression of E-cadherin. While lncRNA DBH-AS1 knockout decreased the expression of ZEB1, snail and fibronectin, it promoted the expression of E-cadherin (Figure 4A-D).

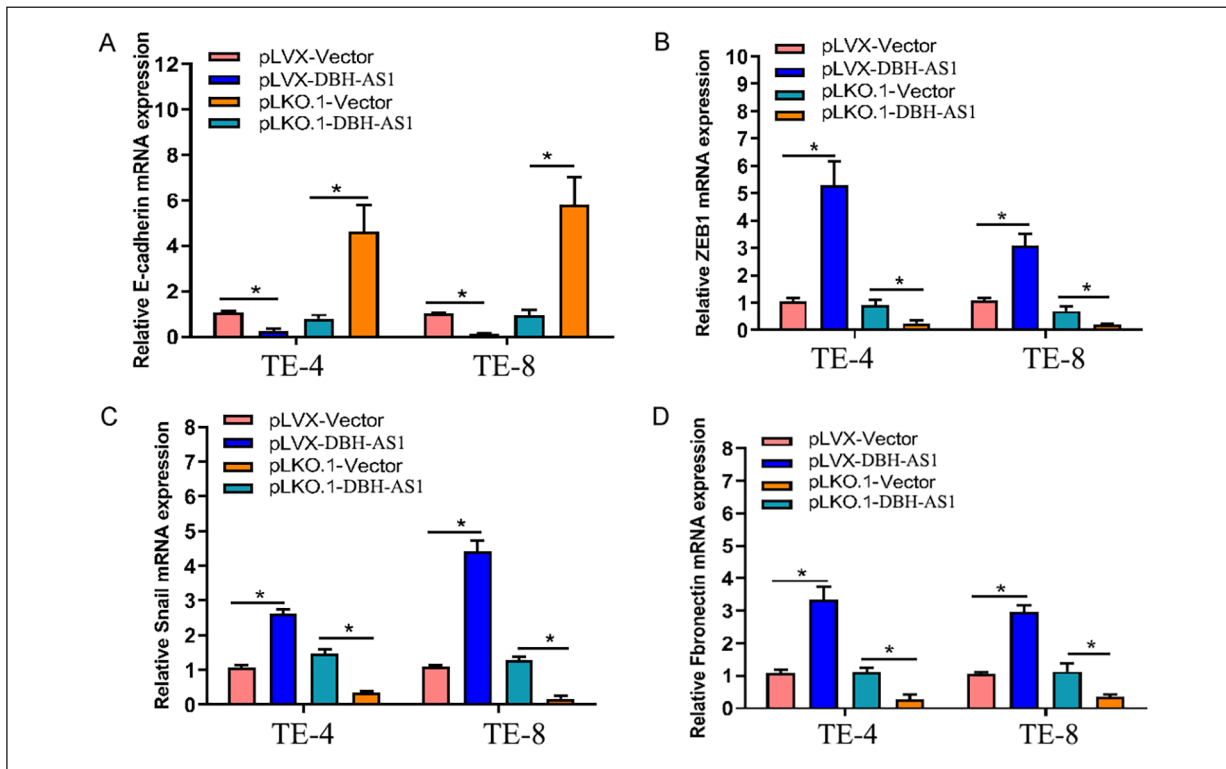
#### ***LncRNA DBH-AS1 Overexpression of Eca Cell Culture Supernatant Can Promote the Angiogenesis of HUVECs***

Transwell analysis showed that the supernatant of ECA cell culture overexpressing lncRNA

DBH-AS1 could promote the migration and invasion of HUVEC, while the supernatant of Eca cell culture downregulated by lncRNA DBH-AS1 could inhibit the migration and invasion of HUVEC (Figure 5A-C). ELISA results showed that overexpression of lncRNA DBH-AS1 increased the expression of vascular endothelial growth factor (VEGF), while knockdown of lncRNA DBH-AS1 inhibited the expression of VEGF (Figure 5D).

#### ***LncRNA DBH-AS1 Regulates the Function of MiR-21***

The pull-down of ms2bs dbh-as1 RNA showed that mir-527, mir-509, mir-562 and mir-21-5p were more enriched in the ms2bs dbh-as1 group than in the ms2bsrlucgroup (Figure 6A). Down regulation of biotin-mir-21-5p RNA showed that lncRNA DBH-AS1 was more enriched in the biotin-mir-21-5p group than in the biotin mirnc



**Figure 4.** LncRNA DBH-AS1 regulated the EMT- related gene in Eca cells. Real time PCR was used to detect the overexpression or knockdown of E-cadherin (A), ZEB1 (B), snail (C) and fibronectin (D) mRNA in Eca cells. \* $p$ -value < 0.05.

group (Figure 6B). Double luciferase reporter gene assay showed that lncRNA DBH-AS1 bound miR-21-5p (Figure 6C). Overexpression of miR-21-5p inhibited Eca cell viability, and knockdown of miR-21-5p promoted Eca cell viability (Figure 6D, E). Conversely, overexpression of miR-21-5p inhibited Eca cell invasion, and knockdown of miR-21-5p promoted ECA cell invasion (Figure 6F, G). However, when miR-21-5p expression was inhibited, the effect of lncRNA DBH-AS1 on Eca cell invasion was significantly reduced (Figure 6H, I).

#### **LncRNA DBH-AS1 Depends on MiR-21 to Regulate yod1 Expression**

Using bioinformatics analysis software, we found that miR-21-5p targeted the 3' untranslated(3'-UTR) region of YOD1 mRNA. qRT-PCR results showed that overexpression and knockout of miR-21-5p did not affect the expression level of YOD1 mRNA (Figure 7A). Double luciferase reporter gene analysis showed that the 3' -UTR regions of YOD1 mRNA bound to miR-21-5p (Figure 7B).

#### **LncRNA DBH-AS1 Is Increased and Correlate with Aggressive Clinicopathologic Features and Poor Survival in Eca**

Compared with healthy tissues, lncRNA DBH-AS1 expression was lower in Eca tissues (Figure 8A), and lower in 96/121 (79.34%) Eca samples (Figure 8B). The expression level of lncRNA DBH-AS1 in T3 + T4 tumor depth was lower than that in T1 + T2 (Figure 8C), and the level of DBH-AS1 in TNM 3 + 4 samples was lower than that in TNM 1 + 2 samples (Figure 8D). The expression level of lncRNA DBH-AS1 in lymph node metastasis positive specimens was also lower than that in non-metastasis specimens (Figure 8E). Table I shows that the low expression of lncRNA DBH-AS1 (according to the median expression of lncRNA DBH-AS1 in Eca tissues: as the critical value) is positively correlated with late TNM ( $p = 0.004$ ), depth of invasion ( $p = 0.008$ ), and lymph node metastasis +ve ( $p < 0.001$ ). KM curve shows that low level of lncRNA DBH-AS1 is associated with poor prognosis, poor OS and low DFS rate of Eca (Figure 8F, G).

**Table I.** The correlation between clinicopathological parameters and DBH-AS1 expression in human esophageal cancer.

Clinical features	Total	DBH-AS1		<i>p</i> -value
		High (N = 61)	Low (N = 60)	
Age (years)				0.210
< 60	36	15	21	
≥ 60	85	46	39	
Gender				0.792
Male	68	35	33	
Female	53	26	27	
Differentiation grade				0.521
Well	41	19	22	
Moderate + Poor	80	42	38	
TNM stage				0.004
I + II	76	46	30	
III	45	15	30	
Depth of invasion				0.008
T1 + T2	57	36	21	
T3 + T4	64	25	39	
Lymph node metastasis				< 0.001
No	80	53	27	
Yes	41	8	33	

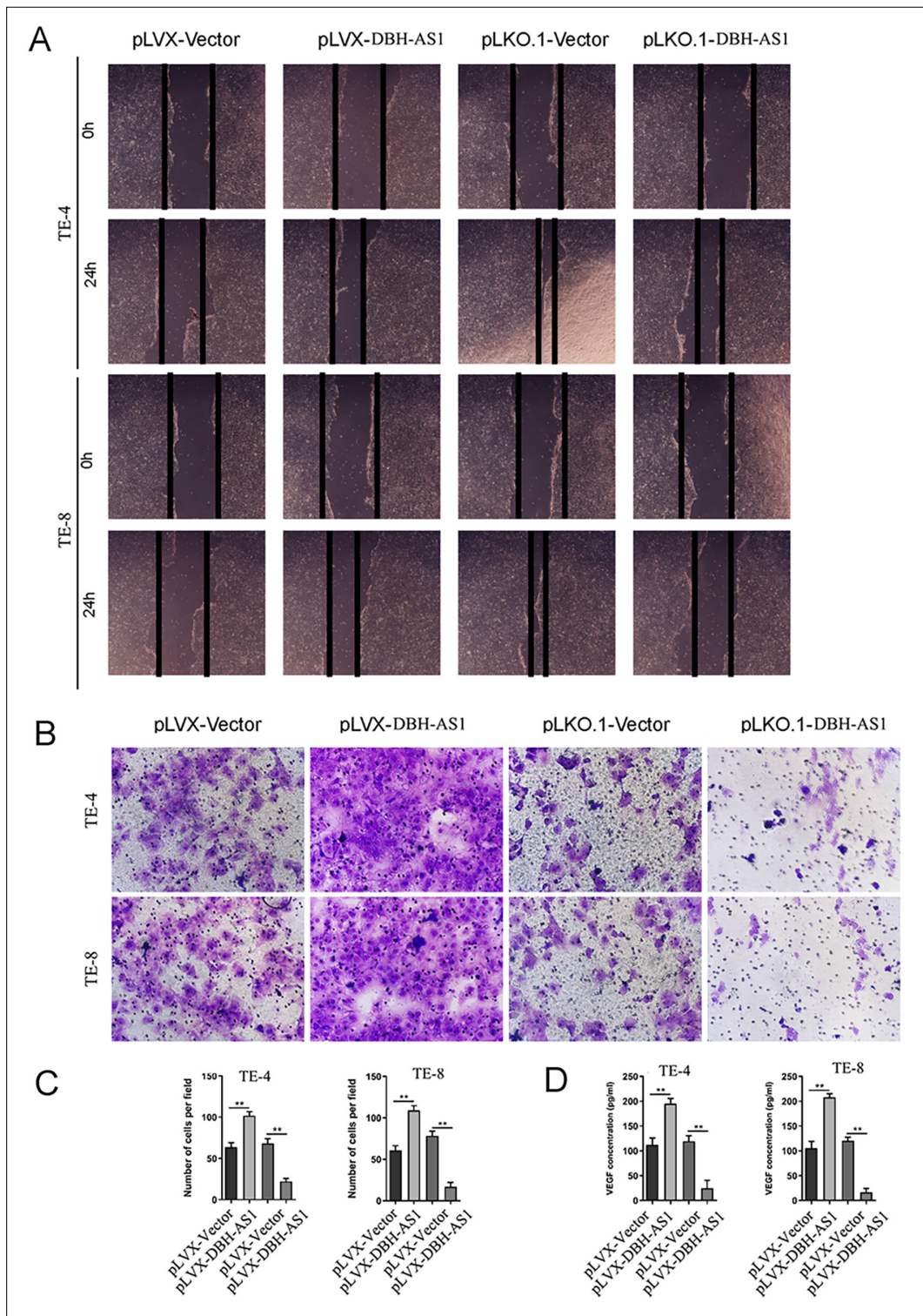
Pearson's Chi-square test was used for comparison between subgroups.

## Discussion

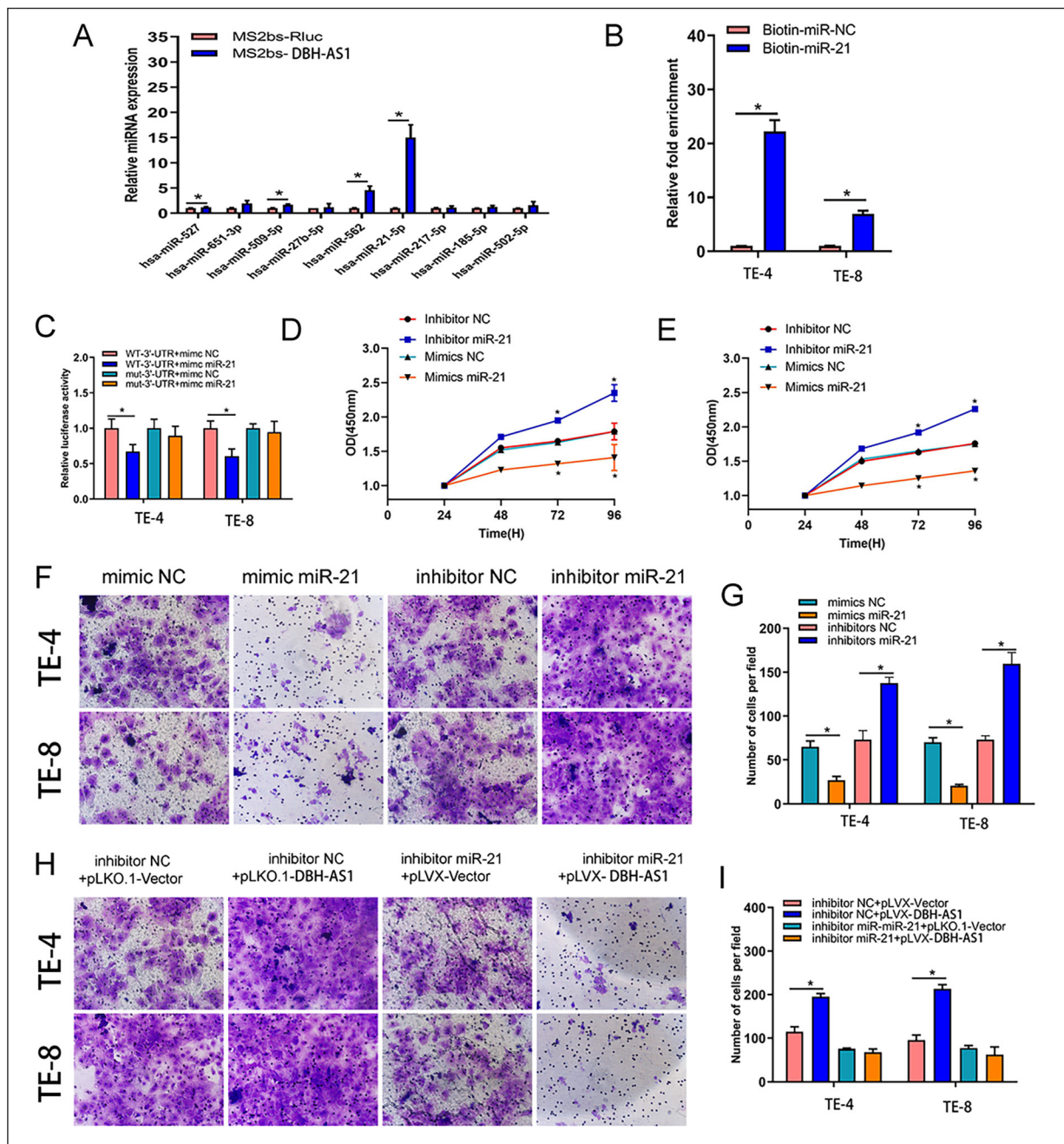
As we all know, esophageal cancer has a high mortality rate in the world, partly due to the late diagnosis of the disease, which is characterized by frequent local/distant metastasis of esophageal cancer and poor subjective symptoms. It has been found that the use of ionizing radiation can increase the resistance of EC cells to PTX, thus making EC treatment more complex<sup>18-20</sup>. In different cancers, such as nasopharyngeal carcinoma and ovarian cancer, lncRNAs have been found to be associated with PTX resistance<sup>18-20</sup>. At present, the mechanism of paclitaxel resistance in Eca has not been clarified. Therefore, exploring the molecular mechanism of paclitaxel resistance is of great significance to improve the sensitivity of paclitaxel treatment and prevent paclitaxel resistance. lncRNAs is a non-coding RNA with more than 200 NT transcripts. In recent years, the research progress of lncRNAs shows that lncRNAs play an important role in tumor drug resistance<sup>21</sup>. Therefore, exploring the relationship between lncRNAs and paclitaxel resistance in Eca has become a new molecular therapeutic target of ECA. In this study, taxol resistant te-4 cells<sup>16</sup> were established according to the methods of previous studies. lncRNA chip was used to detect the differential expression profile

of lncRNAs between parental cell lines and taxol resistant cell lines, and to explore the molecular mechanism of taxol resistance between lncRNA and Eca patients from a new perspective.

So far, the most in-depth research on the related functions of lncRNAs has focused on its role in cancer. Under normal physiological conditions, the expression of lncRNA is strictly regulated. On the contrary, the abnormal expression of lncRNA is related to the occurrence and development of many cancers. lncRNA can not only promote tumor formation as proto-oncogenes, but also inhibit the proliferation and migration of tumor cells as tumor suppressor genes<sup>22</sup>. lncRNA chip analysis showed that the expression level of lncRNA DBH-AS1 was significantly increased in TE-8-taxr cells. The drug resistance of tumor cells to anticancer drugs is the main cause of chemotherapy failure<sup>23,24</sup>. In congenital and acquired drug resistance, the abnormal activation of intracellular signaling pathways is closely related to tumor drug resistance. lncRNA induces drug resistance by affecting tumor cell cycle. At present, many studies have reported that lncRNAs are associated with drug resistance in Eca<sup>25,26</sup>. For example, lncRNA ECAT1 was found to activate Akt and NF-κB signaling pathway promotes castration resistance in Eca patients<sup>27</sup>. In addition, lncRNA



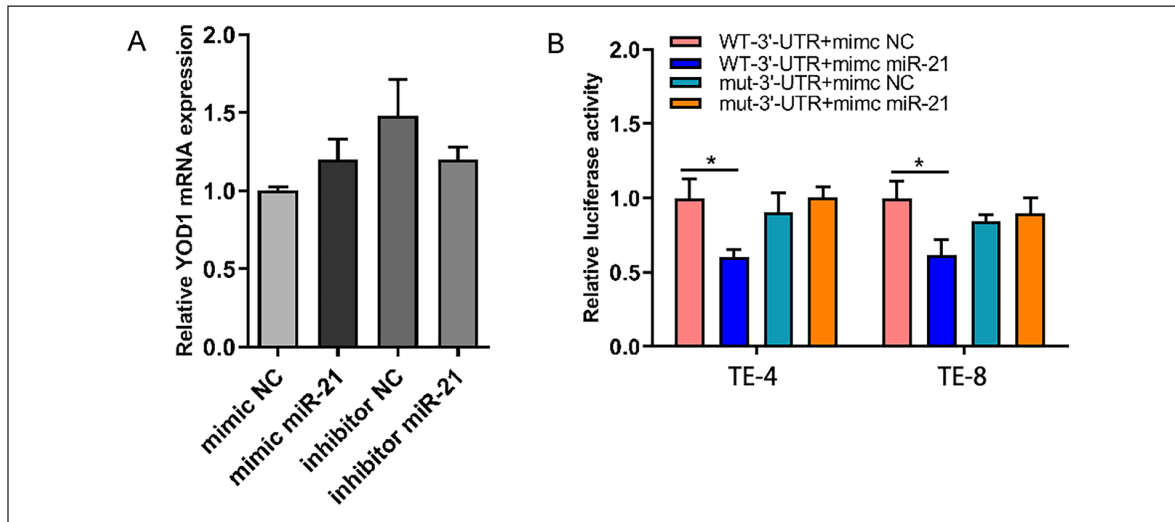
**Figure 5.** The culture supernatant of Eca cells with overexpressed lncRNA DBH-AS1 promoted the angiogenesis of HUVECs. **A**, The migration of HUVECs cultured with lncRNA DBH-AS1 knockdown or overexpressed Eca cell culture supernatant was detected by cell scratch test. Scale: 100  $\mu$ m. **B**, Transwell assay was used to detect the invasive ability of HUVECs cultured in the supernatant of Eca cells with overexpression or down-regulation of lncRNA DBH-AS1. Scale: 100  $\mu$ m. **C**, Transwell quantitative analysis was used to detect the invasive ability of lncRNA DBH-AS1 overexpressed or down regulated Eca cell culture supernatant to HUVECs. **D**, The expression of VEGF in the supernatant of Eca cells overexpressed or down regulated by lncRNA DBH-AS1 was detected by ELISA. Scale: 100  $\mu$ m. \* $p$ -value < 0.05.



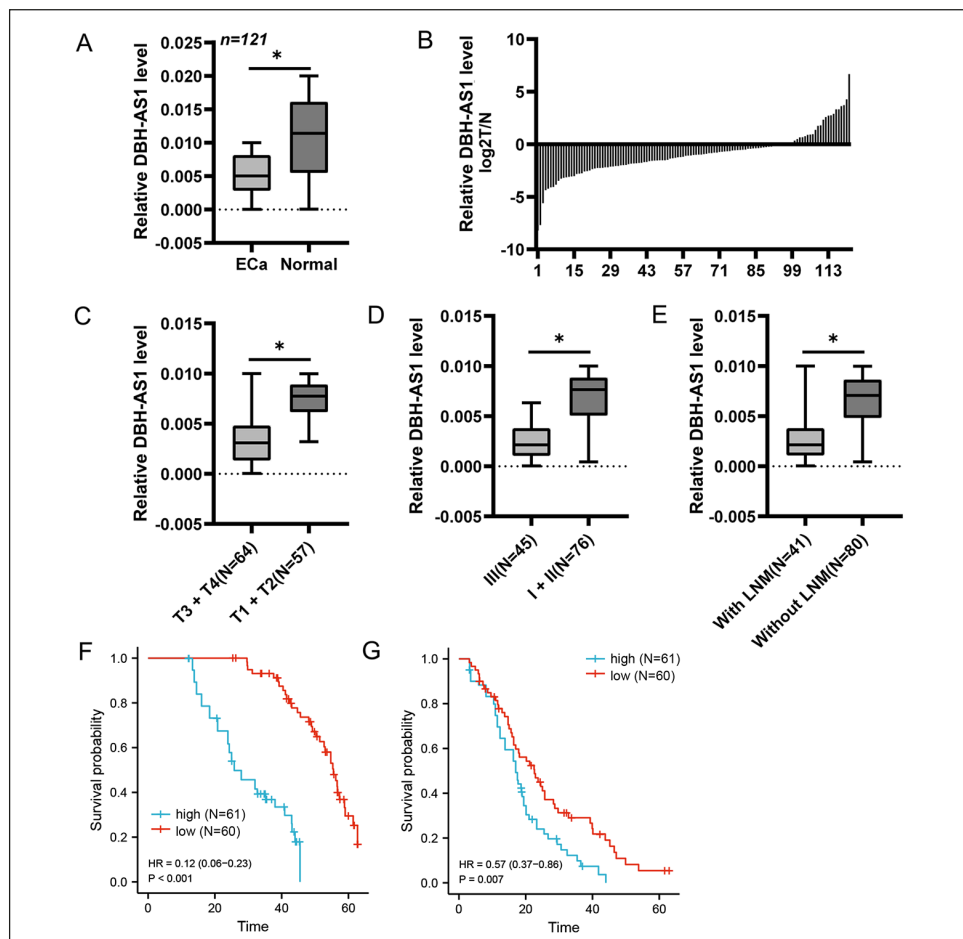
**Figure 6.** LncRNA DBH-AS1 regulated the function of miR-21. **A**, LncRNA DBH-AS1 RNA pull down was used to detect the binding of dbh-as1 to miRNA. **B**, Down regulation of miR-21 RNA detected the binding of dbh-as1 to miR-21. **C**, Luciferase reporter gene analysis detected the binding of dbh-as1 to miR-21. **D**, CCK-8 was used to detect the proliferation of TE-4 cells with miR-21 overexpression or knockdown. **E**, CCK-8 was used to detect the proliferation of te-8 cells with miR-21 overexpression or knockdown. **F**, Transwell assay was used to detect the invasion of Eca cells with miR-21 overexpression or knockdown. Scale: 100  $\mu$ m. **G**, Quantitative analysis of invasion ability of Eca cells with overexpression or overexpression of miR-21 detected by Transwell. **H**, Transwell detected the effect of dbh-as1 on the invasion of miR-21 knockdown Eca cells. Scale: 100  $\mu$ m. **I**, Transwell quantitatively analyzed the effect of dbh-as1 on the invasion of miR-21 knockdown TE-4 and c4-2b cells. Bar, 100  $\mu$ m. \**p*-value < 0.05.

horas5 promotes the castration resistance of Eca to taxane through a BCL2A1 dependent mechanism<sup>28</sup>. lncRNA PCBPI-AS1 mediated ar/ar-V7 deubiquitination enhances ECA taxol re-

sistance<sup>29</sup>. It has been reported that lncRNAs can regulate the function of downstream miRNA through the ceRNA mechanism, thereby regulating the expression of target genes<sup>12,30</sup>.



**Figure 7.** LncRNA DBH-AS1 regulated the expression of YOD1 dependent on miR-21. **A**, Real-time quantitative PCR for detection of YOD1 mRNA expression in Te-4 cells with miR-21 knockdown or overexpression. **B**, Luciferase reporter gene analysis detected the combination of YOD1 3'-UTR and Mir-21. \*Compared with control group,  $p$ -value < 0.05.



**Figure 8.** LncRNA DBH-AS1 is increased and correlates with aggressive clinicopathologic features and poor survival in Eca. **A-B**, RT-PCR showed LncRNA DBH-AS1 levels in 121 Eca tissues and healthy tissues. **C-E**, The loss of LncRNA DBH-AS1 was related to T3 + T4 invasion depth, TNM3 + 4 stage, metastasis. **F-G**, Eca the patient's overall survival and DFS and OS. \* $p$ -value < 0.05.

Bioinformatics software analysis, double luciferase reporter gene and RNA decline analysis showed that DBH-AS1 combined with miR-21-5p to regulate the function of miR-21-5p. Subsequently, through bioinformatics, double luciferase reporter gene and RNA pull-down analysis, we found that mir-21-5p targeted the expression of YOD1. Yod1 is a highly conserved deubiquitinase in the otubain family, which can remove ubiquitin residues in polyubiquitinated proteins. Although yod1 is highly conserved, its function in higher eukaryotes is unclear. Yod1, as a target gene of mir-4429<sup>31</sup>, regulates ovarian cancer metastasis. LncRBA FIRRE mediates the expression of yod1 through the ceRNA mechanism, thereby promoting the progression of gallbladder cancer<sup>32</sup>. It is reported that yod1 may be a biomarker to evaluate the prognosis of Eca<sup>33</sup>. In this study, we found that YOD1 is involved in taxol resistance, which may be a new target for the treatment of taxol resistance.

## Conclusions

In summary, lncRNA DBH-AS1 associated with paclitaxel resistance appears to promote Eca cell proliferation and invasion by acting as a ceRNA and regulating miR-21-5p/YOD1 signaling pathway.

## Conflict of Interest

The Authors declare that they have no conflict of interests.

## Ethics Approval

The Ethics Committee of Fujian Hospital Ethics Committee (No. K2020-03-032) approved the research.

## Informed Consent

Written informed consent was obtained from all the patients.

## Data Availability

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

## Funding

None.

## Authors' Contribution

Z.-X. L, L.-H. Z, J.-Y. H, and Y.-H. contributed significantly to performing the experiments and assisted in writing the manuscript; X L contributed to the conception of the study. All authors read and approved the final study.

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