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# High miR-3648 expression and low APC2 expression are associated with shorter survival and tumor progression in NSCLC

**Yongquan Dong[1]*, Biao Wu[2]*, Xiongxiong Wang[3], Feijie Lu[1], Qianjun Li[1] and Qiong Zhao[4]**

[1]Department of Respiratory Disease, [2]Department of Thoracic Surgery, [3]Department of Critical Care Medicine, Ningbo YinZhou No.2 Hospital, Ningbo and [4]Department of Thoracic Oncology, Shulan Hangzhou Hospital, Affiliated to Shulan International Medical College of Zhejiang Shuren University, Hangzhou, Zhejiang, China

*The first two authors contributed equally to this work

**Summary.** Background. Emerging studies have demonstrated that microRNAs (miRNAs) play crucial roles in the carcinogenesis of many developing human tumors. However, the clinical significance and biological function of microRNA-3648 (miR-3648) in non-small cell lung cancer (NSCLC) have been largely undefined.

Methods. The expression of miR-3648 and the mRNA of adenomatous polyposis coli 2 (APC2) in NSCLC tissues and cell lines were analyzed using quantitative real-time RT-PCR. The prognostic value of miR-3648 and APC2 was examined using the Kaplan-Meier method and Cox regression analyses. Experiments using NSCLC cells were conducted to explore the influences of miR-3648 on tumor cell proliferation, migration and invasion.

Result. Increased expression of miR-3648 was observed in NSCLC tissues and cell lines compared with the corresponding controls (all $P<0.05$). miR-3648 expression was associated with the differentiation, lymph node metastasis and TNM stage (all $P<0.05$) of NSCLC patients, and high expression of miR-3648 was associated with poor overall survival rate. NSCLC cell proliferation, migration and invasion were significantly enhanced by miR-3648 overexpression. The further luciferase reporter assay and expression results showed that the decreased APC2 might also be a prognostic biomarker, and served as a target of miR-3648 in NSCLC.

Conclusion. The findings from the present study indicate that the overexpression of miR-3648 serves as a useful biomarker for the prediction of prognosis in NSCLC, and promotes tumor cell proliferation, migration and invasion. APC2, as another prognosis-related molecule, may be a target of miR-3648 in NSCLC.

**Key words:** miR-3648, APC2, Non-small cell lung cancer, Biological function, Prognosis

*Corresponding Author:* Qiong Zhao, Department of Thoracic Oncology, Shulan Hangzhou Hospital, Affiliated to Shulan International Medical College of Zhejiang Shuren University, No. 848, Dongxin Road, Xiacheng District, Hangzhou, 310003, Zhejiang, China. e-mail: zhaoqiong_shulan@163.com

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## Introduction

Lung cancer ranks first among all malignancies in cancer-related deaths globally (Torre et al., 2016). Approximately 70% of lung cancer cases are diagnosed at advanced stages, mainly owing to the lack of specific symptom and effectual diagnostic tests (Smith et al., 2018). The majority of lung cancer cases, constituting approximately 80 to 85%, are non-small cell lung carcinomas (NSCLC) (Siegel et al., 2013) NSCLC is prevalent and lethal malignant tumors with especially grim prospects due to late-stage detection and chemoresistance (Wei et al., 2019). Despite recent developments in the diagnosis, classification and therapy, the overall survival is still poor (de Sousa and Carvalho, 2018). Currently, the 5-year overall survival rate remains undesirable (Wu et al., 2013). Consequently, accurate prediction of prognosis and efficient therapy are urgently needed to address the significant mortality associated with NSCLC.

MicroRNAs (miRNAs) are short (~22 nucleotides in length) and non-coding RNAs that act as post-transcriptional regulators of gene expression (Lin and Gregory, 2015). Individual miRNAs can have either oncogenic or tumour-suppressive function (Lu et al., 2005). In recent decades, the functional roles of miRNA in tumor progression have been identified in different types of human cancer, which has increased attention regarding the aberrant expression of miRNA in tumor

samples (Dan et al., 2018). miRNAs may potentially to be used in cancer-targeted therapy, and typically exhibit a high diagnostic and prognostic significance in patients with cancer (Deng and Chen, 2017). MicroRNA -3648 (miR-3648) has been reported to be differentially expressed between lung adenocarcinoma tissues and adjacent normal tissues (Lin et al., 2016). Liu et al found that the expression level of miR-3648 in EGFR-mutant NSCLC was significantly changed compared with that in non-EGFR-mutant NSCLC, suggesting that miR-3648 may be involved in NSCLC progression (Liu et al., 2020). In addition, the increased miR-3648 has been reported in bladder cancer, which promoted tumor cell invasion and metastasis (Sun et al., 2019). In the process of osteogenesis, miR-3648 was involved in the regulation of protein arginine methyltransferases mesenchymal stem cell osteogenic commitment and bone remodeling (Min et al., 2019). Adenomatous polyposis coli 2 (APC2) has been identified as a target gene of miR-3648, which played a mediator role in the promoting effects of miR-3648 on prostate cancer cell proliferation (Xing, 2019), and was directly inhibited by miR-3648 that was induced by endoplasmic reticulum stress (Rashid et al., 2017). However, the clinical and functional role of miR-3648 and its target gene APC2 remain elusive in NSCLC.

To investigate the role of miR-3648 in NSCLC further, the present study sought to assess the expression of miR-3648 in NSCLC tissues and cells, evaluate its clinical significance in prognosis, and explore its effects on NSCLC cell function. The clinical and functional role of miR-3648 in NSCLC progression have not been reported previously. The analysis results of this present study may provide novel biomarkers and targets for NSCLC prognosis and treatment.

## Materials and methods

### *Patients and clinical sample collection*

The experimental protocols were approved by the Ethics Committee of the Ningbo YinZhou No.2 Hospital, and signed informed consents were provided by the patients before sampling. A total of 118 patients were included in our cohort, patients who underwent surgical resection at Ningbo YinZhou No.2 Hospital between 2011 and 2014, and were diagnosed with NSCLC through a histopathological examination. Cancerous tissues and matched noncancerous tissues were collected from each patient during radical surgery, and were snap frozen in liquid nitrogen and stored at -80°C for further use. Patients who received preoperative therapy were excluded from our research. All the enrolled patients had complete electronic medical record information, and their clinicopathological characteristics are summarized in Table 1. A 5-year follow-up survey was conducted for each patient after the surgery, and their survival data were recorded.

### *Cell culture and transfection*

NSCLC cell lines (H1299, HCC827, H460, A549) and a normal lung cell line (NHBE) were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). All cells were incubated in Dulbecco's modified Eagle medium (Invitrogen, Thermo Fisher Scientific, Inc, Waltham, Massachusetts) supplemented with 10% fetal bovine serum (FBS, Invitrogen), in a humidified atmosphere with 5% $CO_2$ at 37°C.

Cell transfection was used in this study to achieve the in vitro regulation of miR-3648. The miR-3648 mimic (50 nM), miR-3648 inhibitor (100 nM) and the negative control sequence (50 nM of mimic NC, 100 nM of inhibitor NC) were synthesized from GenePharma (Shanghai, China), and were transfected into H1299 and A549 cells using Lipofectamine 2000 (Invitrogen, CA, USA). The cells transfected with only transfection reagents were set as mock. After 48h of transfection, the cells were used for further analyses.

### *RNA extraction and quantitative real-time RT-PCR (qRT-PCR)*

Total RNA was obtained from the collected tissue samples and cells by TRIzol reagent (Invitrogen, Life Technologies, Paisley, UK), then the RNA was reversely transcribed intro single-stranded cDNA with PrimeScript reverse transcriptase reagent kit (TaKaRa, Shiga, Japan)

**Table 1.** Association of miR-3648 and the clinical characteristics of NSCLC.

| Features | Total No. N=118 | MiR-3648 expression | | P values |
|---|---|---|---|---|
| | | Low (n=56) | High (n=62) | |
| Age (Years) | | | | 0.876 |
| ≤60 | 43 | 20 | 23 | |
| >60 | 75 | 36 | 39 | |
| Gender | | | | 0.893 |
| Female | 45 | 21 | 24 | |
| Male | 73 | 35 | 38 | |
| Smoking | | | | 0.919 |
| Never | 50 | 24 | 26 | |
| Ever | 68 | 32 | 36 | |
| Tumor size (cm) | | | | 0.574 |
| ≤3 | 60 | 30 | 30 | |
| >3 | 58 | 26 | 32 | |
| Differentiation | | | | 0.015 |
| Well/moderate | 62 | 36 | 26 | |
| Poor | 56 | 20 | 36 | |
| Lymph node metastasis | | | | 0.010 |
| Negative | 57 | 34 | 23 | |
| Positive | 61 | 22 | 39 | |
| TNM stage | | | | 0.004 |
| I-II | 53 | 33 | 20 | |
| III-IV | 65 | 23 | 42 | |

following the manufacturer's guidelines. The expression of miR-3648 and mRNA of APC2 was measured by qRT-PCR, which was carried out using a SYBR green I Master Mix kit (Invitrogen, Carlsbad, CA, USA) on a 7500 Real-Time PCR System (Applied Biosystems, USA). U6 was used as an endogenous control for miR-3648, and GAPDH was used as an endogenous control for APC2. Following were the primers used: miR-3648 forward 5'-TCGGCAGGAGCCGCGGGGATC-3', miR-3648 reverse 5'-CTCAACTGGTGTCGTGGA-3'; APC2 forward 5'-CTGTACCGGGTCTCTGCAGTGTTA-3', APC2 reverse 5'-TACGCCGGACAGATGGCTTTA-3'; U6 forward 5'-CTCGCTTCGGCAGCACA-3', U6 reverse 5'-AACGCTTCACGAATTTGCGT-3'; GAPDH forward 5'-GCACCGTCAAGGCTGAGAAC-3', GAPDH reverse 5'-TGGTGAAGACGCCAGTGGA-3'. The final expression value was calculated using the $2^{-\Delta\Delta Ct}$ method.

### *Cell proliferation analysis*

Cell Counting Kit 8 (CCK-8) assay was used to analyze cell proliferation rate. Cells were seeded into a 96-well plate ($2\times10^3$ cells/well) and incubated for 0, 24, 48, and 72 h. Then, 10 μL of CCK-8 reagent (Beyotime, Shanghai, China) was added to each well and further incubated for 2 h. The optical density at 450 nm was analyzed using a microplate reader.

### *Transwell assay*

Transwell chambers (Corning, NY, USA) were applied in this study for the measurement of cell migration and invasion of NSCLC cells. The Transwell chambers precoated with Matrigel (Corning, NY, USA) were used for invasion assay, while the chambers without Matrigel coating were used for migration assay. The transfected cells with a density of $2\times10^5$ cells/chamber were seeded into the upper chambers with serum-free medium, and the low chambers were filed with culture medium supplemented with 10% FBS as chemoattractant. The cells in the lower chambers were stained after 48 h of incubation, and were counted under an inverted microscope (Olympus Corporation, Tokyo, Japan).

### *Luciferase reporter assay*

According to bioinformatic analyses, the potential target genes were predicted from TargetScan (http://www.targetscan.org/vert_72/) (Agarwal et al., 2015). We found that APC2 contained a binding site of miR-3648, and used a luciferase reporter assay to confirm the interaction between miR-3648 and APC2. The wild type (WT) and mutant type (MUT) of APC2 3'-UTR sequences, which contained that binding site of miR-3648, were combined into the luciferase reporter vector pGL3-luciferase basic vector (Promega, Madison, WI, USA). The combined vectors were then co-transfected into tumor cells with miR-3648 mimic, miR-3648 inhibitor or the NCs using Lipofectamine 2000 (Invitrogen). After incubation for 48 h, the relative luciferase activity was analyzed with a Dual Luciferase Reporter Assay System (Promega).

### *Statistical analysis*

Data were presented as mean ± standard deviation (SD) and analyzed with SPSS 21.0 (SPSS, Inc, Chicago, Illinois) and GraphPad 7.0 (GraphPad Software, Inc, USA). Differences between groups were analyzed with Student's t test or one-way analysis of variance and Tukey post hoc test. The relationship between miR-3648 and the clinicopathological characteristics of patients was assessed using Chi-square test. The Kaplan-Meier survival method and Cox regression analyses were adopted to examine the prognostic value of miR-3648 and APC2. Pearson correlation coefficient was used to analyze the correlation between the expressions of miR-3648 and mRNA of APC2. A $P<0.05$ was considered to

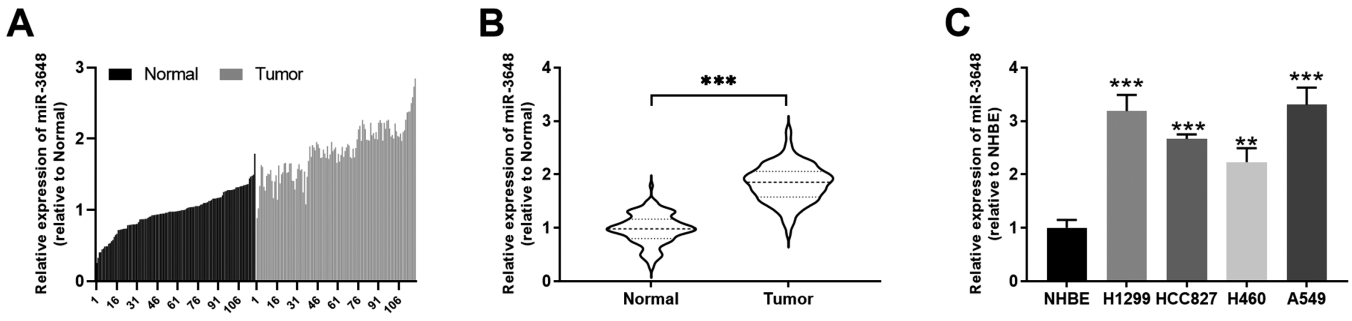


**Fig. 1.** Expression of miR-3648 examined by qRT-PCR in NSCLC. **A.** Expression of miR-3648 in tumor tissues and adjacent normal tissues in 118 NSCLC patients. **B.** Expression of miR-3648 was enhanced in NSCLC tissues compared with the normal tissues (n=118 for each group; ***P<0.001). **C.** Expression of miR-3648 was upregulated in the four NSCLC cell lines compared with the normal cells (data were obtained from 3 repeated experiments; **P<0.01, ***P<0.001).

indicate a statistically significant difference.

## Results

### *Expression of miR-3648 in NSCLC tissues and cell lines*

To further understand the role of miR-3648 in NSCLC, its expression in NSCLC sample and cell lines was quantified by qRT-PCR. We observed that the expression levels of miR-3648 in tissue samples were obviously enhanced in the NSCLC patients compared with the healthy or normal controls (all P<0.001 Fig. 1A,B). Meanwhile, the levels of miR-3648 in NSCLC cell lines were investigated, which also found the higher expression of miR-3648 in NSCLC cell lines (H1299, HCC827, H460, A549) than that in normal NHBE cells (all P<0.01, Fig. 1C).

### *Association between miR-3648 expression and clinicopathological features of patients with NSCLC*

The present study subsequently explored the role of miR-3648 in the development of NSCLC by analyzing the association between miR-3648 expression and the clinicopathological data of the patients. The findings from this analysis showed that miR-3648 expression was associated with lymph node metastasis (P=0.010), differentiation (P=0.015) and TNM stage (P=0.004). In contrast, no association was found between miR-3648 expression and other parameters, such as tumor size, gender, age and smoking history (all P>0.05, Table 1).

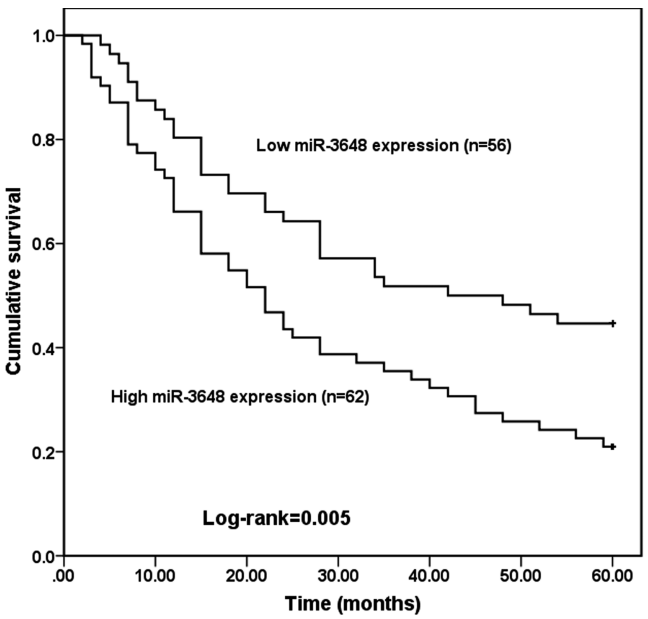


**Fig. 2.** Kaplan-Meier survival curves in the patients with NSCLC and APC2. Patients with high expression of miR-3648 had shorter survival time than those with high miR-3648 expression (Log-rank P=0.005).

### *Clinical significance of miR-3648 in the prognosis of NSCLC*

The association of miR-3648 expression with the overall survival of patients was estimated by plotting the Kaplan-Meier survival curves (Fig. 2), which indicated that patients with low miR-3648 expression levels had better overall survival than those with high miR-3648 expression levels (log-rank P=0.005). Furthermore, our multivariate Cox analysis demonstrated that miR-3648 (HR=1.778, 95% CI=1.059-2.984, P=0.029) and TNM stage (HR=1.733, 95% CI=1.038-2.893, P=0.036) were two independent prognostic factors for the survival of patients with NSCLC (Table 2).

### *Effects of miR-3648 on cell proliferation of H1299 and A549 cells*

To further understand the functional role of miR-3648 in the tumor progression of NSCLC, H1299 and A549 cells were used for cell experiments. Regulation of miR-3648 was achieved by cell transfection in vitro, and the expression of miR-3648 was successfully upregulated by the miR-3648 mimic, and was downregulated by the miR-3648 inhibitor in both H1299 and A549 cell lines (all P<0.001, Fig. 3A,B). After cell transfection, we found that the proliferation of tumor cells was significantly enhance by the overexpression of miR-3648, but was inhibited by the downregulation of miR-3648 when compared with mock group (all P<0.05, Fig. 3C,D).

### *Regulatory effects of miR-3648 on NSCLC cell migration and invasion*

In addition to cell proliferation, the effects of miR-3648 on migration and invasion of H1299 and A549 cells were also investigated. The upregulation of miR-3648 resulted in enhanced cell migration, while the downregulation of miR-3648 led to the opposite result (all P<0.001 Fig. 4A,B). Similarly, the NSCLC cell invasion ability was also promoted by miR-3648 overexpression, but was suppressed by miR-3648 reduction (all P<0.001, Fig. 4C,D).

**Table 2.** Multivariate Cox regression analysis in NSCLC patients.

| Variables | Multivariate analysis | | |
|---|---|---|---|
| | HR | 95% CI | P value |
| miR-3648 | 1.778 | 1.059-2.984 | 0.029 |
| Age | 1.294 | 0.808-2.072 | 0.284 |
| Gender | 1.194 | 0.733-1.944 | 0.476 |
| Smoking | 1.118 | 0.691-1.808 | 0.650 |
| Tumor size | 1.204 | 0.758-1.914 | 0.432 |
| Differentiation | 1.568 | 0.972-2.528 | 0.065 |
| Lymph node metastasis | 1.071 | 0.673-1.705 | 0.773 |
| TNM stage | 1.733 | 1.038-2.893 | 0.036 |

### *APC2 is a direct target of miR-3648 in NSCLC cells*

According to the prediction by the TargetScan, APC2 was predicted as a target of miR-3648 with a complementary sequence at its 3'-UTR (Fig. 5A). The results of luciferase reporter assay showed that the luciferase activity of APC2-WT group was decreased by miR-3648 overexpression, but was increased by miR-3648 reduction in both H1229 and A549 cells (all $P<0.05$, Fig. 5B,C). However, there was no significant luciferase activity change in the MUT group. (all $P>0.05$). The detection of APC2 expression levels in H1229 and A549 cells revealed that miR-3648 mimic significantly inhibited APC2 levels, while the silencing of miR-3648 enhanced APC2 levels (all $P<0.01$, Fig. 5D,E).

### *Expression and prognostic value of APC2 in patients with NSCLC*

Further analysis found that the mRNA expression of APC2 in tumor tissues was significantly lower compare with that in normal lung tissues in NSCLC patients ($P<0.001$, Fig. 6A), and its expression in tumor tissues was negatively correlated with expression of miR-3648 ($r=-0.760$, $P<0.001$, Fig. 6B). The association of APC2 expression with the overall survival of patients was estimated by plotting Kaplan-Meier survival curves (Fig. 6C), which indicated that patients with high APC2 expression levels had better overall survival than those with low APC2 expression levels (log-rank $P=0.017$). It is suggested that the expression of APC2 might also

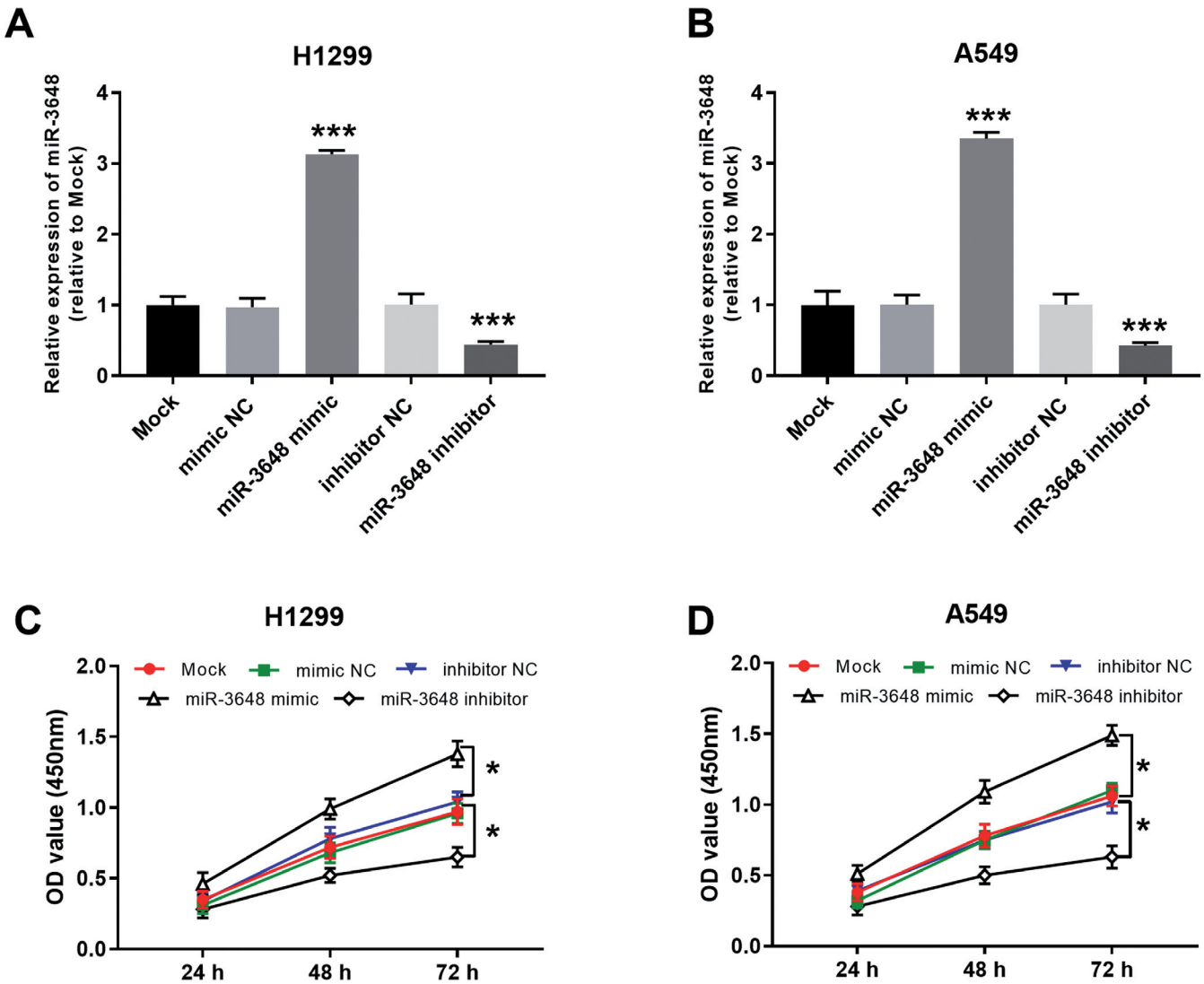


**Fig. 3.** Effects of miR-3648 on cell proliferation of H1299 andA549 cells. **A, B.** Expression of miR-3648 was upregulated by the miR-3648 mimic, but was downregulated by the miR3648 inhibitor. **C, D.** Tumor cell proliferation was promoted by the upregulation of miR-3648, while it was inhibited by the downregulation of miR-3648. (Data were obtained from 3 repeated experiments; *P<0.05, **P<0.001)

serve as a prognostic indicator for the survival of NSCLC.

## Discussion

Lung cancer is the most common cause of cancer-related deaths worldwide (Torre et al., 2015). NSCLC represents approximately 85% of lung cancers, and the 5-year overall survival (OS) rate is about 15% (Goldstraw et al., 2007). Despite advances in early detection and standard treatment, NSCLC is frequently diagnosed at an advanced stage, leading to poor prognosis in cancer patients (Soria et al., 2010). Therefore, to improve NSCLC prognosis and treatment, it is essential to further understand the molecule and related mechanisms involved in NSCLC progression.

MiRNA are critical regulators of gene expression. Abnormal miRNA expression may be involved in the pathogenesis of cancer (Lujambio and Esteller, 2007). For instance, the overexpression of miR-193b in glioma

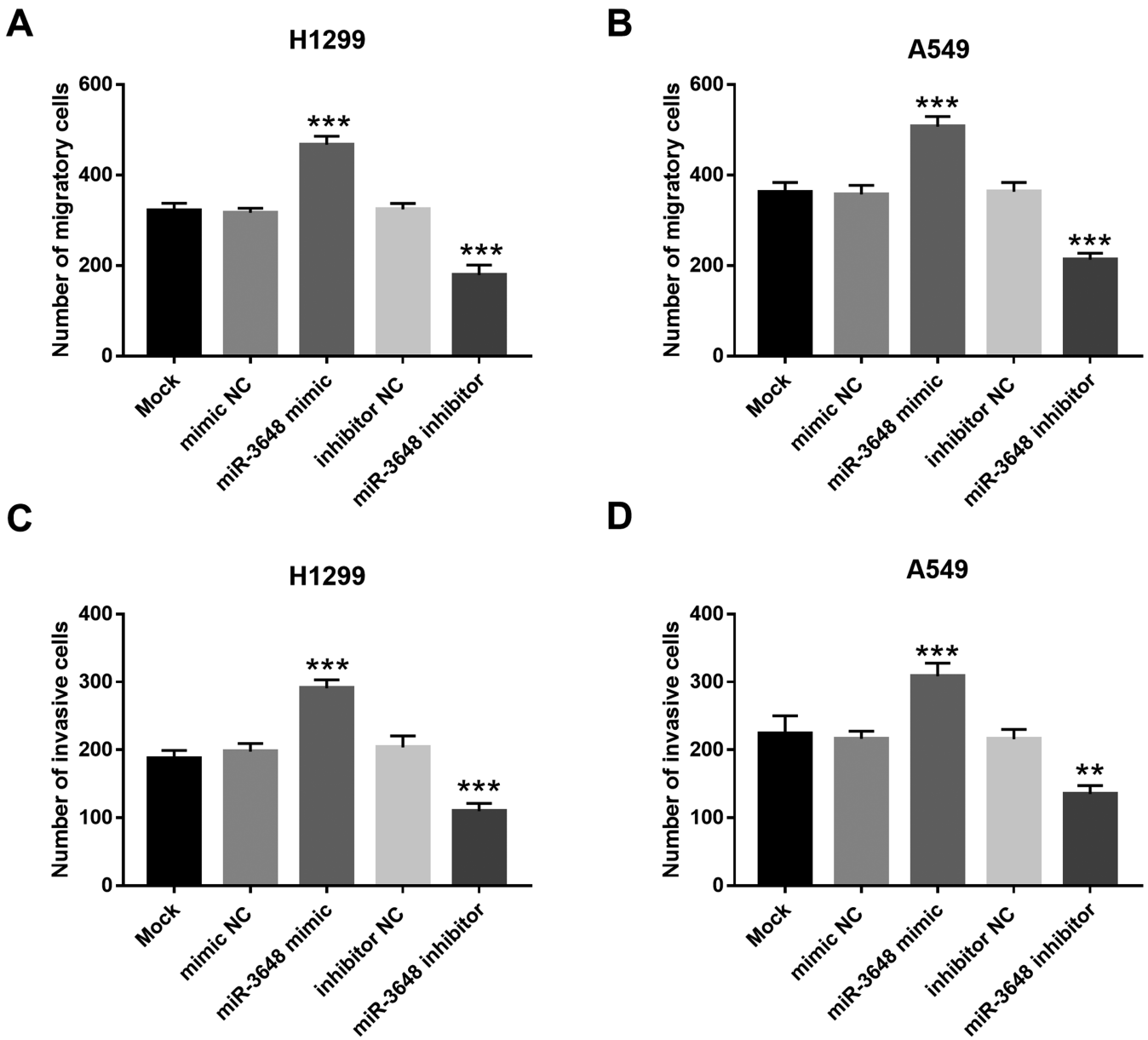


**Fig. 4.** Regulatory effects of miR-3648 on NSCLC cell migration and invasion. **A, B.** The migratory ability H1299 and A549 in NSCLC cell lines was enhanced by the overexpression of miR-3648 but inhibited by downregulation of miR-3648. **C, D.** Similarly, cell invasion was also promoted by miR-3648 overexpression but suppressed by miR-3648 reduction in H1299 and A549 NSCLC cell lines. (Data were obtained from 3 repeated experiments; **P<0.01, ***P<0.001)

cells led to increased proliferation, migration and invasion, whereas its inhibition resulted in the opposite effects on these cell behaviors, indicating the therapeutic potential of miR-193b in glioma (Zhu et al., 2019). The abnormal expression of miR-497 has been confirmed in many other tumor types, including gastric cancer (Feng et al., 2019), colorectal cancer (Zou et al., 2019). These result suggest that miR-497 has a tumor-suppressive role

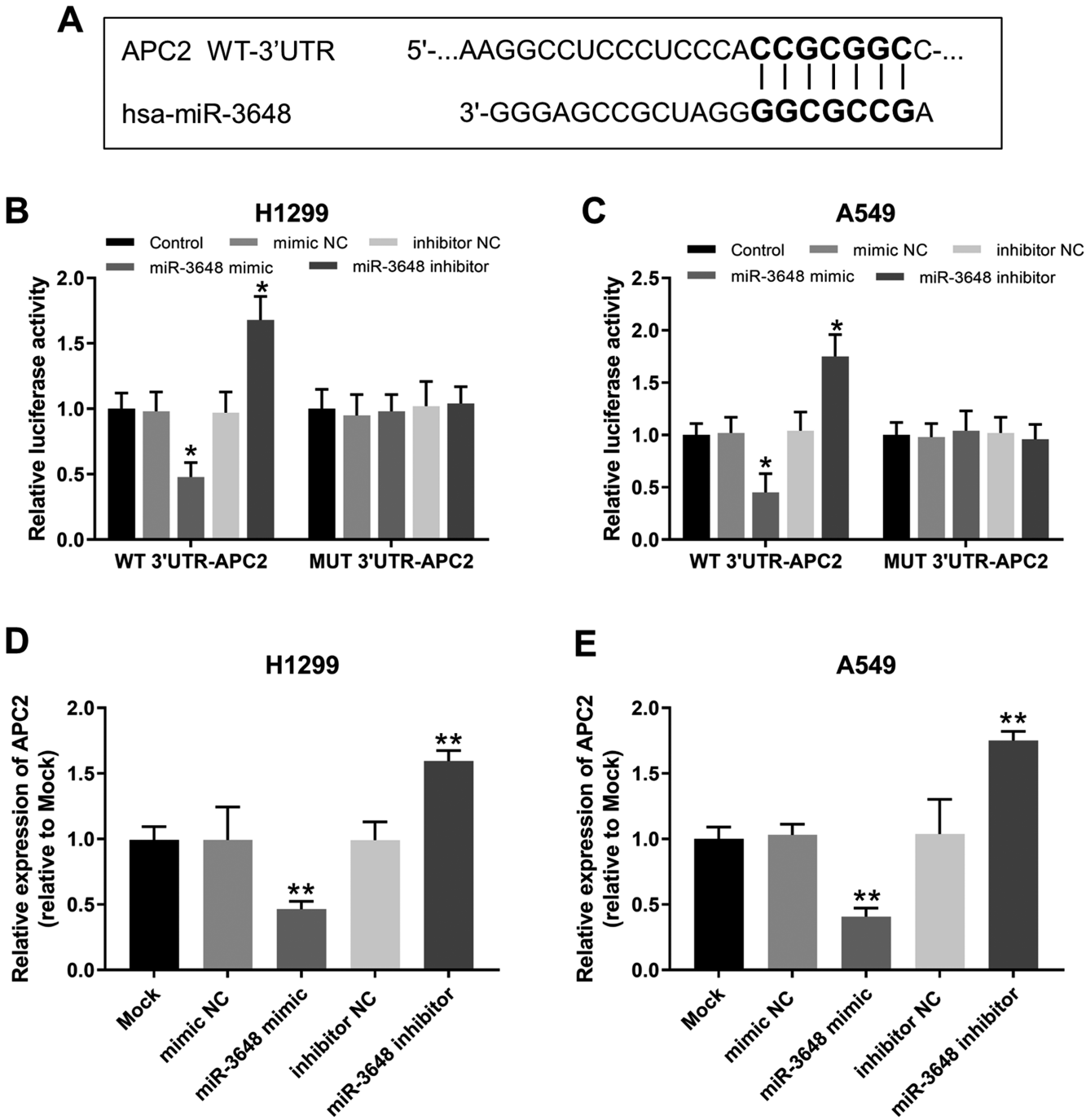


**Fig. 5.** APC2 is a direct target of miR-3648 in NSCLC cells. **A.** The predicted target sequence in the APC2 for miR-3648 binding. **B, C.** In H1299 and A549 NSCLC cell lines, the luciferase activity of theAPC2 -WT was decreased by miR-3648 overexpression but was increased by miR-3648 expression reduction. **D, E.** Expression of APC2 level in H1299 and A549 cells show that miR-3648 mimic inhibited APC2 level, while silencing of miR-3648 enhanced APC2 level. (Data were obtained from 3 repeated experiments; *P<0.05, **P<0.001).

in malignant tumors (Yang et al., 2016). Liu et al found that miR-214 and miR-218 function as tumor suppressors in breast cancer, and may act as biomarkers and potential therapeutic targets in breast cancer (Liu et al., 2016). Hence, novel miRNAs, which can accurately screen cancer cases and predict prognosis, are required to improve the treatment of malignancies in humans.

miR-3648, like other functional miRNA, is also a research hotspot, especially in the impact on tumor cell proliferation, migration and invasion. For example, the results of Sun et al. showed that the overexpression of miR-3648 played an oncogenic role in mediation of breast cancer invasion and metastasis through regulating the TCF21/KISS1 axis, revealing the potential of miR-3648 as a tumor biomarker and therapeutic target (Sun et al., 2019). In prostate cancer, miR-3648 overexpressed in tumor tissues, and promoted the proliferation of prostate cancer cells (Xing, 2019). However, the function and mechanisms of miR-3648 have barely been explored in NSCLC. The present study found the upregulated expression of miR-3648 in NSCLC tissues and cell lines compared with the corresponding normal controls. Because of the dysregulation of miR-3648 in NSCLC, the clinical significance of the abnormal expression of miR-3648 was further investigated in this experiment. From the Kaplan-Meier survival curves and Cox regression analysis, we determined that high miR-3648 expression was associated with poor overall survival, and served as an independent prognostic factor for the overall survival of patients with NSCLC. Moreover, enhanced miR-3648 expression was found to be associated with lymph node metastasis, differentiation, and TNM stage in NSCLC patients, indicating that the upregulated expression of miR-3648 might be related with the development of NSCLC. Collectively, it is postulated that miR-3648 may act as a candidate prognostic biomarker in NSCLC.

To further understand the biological function of miR-3648 in NSCLC progression, function-obtain and –loss cell experiments were conducted using cell transfection with miR-3648 mimic and miR-3648 inhibitor. We found that the downregulation of miR-3648 inhibited the proliferation, migration and invasion of tumor cells, while the upregulation of miR-3648 enhanced the biological behaviors of tumor cells. These results confirm that miR-3648 may play a role in promoting tumorigenesis in the progression of NSCLC. However, the mechanisms underlying the role of miR-3648 in NSCLC remain unclear. In this experiment, the results of luciferase reporter assay revealed that miR-3648 directly bound to APC2 in NSCLC. We suppose that APC2 may be a direct target of miR-3648 in NSCLC. In the study of Zhang et al, it is demonstrated that overexpressed G9a represents a promising therapeutic target, and targeting G9a potentially suppresses growth and Wnt signaling pathway partially through down-regulating HP1α and epigenetically restoring APC2, which is a tumor suppressor that is silenced in NSCLC (Zhang et al., 2018). Another study showed that miR-1205 promoted lung adenocarcinoma cell growth by targeting APC2 protein expression (Dai et al., 2019). Thus, we deduced that miR-3648 might be involved in NSCLC progression by targeting APC2. Meanwhile, the Kaplan-Meier curves showed that patients with low mRNA expression of APC2 exhibited a shorter overall survival time compared with those with high mRNA expression of APC2. Thus, it is suggested that the expression APC2 has certain prognostic value in NSCLC. The biological function of APC2 was not deeply explored in the present study, which is one of the limitations of this study, and further investigations are needed to confirm the role of APC2 and its relationship with miR-3648 in NSCLC progression.

Taken together, all the data in this study indicated that miR-3648 expression is upregulated in NSCLC tissues and cells compared with normal controls. The upregulation of miR-3648 predicts poor prognosis in patients with NSCLC, and may serve as an independent prognostic biomarker. The overexpression of miR-3648 in NSCLC cells results in enhanced cell proliferation,

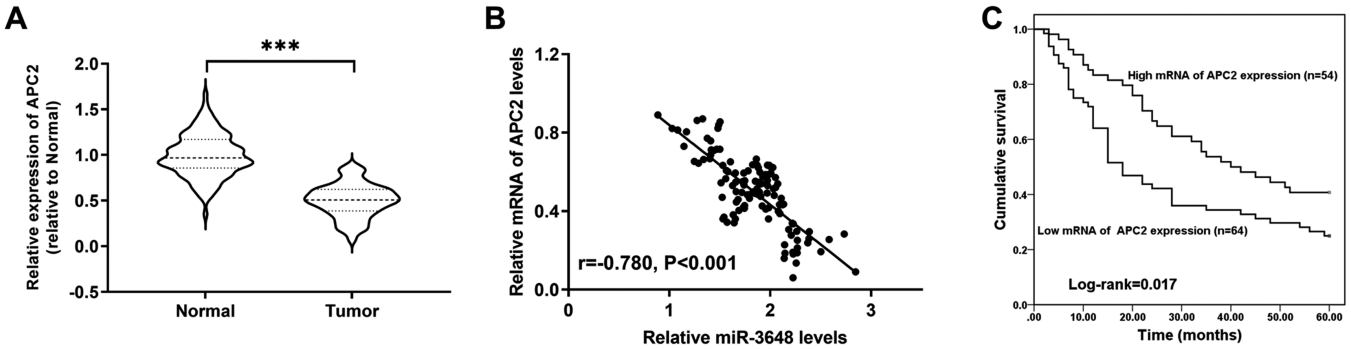


**Fig. 6.** Expression and prognostic value of APC2 in patients with NSCLC. **A.** The expression of APC2 in normal tissue was significantly enhanced compare with NSCLC tissue (n=118 for each group; ***P<0.001). **B.** Expression levels of APC2 were negatively correlated with the level of miR-3648 (r=-0.708, P<0.001). **C.** Patients with low expression of mRNA of APC2 had shorter survival time than those with high mRNA of APC2 expression (Log-rank P=0.017).

migration and invasion, revealing the potential of miR-3648 as a therapeutic target of NSCLC. APC2 is identified as a NSCLC prognosis-related gene, and acts as a target gene of miR-3648 in NSCLC. Finally, the functional role and mechanism of miR-3648 were analyzed only in vitro, which was one of the limitations of our study, and in vivo animal experiments will be necessary in future studies. For the prognostic value of miR-3648 and APC2, the small sample size might limit the accuracy of the results, and a larger study population is necessary to confirm the clinical value results. Additionally, although miR-3648 was identified as a biomarker to predict NSCLC prognosis, the non-specificity of miRNAs needs to be considered, and the combined detection of miRNAs with other tumor-related biomarkers may be more feasible in future clinical practices.

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*Ethics approval and consent to participate.* A signed written informed consent was obtained from each patient and the experimental procedures were all in accordance with the guideline of the Ethics Committee of Ningbo YinZhou No.2 Hospital.

*Consent for publication.* Written informed consent for publication was obtained from each participant.

*Availability of data and material.* All data generated or analyzed during this study are included in this published article.

*Competing interests.* The authors declare that they have no competing interests.

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