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MiRNA-221 negatively regulated downstream *p27Kip1* gene expression involvement in pterygium pathogenesis

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Purpose: MiRNAs are small noncoding RNAs that have been implicated in tumor development. They regulate target gene expression either by mRNA degradation or by translation repression. Activation of β-catenin has been linked to pterygium progression. Here, we hypothesize that β-catenin-associated miRNA, miRNA-221, and downstream *p27Kip1* gene expression are correlated with the pathogenesis of pterygium.
Methods: We collected 120 pterygial and 120 normal conjunctival samples for this study. Immunohistochemistry and real-time reverse transcription (RT)–PCR were performed to determine β-catenin protein localization, miR-221, and *p27Kip1* gene expression. Pterygium cell line (PECs) cell models were used to confirm the effect of β-catenin, miR-221, and *p27Kip1* gene in the proliferation of pterygium cells.
Results: Seventy-two (60.0%) pterygial specimens showed high miR-221 expression levels, which was significantly higher than the control groups (13 of 120, 10.8%, p<0.0001). MiR-221 expression was significantly higher in β-catenin-nuclear/cytoplasmic-positive groups than in β-catenin membrane-positive and negative groups (p=0.001). We also found that *p27Kip1* gene expression in pterygium was negatively correlated with miR-221 expression (p=0.002). In the clinical association, miR-221 expression was significantly higher in the fleshy and intermediate groups than in the atrophic group (p=0.007). The association of miR-221, *p27Kip1* and proliferation of pterygium were also confirmed in the PECs model.
Conclusions: Our study demonstrated that activation of β-catenin in pterygium may interact with miR-221, resulting in *p27Kip1* gene downregulation that influences pterygium pathogenesis.

Pterygium is a chronic condition characterized by the encroachment of a fleshy triangle of conjunctival tissue into the cornea [1]. Pterygium has long been considered a chronic degenerative condition; however, after abnormal expression of the p53 protein was found in the epithelium, pterygium is now considered ultraviolet-related uncontrolled cell proliferation, similar to a tumor [2].

Activation of β-catenin in pterygium pathogenesis has been demonstrated [3,4]. Kato et al. (2007) reported that β-catenin activation and epithelial-mesenchymal transition were involved in the pathogenesis of pterygium [3]. In addition, previous reports indicated that nuclear β-catenin associated with T cell factor/lymphoid enhancer factor (TCF/LEF) family proteins activated target genes, such as *cyclin D1* (Gene ID: 595 OMIM 168461) and *c-myc* (Gene ID: 4609; OMIM 190080) [5,6], which leads to cellular proliferation and division [5-7]. These results were similar to those of our previous report, which indicated that the expression of *cyclin D1*, a downstream gene of β-catenin, was positively correlated with β-catenin nuclear/cytoplasmic expression [4]. Therefore, we hypothesized that the activation of *cyclin D1* by β-catenin nuclear/cytoplasmic expression is involved in pterygium proliferation.

MiRNAs are small noncoding RNAs that have been implicated in tumor development. They regulate target gene expression, either by mRNA degradation or by translation repression [8,9]. Previous reports indicated that β-catenin upregulates and binds miR-221, reducing the stability of the miR-221 target genes, including *Rad 51* (Gene ID: 5888; OMIM 614508), *ER α* (Gene ID: 2099; OMIM 133430), and *p27Kip1* (Gene ID: 1027; OMIM 600778) [10-12]. P27Kip1 is

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a cdk-inhibitory protein with an important role in the proliferation of many cell types [13], and loss of *p27Kip1* gene expression results in cell proliferation [14,15].

In the present study, we hypothesized that β-catenin-associated miRNA, miR-221, and downstream *p27Kip1* gene expression are correlated with pterygium pathogenesis. We used immunohistochemistry and real-time reverse transcription (RT)–PCR methods on 120 pterygial specimens and 120 normal conjunctival samples to analyze the correlation between β-catenin protein and miR-221 expression in pterygium.

METHODS

Study subjects: Pterygial samples were harvested from 120 patients undergoing pterygium surgery from China Medical University Hospital. The controls included normal conjunctival samples collected from the superior 85 conjunctiva of 62 patients and the medial conjunctiva of 58 patients without pterygium and pinguecula; all patients were undergoing cataract or vitreoretinal surgery. There were 78 men and 42 women in the pterygium group (age range 55–90 years, mean 67.9 years) and 60 men and 60 women in the control group (age range 55–75 years, mean 62.8 years). Normal conjunctival samples were collected from bulbar conjunctivas. All pterygial specimens came from primary pterygia. All patients signed a written informed consent form approved by the Institutional Review Board of China Medical University Hospital. Patients in whom the apex of the pterygium had invaded the cornea by more than 1 mm were included in this study. Pterygia were classified into grades 1, 2, or 3 based on slit-lamp biomicroscopic evaluation. Grade 1 ("atrophic") had clearly visible episcleral vessels under the body of the pterygium, grade 2 ("intermediate") had partially visible episcleral vessels under the body of the pterygium, and grade 3 ("fleshy") had completely obscured episcleral vessels underlying the body of the pterygium.

Immunohistochemistry: The formalin-fixed and paraffin-embedded specimens were sectioned at a thickness of 3 µm. All sections were deparaffinized in xylene, sequentially rehydrated through serial dilutions of alcohol, and washed in PBS (1X; 137 mM NaCl, 10 mM NaH_2PO_4, 2.7 mM KCl, and a pH of 7.4). Sections used for β-catenin detection were immersed in citrate buffer (pH 6.0) and heated in a microwave oven twice for 5 min. Mouse anti-β-catenin monoclonal antibodies (at a dilution of 1:200; Santa Cruz: Santa Cruz Biotechnolog, Santa Cruz, CA) were used as the primary antibodies. And then incubated with Mouse anti-β-catenin monoclonal antibodies (at a dilution of 1:200; Santa Cruz) for 90 min at 25 °C. The conventional streptavidin peroxidase method (DAKO, LSAB Kit K675, Copenhagen, Denmark) was performed for signal amplification, and the cells were counter-stained with hematoxylin [4]. Negative controls that did not include the primary antibodies were also prepared. Results were evaluated independently by three observers and were scored for the percentage of positive expression. For β-catenin, which was expressed in the membrane, cytoplasm, and nuclei, we separated the expression site into two groups, membrane expression and cytoplasm/nuclear expression. In this study, three of 120 patients had positive immunostaining in the membrane and the cytoplasm and were placed in the nuclei/cytoplasm groups because the membrane group had only β-catenin expressed in the membrane. We counted the number of epithelial cells and β-catenin-positive cells of the pterygium or normal conjunctiva in three independent fields using a high power field (objective lens 40×). Cells that positively stained for the anti-β-catenin antibody were noted by their labeling index as a percentage (%) in each specimen, and the measurements were averaged. Scores were as follows: score 0, no positive staining; score +, from 1% to 10%; score ++, from 11% to 50%; and score +++, more than 50% positive cells. We considered scores of +, ++, and +++ to represent positive immunostaining, and a score of 0 was classified as negative immunostaining.

Real-time reverse transcription–PCR-based detection of miRNAs and downstream gene expression: Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA). Expression of mature miRNA was detected with the TaqMan miRNA assay (Applied Biosystems, Foster City, CA) and normalized using the $2^{-\Delta\Delta CT}$ method relative to U6-snRNA. All TaqMan PCRs were performed in triplicate. For gene expression analysis, total RNA (3 µg) was reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen) and oligo d(T)15 primer. Real-time quantitative PCR was performed in a final volume of 25 µl containing 1 µl of each cDNA template, 10 pmol of target gene-specific primer, and 12.5 µl of SYBR Green Master Mix. The primers were designed using ABI Prism 7500 SDS software. The PCR consisted of 50 °C for 2 min, 95 °C for 10 min and then 40 cycles of 95 °C for 15 s and 55 °C for 90 s. Quantification was performed with the comparative threshold cycle (CT) method with water as the negative control. An arbitrary threshold was chosen based on the variability of the baseline. CT values were calculated by determining the cycle number at which fluorescence exceeded the threshold limit. The average CT values for the target gene were normalized to an endogenous housekeeping gene encoding 18S rRNA. The definition of high and low expression of individual genes depended on the mean value for all patients. Expression levels higher than

the mean were defined as high expression, while expression levels lower than the mean were defined as low expression.

Pterygium cell lines: The pterygium cell lines (PECs) used in this study were established previously [16]. To confirm whether the established PECs were epithelial cells, the cell type was further confirmed via staining with p63 and pan cytokeratin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

β-catenin protein expression assessed with western blot: Total proteins were extracted from PECs with a lysis buffer (100 mM Tris, pH 8.0, 1% sodium dodecyl sulfate [SDS]) and recovered protein concentrations were determined using the Bio-Rad protein assay kit followed by separation with SDS–polyacrylamide gel electrophoresis (PAGE; 12.5% gel, 1.5 mm thick). After the electrophoretic transfer to a polyvinylidene fluoride (PVDF) membrane, nonspecific binding sites were blocked with 5% nonfat milk in TBS-Tween-20. β-catenin and β-actin were detected by incubating the membrane with anti-β-catenin (Santa Cruz Biotechnology) and β-actin antibodies (Sigma Aldrich, St. Louis, MO) for 60 min at room temperature, followed by subsequent incubation with a peroxidase-conjugated secondary antibody (1:5,000 dilution). Extensive washings with TBS-Tween-20 were performed between incubations to remove non-specific binding. The protein bands were visualized using enhanced chemiluminescence (NEN Life Science Products, Boston, MA).

MiR-221 precursor and inhibitor transfection: Cells were grown to confluence in six-well plates. MiR-221 mimics or miR-221 inhibitor (Life Science Biosystems) and negative control (Life Science Biosystems) cells were transfected using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. Transfection efficiency was evaluated with real-time PCR.

Cell proliferation time: The 10^3 cells per ml of PECs transfected with or without mimic-miR-221 were seeded in a 35 mm dish. After cultured for 24, 48, 72, 96, 120, or 144 h, the cell numbers of each cultured time were counted to calculate the doubling time.

Statistical analysis: Statistical analysis was performed using the SPSS version 13.0 statistical software (SPSS Inc., Chicago, IL). Quantitative data are presented as the standard deviation (SD). Fisher's exact test and χ^2 test were applied for statistical analysis. Results were considered statistically significant at $p<0.05$. The coefficient of determination was detected by Cronbach's alpha analysis.

RESULTS

MiR-221 expression in pterygium and control tissues: To understand whether miR-221 expression is associated with pterygium pathogenesis, we analyzed miR-221 expression in pterygium tissues and conjunctiva controls. As shown in Table 1, after being assigned to one of two groups based on the mean value of miR-221 expression levels, 72 (60.0%) pterygial specimens were found to have high levels of miR-221 expression; these levels were significantly higher than the control groups (10.8%, $p<0.0001$; Table 1) and suggest that the high expression of miR-221 may be associated with pterygium progression.

Association of β-catenin protein localization and miR-221 expression in pterygium tissues: To understand the association of β-catenin protein and miR-221 expression in pterygium tissues, we used immunohistochemistry and real-time PCR to analyze ß-catenin protein and miR-221expression. As shown in Figure 1, the β-catenin protein was detected in the membrane, cytoplasm, and nuclei. In addition, miR-221 levels were associated with β-catenin protein expression (Table 2, $p=0.001$). MiR-221 expression was significantly higher in the β-catenin-nuclear/cytoplasmic-positive groups than in the β-catenin membrane-positive and -negative groups ($p=0.001$).

Correlation of miR-221 and downstream p27Kip1 gene expression in pterygium tissues: Previous reports have shown that ß-catenin can upregulate and bind miR-221 [10-12]. MiR-221 is a negative regulator of *p27Kip1* (CDK

Table 1. MiR-221 expression in pterygial and control conjunctiva analyzed by real-time RT-PCR.

MiR-221	Pterygium		Control	
	N	%	N	%
Low	48	60.0	107	91.2
High	72	6.0	13	10.8
P value	<0.0001			

The χ^2 test was applied for statistical analysis. A $P < 0.05$ was considered to be statistically significant.

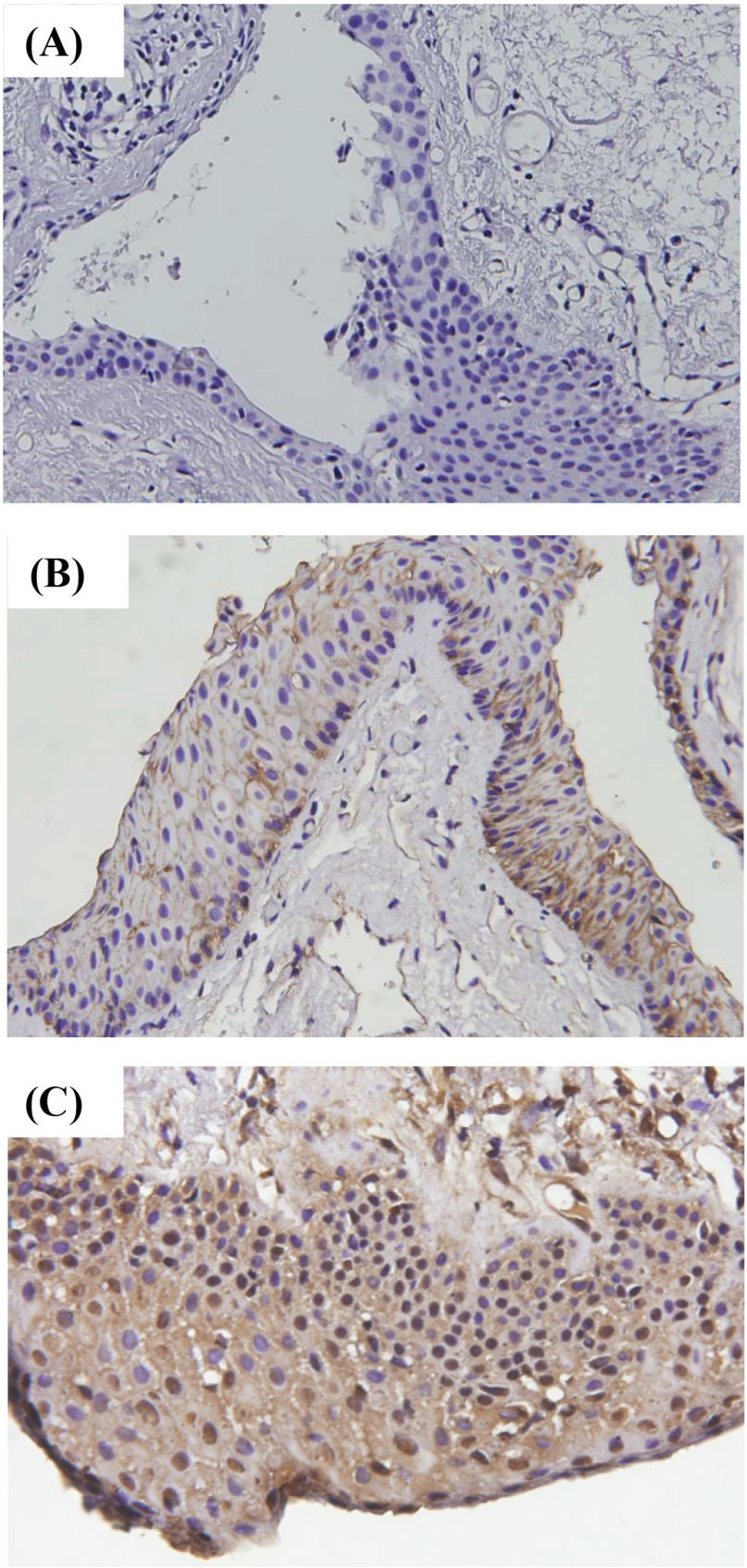

Figure 1. Representative immunostaining results for β-catenin. **A**: The negative control has the first antibody replaced with immunoglobulin. **B**: β-catenin protein expression detected in the membrane (200×), and (**C**) aberrant localization of β-catenin in the cytoplasm/nuclei (200×).

Table 2. Relationship of β-catenin protein localization and miR-221 protein expression in pterygia.

β-catenin	MiR-221		p value
	Low (n=48) (%)	High (n=72) (%)	
Negative/ Membrane (n=94)	45 (93.8)	49 (68.1)	
Nuclei/Cytoplasm (n=26)	3 (6.2)	23 (31.9)	0.001

β-catenin analyzed in this study is based on prior pterygium samples.[6] The Fisher's exact test was applied for statistical analysis. A $P < 0.05$ was considered to be statistically significant. Coefficient of determination (R^2)>0.8.

inhibitor) to promote cell proliferation. Upregulated miR-221 by β-catenin led the downregulation of *p27Kip1* [11,12]. Using the Pearson correlation test, a significantly negative correlation was found between miR-221 and *p27Kip1* gene expression (Figure 2; p=0.011, r^2=–0.232). We therefore further analyzed the association of β-catenin, miR-221, and *p27Kip1* gene expression and found a negative correlation between miR-221 and *p27Kip1* gene expression (p=0.002; Table 3). In addition, *p27Kip1* gene expression in β-catenin-nuclear/cytoplasmic-positive /miR-221 high was significantly higher than in β-catenin-nuclear/cytoplasmic-positive/miR-221 low, membrane-positive and -negative groups/miR-221 high, and membrane-positive and -negative groups/miR-221 low groups (p=0.003), suggesting that the downregulation of *p27Kip1* in pterygium may be associated with ß-catenin-regulated miR-221 upregulation.

Association of miR-221, p27Kip1, and clinical parameters of pterygium patients: To further understand whether the expression of miR-221 and *p27Kip1* is associated with pterygium pathogenesis, the associations of miR-221, *p27Kip1*, and types of pterygium patients were analyzed. We found the expression of miR-221 protein in the fleshy and intermediate types of pterygium was significantly higher than in the atrophic type of pterygium (p=0.007). The expression of p27Kip1 protein in the fleshy group was lower than in the atrophic and intermediate groups (p=0.028; Table 4). We also found that miR-221 expression was positively correlated with age (p=0.004; Table 4). There was no association between the expression of p27Kip1 with other clinical parameters, including age and gender (Table 4).

Effect of miR-221 on p27 (kip) gene expression and cell proliferation in the primary PECs model: We further verified whether the miR-221expression in pterygium downregulates *p27Kip1* gene expression and promotes cell proliferation, establishing PECs lines from the pterygium patients were used. As shown in Figure 3A, miR-221 expression in PECs were positive correlated with β-catenin (Figure 3A). After knockdown, miR-221 expression in miR-221 highly expressed PECs 1, *p27Kip1* gene expression increased (Figure 3B), and cell proliferation (Figure 3C) decreased. The reversed

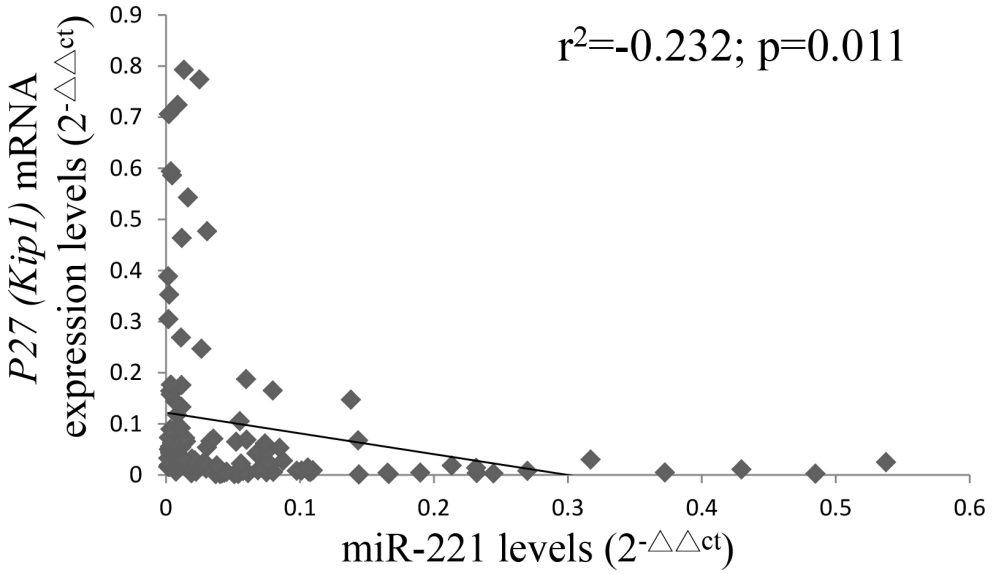

Figure 2. Correlation of miR-221 and p27Kip1 mRNA expression in pterygium tissues.

Table 3. Relationship of β-catenin protein localization, miR-221 and *p27 (kip1)* gene expression in pterygia.

Parameters	*p27 (kip1)* mRNA		p value
	Low (n=78) (%)	High (n=42) (%)	
	miR-221		
Low (n=48)	23 (29.5)	25 (59.5)	
High (n=72)	55 (70.5)	17 (40.5)	0.002
	β-catenin/miR-221		
Negative/membrane/low (n=45)	20 (25.6)	25 (59.5)	
Negative/membrane/high (n=49)	37 (47.4)	12 (28.6)	
Nuclei/Cytoplasm/low (n=3)	3 (3.8)	0 (0)	
Nuclei/Cytoplasm/high (n=23)	18 (23.2)	5 (11.9)	0.003

β-catenin analyzed in this study is based on prior pterygium samples.[13] The Fisher's exact test and χ^2 test were applied for statistical analysis. A $P < 0.05$ was considered to be statistically significant. Coefficient of determination (R^2)>0.8.

correlation was also found in mimic-miR-221 transfected miR-221 low expressed PECs 3 ($p<0.001$; Figure 3B,C). Thus, miR-221 promotes PECs proliferation via inhibiting *p27Kip1* gene expression.

DISCUSSION

Pterygium has been considered a degenerative process of the corneal limbus characterized by the invasion of a fleshy triangle of conjunctival tissue onto the cornea. Shimmura et al. demonstrated increased telomerase activity in pterygial epithelial cells, indicating their hyperproliferative nature [17], and increased expression of Ki67, a cell proliferation marker, has been noted in pterygia [18]. We assume that the proliferative capacities of pterygial cells give pterygia the appearance of having a mechanism similar to tumorigenesis.

MiR-221 is one of the miRNAs that were discovered relatively early and are widely expressed in human tissues and cells. It functions in promoting cell proliferation and inhibiting apoptosis [19-21]. Promoting cell proliferation by miR-221 high expression were found in several types

Table 4. Correlation of miR-221, p27 mRNA expression and clinical parameters of pterygium.

Parameters	miR-221		p27 (kip1) mRNA	
	Low (n=48)	High (n=72)	Low (n=78)	High (n=42)
	Gender			
Female (n=42)	12	30	30	12
Male (n=78)	36	42	48	30
P value	0.079		0.320	
	Age (years)			
<68 (n=50)	28	22	30	20
>68 (n=70)	20	50	48	22
P value	0.004		0.340	
	Type			
Atrophic (n=40)	24	16	22	18
Intermediate (n=52)	16	36	33	19
Fleshy (n=28)	8	20	24	4
P value	0.007		0.028	

The Fisher's exact test and χ^2 test were applied for statistical analysis. A $P < 0.05$ was considered to be statistically significant. Coefficient of determination (R^2)>0.8.

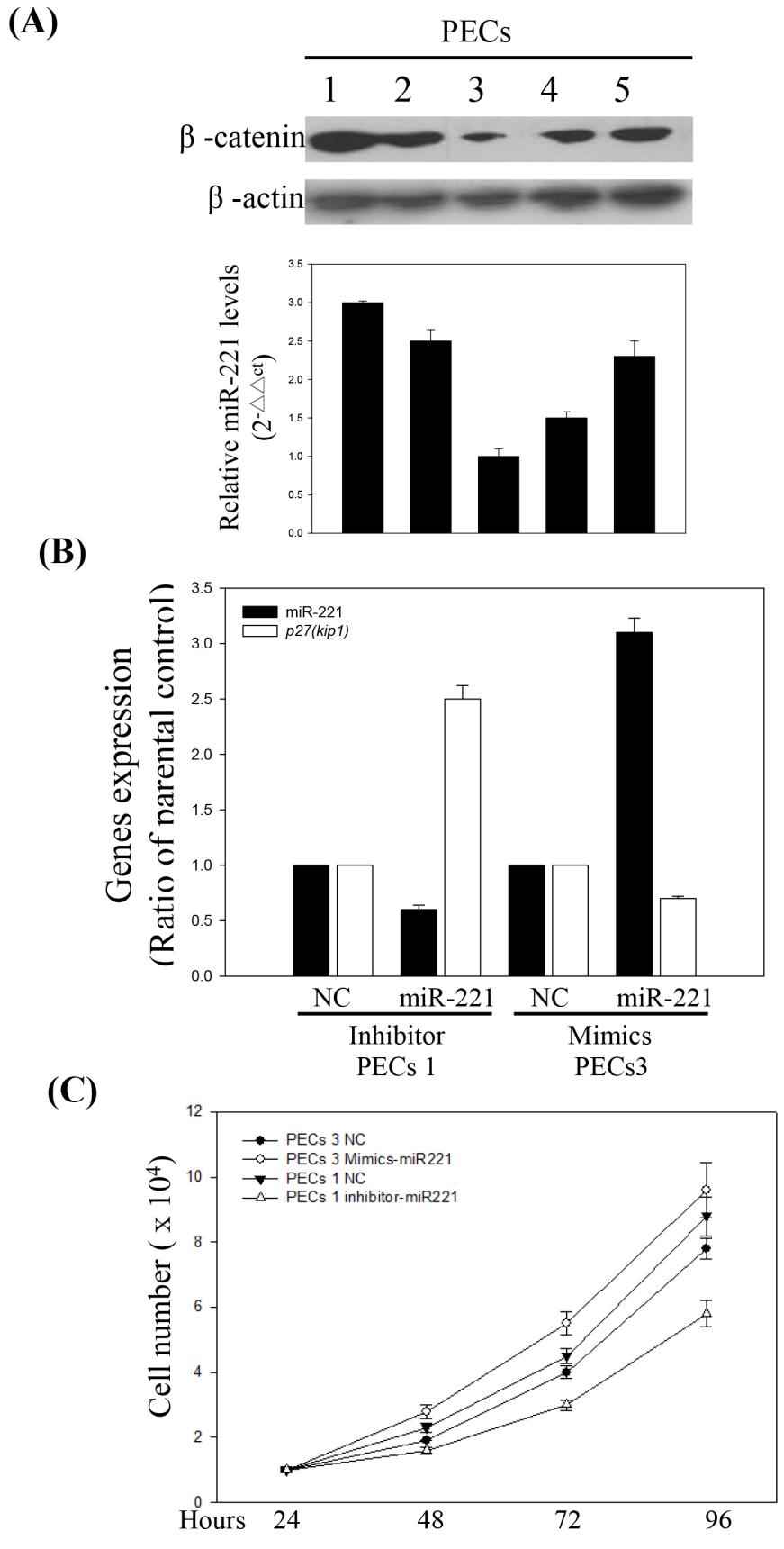

Figure 3. Effect of miR-221 in cell proliferation. **A**: β-catenin protein and miR-221 expression in pterygium cell line (PECs) analyzed with western blot and real-time reverse transcription (RT)–PCR. The PECs were cultured from five patients. **B**: MiR-221 and p27Kip1 mRNA expression in PECs treated with the miR-221 inhibitor in miR-221 high expressed PECs 1 cells and mimic-miR-221 in miR-221 low expressed PECs 3 cells. **C**: Cell proliferation ability of PECs decreased or increased after transfection of miR-221 inhibitor in miR-221 high expressed PECs 1 cells or mimic-miR-221 in miR-221 low expressed PECs 3 cells. NC: negative control, cells not treated with miR-221 inhibitor or mimic-miR-221.

of cancer, including breast, prostate, and colorectal cancer [20-25]. High levels of miR-221 have been shown to promote proliferation of these cells through binding to the 3′ untranslated region (UTR) of the cell cycle inhibitor and tumor suppressor *p27Kip1* and inhibiting its expression [26,27]. In the present study, we found that miR-221 expression in pterygium tissues was significantly higher than that of the control groups (Table 1, p<0.001). We also found a negative association between miR-221 and *p27Kip1* in the pterygium groups. In addition, we found that the expression of miR-221 in the intermediate and fleshy types of pterygium was significantly higher than in the atrophic type of pterygium (p=0.007) and that the expression of *p27Kip1* mRNA in the fleshy group was significantly lower than in the atrophic and intermediate groups (p=0.028). In addition, in our PECs models, miR-221 expression was positively correlated with ß-catenin protein expression (Figure 3A). After miR-221 expression was inhibited, downstream *p27Kip1* mRNA expression increased, and PECs proliferation decreased (Figure 3C). Thus, we consider that miR-221/ *p27Kip1* signaling is involved in the cell proliferation of pterygium.

A previous study showed that β-catenin upregulates and binds miR-221 in the cytoplasm, reducing the stability of the miR-221 targets *Rad51* and *ERa* mRNAs in breast cancer stem cells [10]. In our previous study, we used immunohistochemistry to demonstrate that the cyclin D1 protein activated through β-catenin protein nuclear/cytoplasmic expression contributes to cell proliferation in pterygium [4]. In the present study, miR-221 levels in pterygium were positively correlated to β-catenin protein nuclear/cytoplasmic expression (Table 1). MiR-221 expression was significantly higher in the β-catenin-nuclear/cytoplasmic-positive groups than in the β-catenin membrane-positive and -negative groups (p=0.001). We also found that *p27Kip1* gene expression in the β-catenin-nuclear/cytoplasmic/miR-221 high groups was significantly lower than in the other three groups (Table 3, p=0.003). Therefore, we believe that cytoplasmic ß-catenin can upregulate and bind miR-221 to reduce the stability of the miR-221-targeted *p27Kip1* mRNAs in pterygium cells.

In conclusion, our study is the first to provide evidence that miR-221 may be activated through β-catenin protein nuclear/cytoplasmic expression to downregulate *p27Kip1* gene expression, thus promoting cell proliferation. Our data demonstrate that changes in miR-221/*p27Kip1* signaling may contribute to cell proliferation in pterygium.

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