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MiR-221 promotes trastuzumab-resistance and metastasis in HER2-positive breast cancers by targeting PTEN

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HER2-overexpressing breast cancers are characterized by frequent distant metastasis and often develop resistance after short-term effective treatment with the monoclonal antibody drug, trastuzumab. Here, we found that the oncogenic miRNA, miR-221, inhibited apoptosis, induced trastuzumab resistance and promoted metastasis of HER2-positive breast cancers. The tumor suppressor PTEN was identified as a miR-221 target; overexpression of PTEN abrogated the aforementioned miR-221-induced malignant phenotypes of the cells. These findings indicate that miR-221 may promote trastuzumab resistance and metastasis of HER2-positive breast cancers by targeting PTEN, suggesting its role as a potential biomarker for progression and poor prognosis, and as a novel target for trastuzumab-combined treatment of breast cancers. [BMB Reports 2014; 47(5): 268-273].

INTRODUCTION

Human epidermal growth factor receptor 2 (HER2) is a transmembrane receptor tyrosine kinase of the epidermal growth factor (EGF) receptor family, playing a key role in normal cell growth and differentiation. HER2 overexpression, which was found in 25-30% metastatic breast cancers (MBC) (1, 2), leads to a more aggressive tumor phenotype featured by frequent distant metastasis and often acquired resistance to tumoricidal drugs during one year (3-7). However, the mechanism underlying these malignant phenotypes of HER2-positive breast cancers remains elusive (3, 8, 9).

MicroRNAs (miRNAs) are a class of conservative 22-25 nucleotide RNA molecules that negatively regulate gene expression (10). By base-pairing with the complementary sites in the 3'untranslated region (3'UTR) of the mRNA, miRNAs control mRNA stability and translation efficiency. Frequent deregulation of miRNAs has been detected in breast cancers, and some are associated with breast cancer metastasis and poor prognosis, suggesting an important role of miRNAs in breast oncogenesis and cancer progression (11, 12). The oncogenic miRNA, miR-221, is one of the few miRNAs that are consistently up-regulated in malignancies of various tissue origins, including the glioblastomas (13), prostate carcinoma (14), gastric carcinoma (15), melanoma (16), luminal breast cancer cells (17). Consistent with these reports, our previous study suggested that miR-221 was upregulated in breast cancer cells resistant to the therapeutic HER2 antibody, trastuzumab (Gene Expression Omnibus, assigned accession #: GSE47011). Here, we established that miR-221 promotes the invasiveness and trastuzumab resistance of HER2-positive breast cancers by targeting the tumor suppressor gene PTEN. The effect of restored PTEN expression on the malignant phenotypes of breast cancers was also addressed.

RESULTS

MiR-221 conferred trastuzumab resistance in HER2-positive cell line

We previously generated recombinant lentiviruses of a miR-221 precursor, which achieved efficient delivery and expression of pre-miR-221 in HER2-positive cell line- SK-BR-3 (18). Conversely, transfection of cells with a synthesized miR-221 inhibitor caused dramatic downregulation of miR-221 (Fig. 1A). Then MTT assay showed that overexpression of pre-miR-221 increased cell growth in the presence of trastuzumab; cells transfected with miR-221 inhibitors showed a significant decrease of surviving cells (Fig. 1B). We next used flow cytometry to test the role of miR-221 in apoptosis. MiR-221 suppression triggered massive apoptosis of SK-BR-3 cells when exposed to trastuzumab, whereas in-

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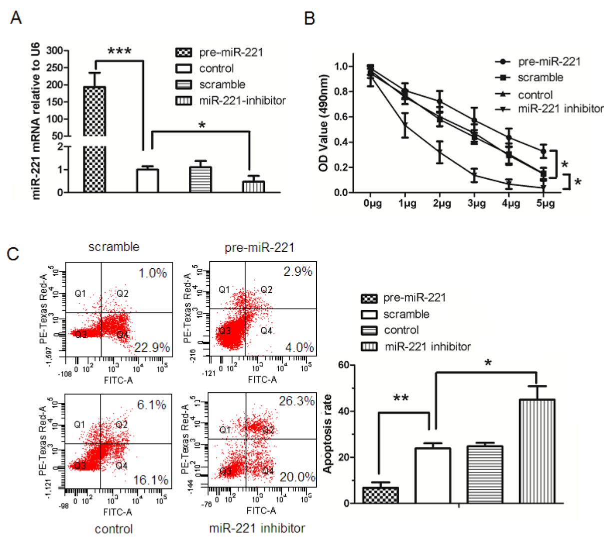

Fig. 1. MiR-221 conferred trastuzumab resistance in HER2-positive cell line. (A) qRT-PCR assay was performed for miR-221 expression in SK-BR-3 cells infected with pre-miR-221 lentiviruses, control, scramble, or miR-221 inhibitor. Data were compared with control transfected cells. All bars and error bars represent means ± SD (n = 3) (*P < 0.05 and ***P < 0.001). (B) SK-BR-3 cells were treated with increasing concentrations of trastuzumab. MTT assays for SK-BR-3 cells transfect with pre-miR-221 expressing lentivirus, scramble, control, or miR-221 inhibitor. All bars and error bars represent means ± SD (n = 3) (*P < 0.05). (C) SK-BR-3 cells transfect with pre-miR-221 expressing lentivirus, scramble, control, or miR-221 inhibitor were treated with trastuzumab (5 μg/ml) for 24 h, and then stained with FITC-conjugated Annexin V and PE-labeled PI. Apoptosis was analyzed by flow cytometry. All bars and error bars represent means ± SD (n = 3) (*P < 0.05 and **P < 0.01).

troduction of pre-miR-221 significantly decreased the number of cells undergoing apoptosis (Fig. 1C).

MiR-221 promotes cell motility and HER2-positive breast cancer metastasis

Enforced overexpression of pre-miR-221 promoted the mobility of breast cancer cells in a wound-healing assay and dramatically enhanced the invasion capacity of cells on a matrigel (Fig. 2A and B) as compared with control cells or cells modified to express a scramble miRNA. In contrast, miR-221 suppression by transfection with a miR-221 inhibitor resulted in a pronounced decrease in cell motility and invasion as compared with control cells (Fig. 2A and B).

A metastatic tumor model was further generated by challenging the nude mice in the tail vein with SK-BR-3 cells modified to stably express pre-miR-221 or a scramble pre-miRNA. At a time point allowing for lung metastasis development in our pilot experiment (5 weeks), mice were sacrificed. Metastatic nodules were observed on the surface of the lung in mice inoculated with cells expressing pre-miR-221, but not in those challenged with cells modified to express a scramble miRNA (Fig. 2C). Taken together, these results indicated that miR-221 could effectively promote the motility and invasion of cultured breast cancer cells, and expedite metastasis of breast cancers *in vivo*.

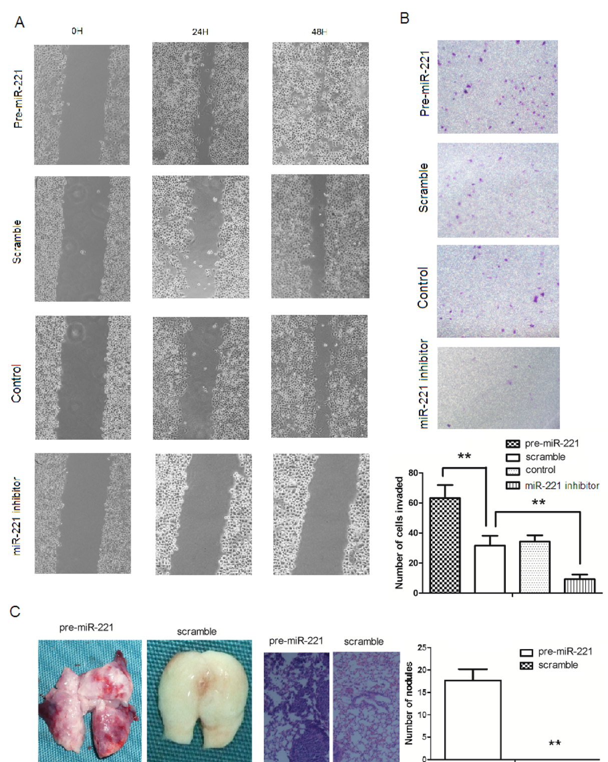

Fig. 2. MiR-221 overexpression promoted cell migration, invasion and metastasis *in vitro* and *in vivo*. (A) Wound-healing assay of SK-BR-3 cells infected with pre-miRNA expressing lentivirus, scramble, control, or miR-221 inhibitor. Representative images were taken at indicated time after scratching. (B) Transwell assay of SK-BR-3 cells infected with pre-miRNA expressing lentivirus, scramble, control, or miR-221 inhibitor. U6 was used to normalize the qRT-PCR data. All bars and error bars represent means ± SD (n = 3) (**P < 0.01). (C) Representative photos of the lungs and haematoxylin and eosin staining sections showing treated lung from mice injected with pre-miR-221 SK-BR-3 cells and scramble transfected cells. Metastatic nodules was counted. M, with metastasis; N, no metastasis (**P < 0.01).

PTEN is a direct target gene of miR-221 in HER2-positive breast cancer

MicroRNAs posttranscriptionally silence specific genes via pairing to the target mRNAs. We next searched for potential targets by prediction using the PicTar, miRanda and TargetScan Database (data not shown), and identified PTEN, a widely expressed tumor suppressor, as a candidate for miR-221 targeting. Indeed, enforced expression of pre-miR-221 in SK-BR-3 cells caused significantly reduced protein levels of PTEN (Fig. 3A) and mRNA (Fig. 3B), while inhibition of miR-221 resulted in upregulation of PTEN. The 3'-UTR of PTEN (200 bp) containing the po-

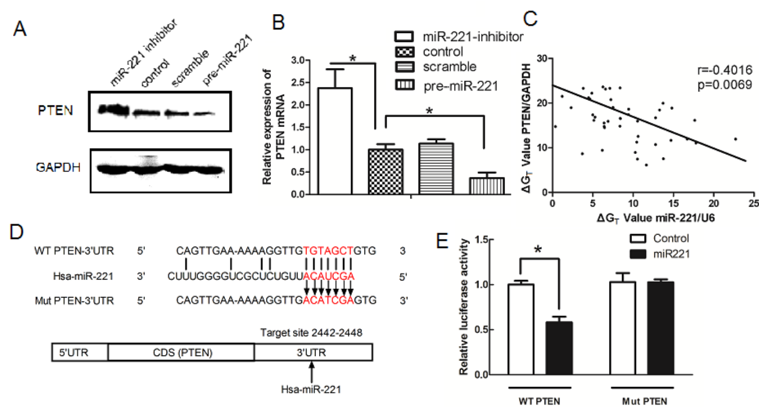

Fig. 3. PTEN is a direct target gene of miR-221 in HER2 positive breast cancer. (A) Western blot analysis of endogenous PTEN expression in SK-BR-3 cells transfected with pre-miR-221 lentivirus, scramble, control, or miR-221 inhibitor. (B) Quantitative reverse transcription-PCR analysis of PTEN mRNA expression in SK-BR-3 cells were treated as described in panel A. GAPDH was used to normalize the qRT-PCR data. All bars and error bars represent means ± SD (n = 3) (*P < 0.05). (C) Pearson's correlation analysis of the relative expression levels of miR-221 (normalized to U6) and the relative expression levels of PTEN mRNA (normalized to GAPDH) determined using qRT-PCR in 40 human breast cancer tissue samples (P = 0.0069). (D) Schematic representation of the miR-221-binding sequence in the 3′-UTR of PTEN mRNA. Mutations were generated in the miR-221-binding sequence of the PTEN 3′-UTR as indicated. (E) SK-BR-3 cells were co-transfected with pGL3 constructs containing intact or mutant 3′UTR of PTEN, an internal control vector (pGL4.73), and synthetic miR-221 mimics. 24 h after transfection, luciferase activity was measured. Data were shown as the luciferase activity relative to that of vehicle transfection. All bars and error bars represent means ± SD (n = 3) (*P < 0.05).

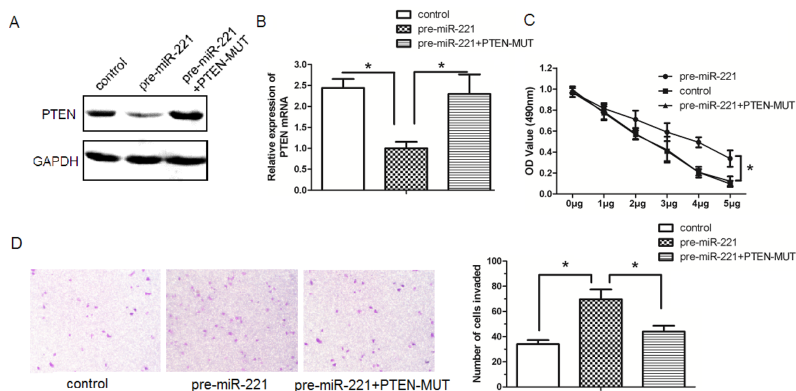

Fig. 4. Overexpression of PTEN rescues miR-221-induced malignant phenotype in HER2 positive breast cancer cell. (A) SK-BR-3 cells were transfected with pre-miR-221, scramble oligonucleotide (Scr) or co-transfected with pre-miR-221 lentivirus and pcDNA3.1-PTEN-mut. The scramble group was used as a control. The expression of PTEN was analyzed by Western blot. GAPDH was used as a loading control. (B) Quantitative reverse transcription-PCR analysis of PTEN mRNA expression in SK-BR-3 cells treated as described in panel A. All bars and error bars represent means ± SD (n = 3) (*P < 0.05). (C) SK-BR-3 cells were treated as described in panel A. Then the cells were treated with increasing concentrations of trastuzumab for 24 h and viability evaluation were examined. All bars and error bars represent means ± SD (n = 3) (*P < 0.05). (D) SK-BR-3 cells were treated as described in panel A. Transwell assay was used to investigate the invasion ability. All bars and error bars represent means ± SD (n = 3) (*P < 0.05).

tential miR-221 binding site was cloned for a firefly luciferase reporter assay (Fig. 3D). Luciferase activity was reduced by approximately 50% in miR-221-expressing cells compared with the control transfectants (Fig. 3E). In clinical breast cancer samples, the expression of miR-221 was found to correlate inversely with PTEN levels (Fig. 3C). These data suggest that PTEN is the direct target of miR-221 in breast cancer cells.

Overexpression of PTEN rescues miR-221-induced malignant phenotype in HER2-positive breast cancer cell

We next evaluated the role of PTEN overexpression in counteracting miR-221 to repress the invasiveness and trastuzumab resistance of HER2-positive breast cancer cells. A construct of miR-221-refractory PTEN mutant lacking the 3′-UTR, pcDNA3.1-PTEN-mut, was generated. Transfection of SK-BR-3 cells with

pcDNA3.1-PTEN-mut dramatically increased cellular PTEN level, which was not affected by the co-introduction of pre-miR-221 recombinant letiviruses (Fig. 4A and B). Enforced overexpression of PTEN significantly suppressed the invasion of SK-BR-3 cells induced by pre-miR-221 lentivirus (Fig. 4D), and restored trastuzumab sensitivity to a level comparable to control SK-BR-3 cells (Fig. 4C). These results indicate that miR-221 maintains the malignant phenotypes of HER2-positive breast cancers, e.g. high invasiveness and trastuzumab resistance, mainly by targeting and silencing the tumor suppressor PTEN.

DISCUSSION

The past decades has witnessed the considerable progresses in understanding the molecular machinery regulating the occurrence and metastasis of breast cancers. In particular, mutations in genes that constitute the phosphatidylinositol 3-kinase (PI3K) pathway occur in >70% of breast cancers (19). The PI3K pathway is regulated by numerous molecules. Of note is the well-documented tumor suppressor PTEN, which dephosphorylates phosphatidylinositol-3,4,5-triphosphate (PIP3), thereby opposing the action of the phosphatidylinositol-3 kinase (PI3K) (20). Considering the relatively low incidence of PTEN gene mutation, e.g. no more than 7% in invasive breast cancers (21), the aberrant PTEN expression due to posttranscriptional gene silencing in breast cancers may be critically involved, which is consistent with recent findings of several miRNAs like miR-22 (22), miR-21 (23), miR-153 (24) that targeting PTEN. Here, we demonstrated that PTEN was directly targeted and inhibited by miR-221, leading to elevated motility and invasiveness of HER2-positive breast cancer cells. Suppression of miR-221 or restoration of PTEN expression reversed the malignant phenotypes of HER2-positive breast cancer, suggestive of a pivotal role of key miRNA(s) in regulating HER2-positive breast cancer progression.

Breast cancers overexpressing HER2 represent a pathological type with high metastasis incidence and poor prognosis (25, 26). Despite the potent tumor suppressive effect of the monoclonal HER2 antibody, trastuzumab, the primary and acquired resistance has become the major obstacle of its clinical application. We found that silencing of PTEN by miR-221 conferred trastuzumab resistance of HER2-positive breast cancers. These results are also consistent with previous findings that miR-221 is involved in TRAIL resistance of liver and lung cancers (27). Unlike chemotherapeutics, resistance to antibody drugs is attributed to both behaviors of cancer cells and their interaction with the immune cells. In this regard, miR-221-mediated PTEN downregulation represents an autonomous mechanism of breast cancer cells, although a precise role of miR-221 in trastuzumab resistance of clinical breast cancers remains to be dissected. Whereas further investigations are needed to decipher a molecular link between miR-221 and HER2 signaling, combined HER2 and miR-221 targeting will be beneficial to improving the treatment of HER2-positive breast cancers.

Distal metastasis and drug resistance are hallmarks of advanced breast cancers and account for most morbidity of patients. Interestingly, these features can be concurrent and causative to each other. For instance, central nervous system metastasis of breast cancers is particularly common in patients receiving trastuzumab treatment (28). Although the molecular connection between these malignant phenotypes remains to be defined, they are reminiscent of the features of cancer stem cells. Therefore, it is worth further investigation whether miR-221 correlates with the expression of the established biomarkers of breast cancer-initiating cells. Collectively, this study revealed the critical involvement of miR-221 and the deregulation of PTEN in maintaining the malignant phenotype of HER2-positive breast cancers.

MATERIALS AND METHODS

Clinical samples collection

Breast cancer samples were collected directly after surgical resection at Xijing Hospital affiliated to the Fourth Military Medical University, Xi'an, China. The tissues were immediately frozen in liquid nitrogen after surgical removal and stored at -80°C. Sample collection was approved by the Ethics Committee of the Fourth Military Medical University.

Plasmid construction and preparation of lentivirus

Lentivirus packaging and infection were performed according to standard protocols as recommended by the provider's online protocol. Primers used for PCR amplification of pre-miR-221 coding sequence are as follows: 5'-GAATTCTAGGTAAGTC CCAGCAT-3' and 5'-CTCGAGTTTGGCTTCTATTTCTG- 3'. The PCR fragment was cloned into the vector pMD-18T (Takara, Japan) and confirmed by DNA sequencing. The pre-miR-221 coding sequence was further subcloned into the lentivirus-based expression plasmid pLenti6/V5 (Invitrogen, USA), and virus packaging and infection were performed according to protocols as recommended by the manufacturer. Wild-type PTEN lacking the 3'UTR region was constructed into the pcDNA vector (pcDNA3.1-PTEN-mut) by Genesil Biotechnology Co. Ltd. (Wuhan, China).

miR-221 mimics, miR-221 inhibitor and negative controls were purchased from Shanghai Genechem Inc. (Shanghai, China). Transfection with 50 nM miRNAs were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Luciferase reporter assay

The intact 3'UTR of the PTEN and a mutant with replaced nucleotides in the putative binding site corresponding to the seed-sequence of miR-221 (Fig. 3C) were cloned downstream of the firefly luciferase gene in the pGL3 vector. Primers used for PCR amplification of PTEN 3'UTR were as follows: wild-type, 5'-TCTAGAGACTCTGATCCAGAGAATGAACC-3' and 5'-TCTA GAGTTGCCACAAGTGCAAAGGGGTAGGATGTG-3'; Mutant, 5'-GTTGAAAAAAGGTTGACATCGAGTGTCATGTATATAC-3'

and 5′-TATATACATGACACTCGATGTCAACCTTTTTTCAAC-3′. SK-BR-3 cells were co-transfected with reporter constructs, an internal control vector (pGL4.73), and synthetic miR-221 mimics. Cells were rinsed in phosphate buffered saline (PBS) 48 h later, and luciferase activity was assessed using the Dual-Luciferase Reporter Assay System with a luminometer (Promega, USA). The luciferase activity of each lysate was measured and normalized to the activity of Renilla luciferase driven by the constitutively expressing promoter in the phRL vector. Basal promoter activity was measured as the fold change relative to the activity observed with the basic pGL3 vector alone.

Quantitative RT-PCR for miRNAs and protein-coding genes

Total RNA from each cell line was extracted by Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. Reverse transcription reaction was performed with SuperScriptTM II Reverse Transcriptase (Invitrogen, USA). cDNA was detected using SYBR@ Premix Ex TaqTM (TaKaRa, Japan). To quantify miRNAs, a miScript Reverse Transcription Kit (Qiagen, Germany) was used for reverse transcription, followed by amplification using SYBR@ Premix Ex TaqTM (Takara, Japan). GAPDH and U6 RNA were used as internal loading controls for mRNAs and miRNA, respectively. The following primers were used for PCR amplification: universal primer (UP) in the miScript Reverse Transcription Kit (Qiagen, Germany) and 5′-AGCT ACATTGTCTGCTGGGTTTC-3′ or 5′- GTGCTCGCTTCGGCAG CACATAT-3′ for miR-221 or U6 RNA, respectively; 5′-TGGATT CGACTTAGACTTGACCT-3′ and 5′-GGTGGGTTATGGTCTT CAAAAGG-3′ for PTEN and 5′-GCCCAATACGACCAAATCC-3′ and 5′-AGCCACATCGCTCAGACAC-3′ for GAPDH.

Proliferation assay

Cells (3,000/well) were seeded in 96-well plates and treated with increasing concentrations of trastuzumab in complete medium after overnight serum starvation. Briefly, after transfection for 48 h, the transfection medium in each well was replaced by 100 μl fresh serum-free medium with 0.5 g/l MTT. After incubation at 37°C for 4 h, the MTT medium was removed by aspiration and 50 μl DMSO was added to each well. After incubation at 37°C for a further 10 min, the A490 values of each sample were measured with a plate reader.

Western blot analysis

Cells were washed in PBS twice before proteins were extracted, and proteins were separated on an SDS/PAGE gel, transferred onto a PVDF membrane and subjected to immunoblot analysis. Blotting was performed with antibodies against PTEN (Cell Signaling Technology, USA), GAPDH (Epitomics, USA). Goat anti-rabbit and goat anti-mouse immunoglobulin horseradish peroxidase-linked $F(ab)_2$ fragments (ZB-2305, Zhong Shan Jin Qiao, China) were used as secondary antibodies.

Apoptosis assay

Cells were plated in 6-well plates (4×10^5 cells/well), and were transfected with pre-miRNAs or miRNA antisense using Lipofectamine 2000 (Invitrogen, USA). SK-BR-3 cells were treated with trastuzumab in a final concentration of 5 μg/ml, respectively. 24 hours after the treatment of trastuzumab, cells were stained with AnnexinV-FITC and PI, and flow cytometry was performed to detect apoptosis of the transfected cells.

Cell motility and invasion assay

For wound-healing assay, wound closure was observed by taking photos under a microscope at 0, 24 and 48 h after scratching. Invasion was performed with Matrigel (BD Biosciences, USA) following the manufacturer's instructions. Photographs of three randomly selected fields of the fixed cells were taken and cells were counted in high power fields (hpf) by light microscopy. Invasive potential was calculated as follows: % Invasion = (Mean cells invading through matrigel insert membrane) / (Mean cells migrating through control insert membrane) × 100%. Cell invasion rates were plotted as mean ± SD. Experiments were repeated three times independently.

Metastasis assay in nude mice

Briefly, 1×10^6 cells were injected through the tail vein into nude mice. 5 weeks later, the number of tumour nodules formed on the lung surfaces was counted. Lungs were excised and fixed in 10% formaldehyde, followed by preparation of 0.3 μm paraffin-embedded sections and subsequent haematoxylin and eosin (HE) staining.

Statistical analysis

Statistical analysis was carried out by SPSS 16.0 for Windows. Student's t test was used for analysing the results expressed as mean ± SD. The c2 test or Fisher's exact test was used to analyze the association between miR-221 and PTEN expressions. Differences were considered significant when the P value was less than 0.05.

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