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MiR-21 Suppresses Anoikis through Targeting PDCD4 and PTEN in Human Esophageal Adenocarcinoma

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Summary: Anoikis is a form of apoptosis induced upon cell detachment from extracellular matrix. It has been determined that acquisition of resistance to anoikis is a critical step for tumor cell metastasis. MiR-21, the most prominent oncomiR, plays an important role in tumor progression. In this study, we revealed that up-regulation of miR-21 in human esophageal adenocarcinoma (EA) is associated with lymph node metastasis and poor survival rate. Because of the established anti-apoptosis effect of miR-21, it is tempting to speculate that miR-21 might contribute to tumor metastasis by regulating anoikis. qRT-PCR analysis demonstrated that miR-21 expression in OE33/AR cells (subpopulation of human EA OE33 cells that acquired resistance to anoikis) was significantly increased. Also, transfection of miR-21 mimics provided OE33 cells resisting to anoikis. By luciferase assays, we verified that PDCD4 and PTEN were the functional targets of miR-21. In mouse model, via tail vein injection experiment, we showed that the metastasis formation of OE33 cells *in vivo* could be mediated by changing the miR-21 expression pattern. Taken together, our findings suggested that miR-21 was involved in the regulation of anoikis in human EA cells. Targeting miR-21 may provide a novel strategy to prevent metastasis.
Key words: miR-21; anoikis resistance; PDCD4; PTEN; esophageal adenocarcinoma

Anoikis ('homelessness' in Greek) is a mode of apoptotic cell death, following the detachment of cells from the appropriate extracellular matrix (ECM)[1]. It has been distinguished that anoikis plays a critical role in many physiologic and pathologic processes, such as embryogenesis, tissue organization and tumor metastasis[2, 3]. The metastatic spread of tumor cells from the primary site is responsible for most cancer deaths. To achieve metastasis, tumor cells must carry out multistep processes, including detachment from the primary tumor, intravasation into lymphatic or blood vessels, survival in the detached condition, extravasation out of the vessels, and attachment and proliferation at the metastatic site[4, 5]. Detachment from the primary tumor is the early obligatory step in metastasis formation. As a consequence of detachment from ECM, tumor cells will undergo anoikis. Therefore, anoikis is considered as a mechanism of preventing metastasis. However, a subpopulation of tumor cells exhibit anoikis resistance, allowing them to survive after detachment and invade other organs.

MicroRNAs (miRNAs) are endogenous small non-coding RNAs that control gene expression by degrading or suppressing the translation of target mRNAs. Increasing evidence has demonstrated that miRNAs play vital roles in multiple biological processes, such as cell growth, differentiation and apoptosis[6–8]. MiR-

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21 is one of the most commonly observed aberrant miRNAs in human cancers, and represents one of the earliest miRNAs identified as an oncomiR. Numerous researches have revealed that miR-21 could promote carcinogenesis and tumor progression by targeting genes including PDCD4, PTEN, BTG2, FasL, RECK, TGF-β1, FBXO11 and TIMP3[9–11]. Importantly, a large number of studies have explored the function of miR-21 as a biomarker for cancer diagnosis and prognosis[12–14].

To date, only a limited number of studies have ever explored the role of miRNAs in the regulation of anoikis. In this study, we investigated the relationship between miR-21 expression and clinicopathologic factors in human esophageal adenocarcinoma (EA), and explored the possible pathway that links aberrant miR-21 expression with acquisition of anoikis resistance and subsequential metastasis.

1 MATERIALS AND METHODS

1.1 Ethics Statement

All experimental procedures were approved by the Institutional Review Boards of Zhongnan Hospital of Wuhan University and General Hospital of Air Force. Written informed consent was obtained for all patient samples. Animal experiments were performed with the approval of the Institutional Committee for Animal Research and in conformity with national guidelines for the care and use of laboratory animals.

1.2 Clinical Samples

Thirty patients who had undergone esophagectomy with lymph node dissection for EA at Zhongnan Hospital or General Hospital of Air Force between January 2010 and December 2014 were included in this study. No patients received neoadjuvant chemotherapy or radiation therapy before surgery. The resected specimens were histologically examined by HE staining. Total RNA from paraffin-embedded tumor tissues and corresponding non-tumor mucosa were collected for each patient using the paraffin-embedded tissue microRNA extraction Kit (Bioteke, China) according to the manufacturer’s instructions.

1.3 Cell Culture

The OE33 cells (human Barrett’s adenocarcinoma derived cell line) used in this study was kindly provided by Dr. Xiaoxin Chen (North Carolina Central University, USA). To obtain a culture condition for suspension, OE33 cells were seeded on plates pre-coated with poly (2-hydrocyethyl methacrylate) (Poly-HEMA, Sigma Aldrich, USA)[15]. Via suspension culture, a subpopulation of OE33 cells that acquired resistance to anoikis was designated as OE33/anoikis-resistance (AR) cells.

1.4 Cell Transfection

Cells were transfected with miR-21 mimics and the negative control duplexes (Ambion, USA) using Lipofectamine 2000 (Invitrogen, USA). After 24-h transfection, cells were collected for qRT-PCR analysis or further processing.

1.5 qRT-PCR

TaqMan stem-loop RT-PCR method was used to detect the expression of miR-21. The real-time PCR results, recorded as threshold cycle numbers (Ct), were normalized against an internal control (U6). For relative expression levels, the 2(-DCt) method was used[16]. Experiments were carried out in triplicate for each data point, and data analysis was done by using Bio-Rad IQ software.

1.6 Anoikis Analysis

Anoikis analysis was performed by flow cytometry (Coulter Epics XL-MCL). OE33 cells in exponential phase were harvested and then were cultured in plates pre-coated with Poly-HEMA for 48 h. After that, cells were collected and the apoptotic (anoikis) cells were measured by annexin-V/PI analysis with flow cytometry. For OE33/AR cells in suspension, after 48 h culture in normal cell plates, the cells were re-cultured in plates pre-coated with Poly-HEMA for 48 h and then the anoikis was analyzed.

1.7 Western Blot Analysis

Total cell lysates were prepared in 1×SDS buffer. Proteins were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were then blotted with antibodies specific for PDCD4, PTEN, FasL and β-actin (Sigma-Aldrich). Antigen-antibody complexes were visualized using enhanced chemiluminescence (Sigma-Aldrich).

1.8 Luciferase Assay

The 3’-UTR segments of PDCD4/PTEN mRNA containing the miR-21 binding sites were amplified by PCR from human genomic DNA and inserted into the pMIR-REPORT luciferase reporter vector (Ambion) and named pMIR-PDCD4-3′UTR and pMIR-PTEN-3′UTR respectively. The corresponding mutant versions were also generated and named pMIR-mut-PDCD4-3′UTR and pMIR-mut-PTEN-3′UTR, respectively. The recombinant reporter vectors with wild-type or mutant PDCD4/PTEN 3′-UTR were then co-transfected with miR-21 mimics or control into OE33 cells, respectively, using LipofectamineTM 2000. The luciferase assay was performed according to the manufacturer’s instructions.

1.9 Animal Study

The effect of miR-21 on *in vivo* tumorigenesis and metastasis was evaluated by constructing stable miR-21-expressing (increased or decreased) OE33 cells. Lentiviral vectors containing pre-miR-21 (Lenti-miR-21) or anti-miR-21 (lenti-anti-miR-21) were constructed. By lentivirus infection, we generated the subclones of OE33 cells stably over-expressing miR-21 or stably expressing reduced miR-21, respectively. Lentivirus-transduced OE33 cells in exponential phase

were harvested and then were cultured in plates pre-coated with Poly-HEMA for 48 h. After that, cells were resuspended in PBS at a concentration of 10^7 cells/mL and 4- to 6-week-old athymic female BALB/c nu/nu mice were injected with 100 µL of each cell clone via tail vein[17]. All mice were sacrificed and autopsied 3 weeks after injection. The liver tumor nodules distinguished by HE staining were counted to evaluate the tumorigenesis and metastasis of OE33 cells.

1.10 Statistical Analysis

Data were expressed as $\bar{x}\pm s$. Differences were compared by one-way ANOVA analysis followed by group comparison with *t*-test. Survival curves were plotted by Kaplan-Meier method and log-rank test was carried out to compare the differences. All statistical analyses were performed using SPSS 21.0 software (USA). $P<0.05$ was considered as statistically significant.

2 RESULTS

2.1 Association of High miR-21 Expression with Lymph Node Metastasis and Poor Prognosis in Human EA Patients

MiR-21 is one of the most frequently over-expressed miRNAs in human cancers, e.g. lung cancer, colorectal cancer and pancreatic cancer[9–11]. In this study, we performed qRT-PCR analysis to detect the expression of miR-21 in 30 EA samples and 30 paired adjacent mucosae. The term -DCt was used to describe the miR-21 expression level. As illustrated in fig. 1A, the miR-21 expression in EA tissues was significantly up-regulated as compared with that in adjacent mucosae (5.33±1.61 *vs*. 1.78±0.69, $P<0.01$, paired *t*-test). Correlations between the miR-21 expression level and clinicopathologic characteristics of EA are summarized in table 1. Statistically significant association between the miR-21 expression level and lymph node (LN) metastasis was observed.

The median expression of miR-21 was 5.94±1.66 in the 18 cases with LN metastasis, and that was 4.53±1.16 in the 12 cases without LN metastasis ($P<0.01$, *t*-test, fig. 1B). Moreover, we investigated whether the miR-21 expression level was associated with survival in EA patients. Patients were subsequently divided into high expression (n=15) and low expression groups (n=15) based on miR-21 levels greater or less than the median value (4.93) (fig. 1C). Kaplan-Meier survival analysis revealed that patients whose primary tumors displayed

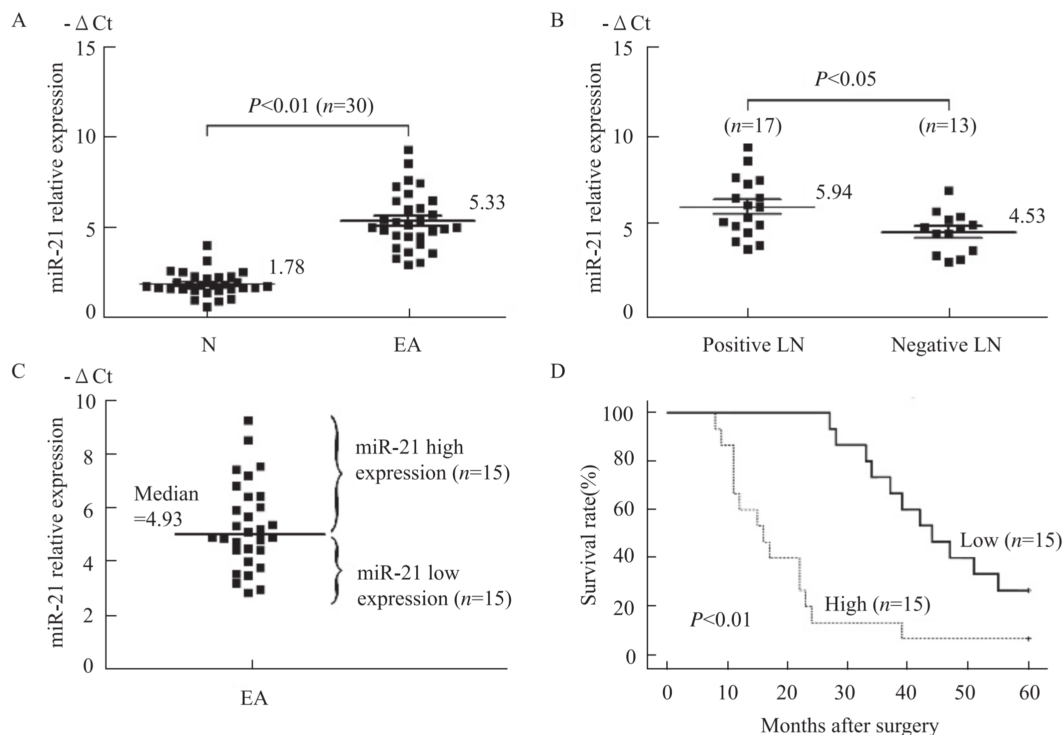

Fig. 1 MiR-21 expression in clinical EA specimens

A: miR-21 was differentially expressed between EA and corresponding non-neoplastic mucosa (N); B: miR-21 expression was significantly higher in cases with LN metastasis than in those without LN metastasis; C: Patients were divided into two groups according to the mean expression of miR-21 (4.93); D: Kaplan-Meier survival curve and log-rank test for EA patients between high and low miR-21 expression

high expression of miR-21 had a shorter median survival time (P<0.01, log-rank test, fig. 1D).

Table 1 The correlation between clinicopathological parameters and miR-21 expression in human esophageal adenocarcinoma

Variables	n (%)	MiR-21 expression	P-value
Age (years)			
≥60	18	5.52±1.76	0.443
<60	12	5.05±1.38	
Gender			
Male	20	5.29±1.63	0.863
Female	10	5.40±1.65	
Differentiation			
Well/Moderate	12	5.13±1.78	0.591
Poor	18	5.46±1.52	
TNM stage			
Stage I/II	11	4.60±1.19	0.058
Stage III/IV	19	5.75±1.69	
Invasive depth			
T1/T2	11	5.23±1.18	0.810
T3/T4	19	5.38±1.84	
Lymph node metastasis			
Negative	13	4.53±1.16	0.015
Positive	17	5.94±1.66	

2.2 Involvement of MiR-21 in Regulation of Anoikis Resistance in Human EA OE33 Cells

Acquisition of anoikis resistance has been considered a critical step in the progression of tumor metastasis. In this study, we generated an anoikis-resistant cell line using suspension culture of OE33 cells, named OE33/AR (fig. 2A and 2B). The qRT-PCR analysis verified that the miR-21 expression level in OE33/AR cells was significantly increased compared to the primary OE33 cells (2.4-fold increase, P<0.05, fig. 2C). Furthermore, we investigated the phenotypic change of anoikis in OE33 cells after transfection of miR-21 mimic, and revealed that the enforced miR-21 expression could induce anoikis resistance (fig. 2D and 2E).

2.3 PDCD4 and PTEN: the Functional Targets of miR-21 in OE33 Cells

PDCD4 and PTEN, both of which play a regulatory role in cell apoptosis, have been determined to be the direct targets of miR-21 in multiple cell types such as mesenchymal stem cells, nerve cells, and cancer cells (melanoma, cervical cancer, gastric cancer, etc.)[9–11]. FasL, which induces apoptosis in Fas-positive cells, is another reported target of miR-21[18]. In this study, we first analyzed the expression of PDCD4, PTEN and FasL in OE33 cells and anoikis-resistant OE33/AR cells. As shown in fig. 3A, the protein levels of PDCD4 and PTEN (but not FasL) in OE33/AR cells were lower than those in OE33 cells. Furthermore, we observed that the protein levels of PDCD4 and PTEN (but not FasL) were markedly decreased when OE33 cells were transfected with miR-21 mimics. We thus hypothesized that PDCD4 and PTEN are the direct miR-21 targets in OE33 cells. To confirm this, we constructed luciferase-reporter plasmids that contained the wild-type or mutant 3′-UTR segments of PDCD4 and PTEN, respectively (fig. 3B). Luciferase reporter assays showed that co-transfection of miR-21 strongly inhibited the luciferase activity from the reporter construct containing the 3′-UTR segment of PDCD4 and PTEN, whereas no effect was observed with their corresponding mutant constructs (fig. 3C and 3D). Therefore, we concluded that in OE33 cells PDCD4 and PTEN are the functional targets of miR-21.

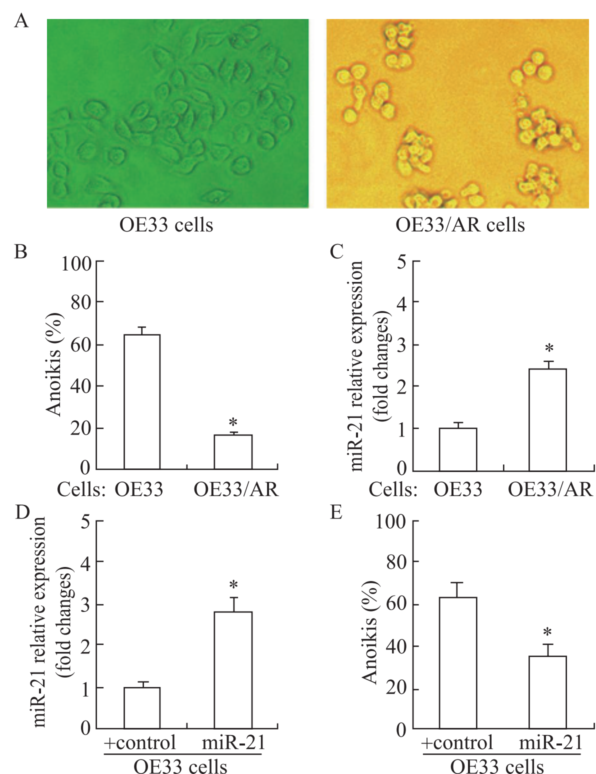

Fig. 2 Effects of miR-21 expression on anoikis resistance (AR) in human EA OE33 cells

A: OE33 cells cultured in normal cell plates and OE33/AR cells cultured in plates pre-coated with Poly-HEMA. B: Anoikis after detachment in OE33/AR cells was significantly decreased compared to OE33 cells. C: miR-21 relative expression in OE33/AR cells was significantly increased compared to OE33 cells. D: miR-21 relative expression was significantly increased in OE33 cells after miR-21 mimics transfection. E: The miR-21 mimics transfection in OE33 cells led to decreased anoikis.

2.4 Regulation of Metastasis Formation of OE33 Cells *In Vivo* by Controlling miR-21 Expression

To further examine the effect of miR-21 on metastasis *in vivo*, the metastatic potential of stably miR-21 and anti-miR-21-transfected OE33 cells were injected into BALB/c nude mice via the tail vein. Fig.

4A showed that after lentivirus-mediated transfection of miR-21 (lenti-miR-21) and anti-miR-21 (lenti-anti-miR-21), the expression levels of miR-21 in OE-33 cells were significantly increased or decreased, respectively (P<0.05). Consistently, the anoikis was significantly decreased in OE33 cells transfected with lenti-miR-21, and significantly increased in OE33 cells transfected with lenti-anti-miR-21, as compared with OE33 cells transfected with blank lentivirus, respectively (P<0.05, fig. 4B). Fig. 4C and 4D showed that intravenous injection of lenti-miR-21-transfected OE33 cells induced marked liver tumor formation *in vivo*, whereas intravenous injection of OE33 cells transfected with lenti-anti-miR-21 induced fewer liver metastases (P<0.05).

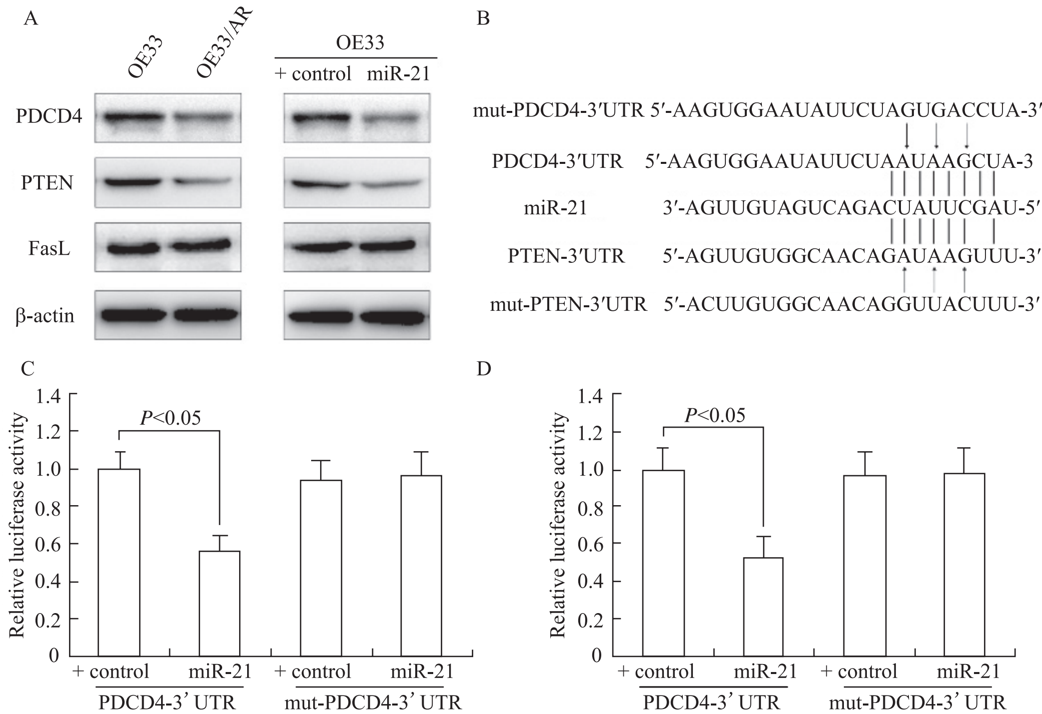

Fig. 3 PDCD4 and PTEN: the direct targets of miR-21 in OE33 cells

A: the protein levels of PDCD4, PTEN and FasL detected by Western blot analysis; B: schematic diagram illustrating luciferase reporter constructs used in C and D. C and D: Luciferase reported assays confirmed that PDCD4 and PTEN were directly targeted by miR-21 in OE33 cells. mut: mutant

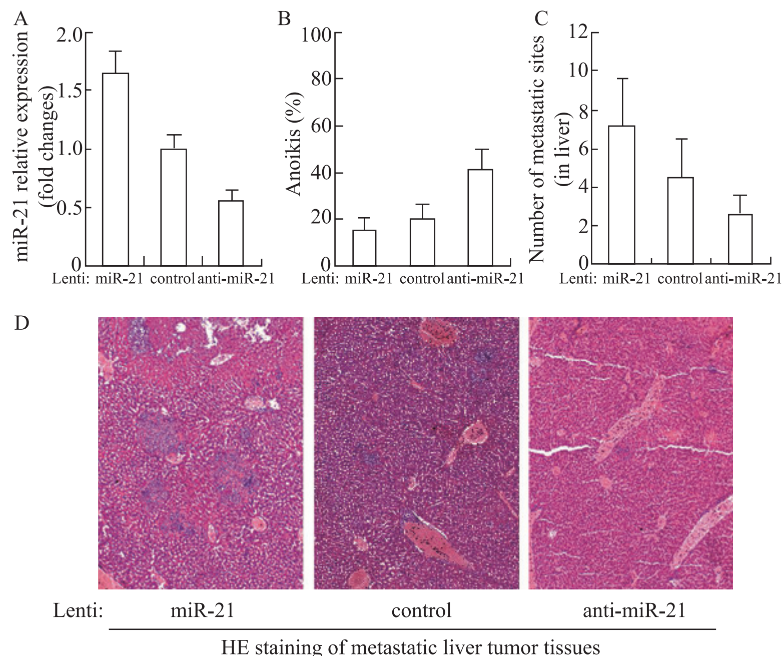

Fig. 4 *In vivo* experiment

A: OE33 cells were transfected with lenti-miR-21, lenti- anti-miR-21 and lenti-control, respectively. B: Effects of lentivirus-mediated transfection on anoikis in OE33 cells *in vitro*. C and D: Effects of lentivirus-mediated transfection on metastasis formation in OE33 cells *in vivo*. D: HE staining, 40×

3 DISCUSSION

MiR-21 is one of the most commonly and dramatically up-regulated miRNAs in human malignancy, including lung cancer, breast cancer, gastric cancer, hepatocellular carcinoma, and some other solid tumors. Feber *et al* first found that miR-21 expression in EA tissues was significantly higher than that in adjacent non-cancerous squamous epithelium tissues[19]. Subsequently several studies reported similar results. However, there are contradictory results about the correlation between miR-21 expression and survival time of the EA patients. Meng *et al* reported that no relationship was found between miR-21 expression and survival[20]. Winther *et al* conversely reported that miR-21 was an independent prognostic biomarker for EA patients, but not for patients with esophageal squamous cell carcinoma[21]. In the present study, we confirmed that miR-21 expression was significantly up-regulated in human EA tissues, and the increased miR-21 expression was associated with poor prognosis. Additionally, we found that miR-21 expression correlates with lymph node status, indicating that miR-21 was involved in the regulation of EA metastasis.

Acquisition of anoikis resistance is critical for tumor cells surviving after detachment and traveling to distant sites during metastasis. Increased miR-21 expression has been determined to contribute to apoptosis suppression by reducing gene expression such as PDCD4, PTEN and FasL[9–11, 22]. PDCD4, a novel tumor suppressor, is involved in cell apoptosis, transformation, tumor promotion and progression[23, 24]. It has been established that miR-21 exerts its oncogenic activity by directly targeting the 3′-UTR region of PDCD4 and inhibiting its expression[23, 24]. PTEN, also regarded as a tumor suppressor gene, has been revealed to be involved in the regulation of many basic cellular functions including cell apoptosis. Up-regulation of PTEN increases apoptosis, whereas its inactivation activates the Akt signaling and reduces apoptosis[25, 26]. Several studies have determined that increased miR-21 expression suppresses apoptosis by inhibiting PTEN/Akt signaling pathway[27, 28]. In this study, we revealed that in EA OE33 cells both PDCD4 and PTEN are the direct functional targets of miR-21. Up-regulation of miR-21 in OE33 cells results in the inhibition of PDCD4 and PTEN, which subsequently give cells better ability of anoikis resistance. FasL, which triggers apoptosis in cells expressing Fas antigen, is another reported target of miR-21[22]. However, in EA OE33 cells, we found no effect of changed miR-21 expression on the protein level of FasL.

Restoring or inhibiting the expression of specific miRNA could be a potential approach to treat cancer. As the most known oncomiR, development of a targeted therapeutic that aims to silence miR-21 seems to be very promising. Numerous studies have reported successful therapeutic use of anti-miR-21 *in vitro* and *in vivo*. Gaudelot *et al* reported that targeting miR-21 is an effective therapeutic strategy to improve chemotherapy efficacy in renal carcinoma cells[29]. He *et al* demonstrated that miR-21 suppression in combination with 5-fluorouracil and pirarubicin treatment significantly improved the inhibition of tumor growth in a subcutaneous xenograft hepatocellular carcinoma mouse model[30]. Yan *et al* reported that knockdown of miR-21 by locked nucleic acid (LNA) suppressed cell growth and proliferation of MCF-7 cells *in vitro*, and suppresses MCF-7 xenograft growth[31]. Sicard *et al* revealed that miR-21 depletion by lentiviral infection impeded proliferation and induced apoptosis of pancreatic cancer cells *in vitro* and *in vivo*[32]. In the present study, we evaluated the effect of miR-21 expression on distant metastasis *in vivo*. OE33 cells with stably increased or decreased miR-21 expression were established by lentiviral infections and were injected into BALB/c nu/nu mice via the tail vein. Pathological examination of hepatic tissues showed that the formation of distant metastases of OE33 cells was associated with miR-21 expression level. Because increased miR-21 expression contributes to anoikis resistance, which is critical for the survival and distant metastasis of detached tumor cells, the *in vivo* result further validated the involvement of miR-21 in the anoikis regulation of human EA OE33 cells. Meanwhile, in this mouse experiment, inhibition of miR-21 expression led to less metastasis formation, indicating the potential therapeutic value of miR-21 in the treatment of human EA.

In summary, results of the present study establish a role for miR-21 in the acquisition of anoikis resistance. MiR-21 promotes anoikis resistance and metastasis via targeting PDCD4 and PTEN in human EA cells. By targeting miR-21, a therapeutic approach for preventing metastasis could be applied in patients with EA.

Conflict of Interest Statement

The authors declare that there is no conflict of interest with any financial organization or corporation or individual that can inappropriately influence this work.

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