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Original Article

Expression and Correlation Research of MicroRNA-10a-5p and PIK3CA in Middle Ear Cholesteatoma

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BACKGROUND: This study aimed to examine the roles of miR-10a-5p and phosphatidylinositol-4,5-bisphosphonate 3-kinase catalytic subunit α in the pathogenesis of middle ear cholesteatoma.

METHODS: We enrolled 27 patients with middle ear cholesteatoma and collected samples of intraoperative cholesteatoma and normal posterior ear skin tissues. The mRNA expression levels of miR-10a-5p and *PIK3CA* were detected using real-time quantitative polymerase chain reaction. PIK3CA protein expression was measured by immunohistochemistry and western blotting.

RESULTS: Middle ear cholesteatoma tissues showed significantly lower miR-10a-5p expression levels and significantly higher PIK3CA expression levels than normal posterior ear skin tissues (both $P < .05$). Furthermore, the miR-10a-5p and *PIK3CA* expression levels were significantly negatively correlated in middle ear cholesteatoma tissues ($r = -0.926$, $P < .001$).

CONCLUSION: Low miR-10a-5p expression levels in middle ear cholesteatoma tissues may inhibit the growth and proliferation of cholesteatoma, whereas high *PIK3CA* expression level may promote its growth and proliferation. In addition, miR-10a-5p may affect the proliferation and differentiation of cholesteatoma by negatively regulating its target gene, *PIK3CA*.

KEYWORDS: Cholesteatoma, gene expression, microRNAs, middle ear

INTRODUCTION

Middle ear cholesteatoma, a benign cystic lesion, is a frequently occurring condition in otorhinolaryngology.[1] It is characterized by abnormally growing keratinized squamous epithelium within the temporal bone, which can gradually destroy the surrounding bone. Middle ear cholesteatoma can be associated with intracranial and extracranial complications, including hearing loss, vertigo, facial paralysis, and brain abscess.[2,3] Surgical resection is the only effective method for treating middle ear cholesteatoma. However, surgery is associated with certain risks, including those of facial paralysis, vertigo, and recurrence.[4] Therefore, it is necessary to explore nonsurgical treatment options for cholesteatoma.[5]

Recently, more studies have investigated the role of microRNA (miRNA) in the occurrence and development of middle ear cholesteatoma. Xie et al[6] used miRNA microarray technology to study expression profiles of ear cholesteatoma and normal skin tissues and found that the expression of miR-10a-5p was significantly different between these tissues. They also revealed that phosphatidylinositol-4,5-bisphosphonate 3-kinase catalytic subunit α (*PIK3CA*) might be the most important target gene of miR-10a-5p. *PIK3CA*, a catalytic subunit of PI3K, is a key activation molecule in the EGFR-PI3K-Akt-CyclinD1 signaling pathway, which is important in

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regulating cholesteatoma.[7] The involvement of *PIK3CA* mutations has been reported in the progression of breast cancer,[8] kidney cancer,[9] and squamous cell carcinoma in the head and neck,[10] among others.

Middle ear cholesteatoma is benign yet exhibits patterns of proliferation, invasion, and migration, similar to those observed in malignant tumors.[11] We speculate that miR-10a-5p and *PIK3CA* play important roles in the development and growth of cholesteatoma. Currently, to the best of our knowledge, there is no literature regarding the mechanisms of action of miR-10a-5p and *PIK3CA* in middle ear cholesteatoma. Therefore, we chose miR-10a-5p as the research object and aimed to examine and compare the expression of miR-10a-5p and *PIK3CA* in cholesteatoma and normal skin tissues from behind the ear; to explore the effects of their expression on cholesteatoma; and to provide insights for nonsurgical treatment options of middle ear cholesteatoma.

METHODS

Participants

In this study, we enrolled 27 patients [18 female and 9 male participants aged 5–76 years (mean ± standard deviation, 40.4 ± 16.6 years)] with acquired secondary middle ear cholesteatoma diagnosed at Zhuhai People's Hospital (Zhuhai Hospital Affiliated with Jinan University) from June 2020 to January 2021. The participants were receiving their initial surgical treatment during the study period. This study was conducted according to tenets of the Helsinki Declaration of 1975, as revised in 2008, and was approved by the Ethics Committee of Zhuhai People's Hospital (No. 69 of 2020). All 27 patients or their guardians provided written informed consent. The study adhered to the TREND guidelines for nonrandomized public behavior.

Research Methods

Reagents

The total RNA extraction reagent (TRIzol® Reagent) and RT Reagent Kit were purchased from Takara (Kyoto, Japan); the Bulge-Loop™ miRNA qRT-PCR Starter Kit from Guangzhou RiboBio Co., Ltd. (Guangzhou, China); *PIK3CA* primary antibody from Abcam Trading Co., Ltd. (Shanghai, China); general SP kit and diaminobenzidine (DAB) color development kits from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd. (Beijing, China); WB Super RIPA Lysis Buffer, bicinchoninic acid (BCA) protein quantification kits, and Benzonase® Nuclease from HaiGene (Harbin, China); and gel preparation kit, western primary and secondary antibody dilutions, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein loading buffer from Beyotime Biotechnology Co., Ltd. (Shanghai, China).

Total RNA Extraction, Reverse Transcription, and Real-Time Quantitative Polymerase Chain Reaction

Total RNA was extracted from the matrix of middle ear cholesteatoma and retroauricular normal skin tissues according to the Trizol test method.[12] RNA content was measured using UV spectrophotometry, and its integrity was evaluated using agarose gel electrophoresis. The extracted total RNA was reverse-transcribed into cDNA and amplified by polymerase chain reaction (PCR), according to the instructions of the real-time quantitative reverse transcription PCR (qRT-PCR) detection kit. We subsequently used the Bulge-Loop™ miRNA qRT-PCR Starter Kit to perform PCR. *U6* was used as the internal reference gene for detecting miR-10a-5p, and single well system for each qRT-PCR reaction was 20 μL. We used *GAPDH* as the internal reference gene for detecting *PIK3CA*. The primer sequences were as follows: hsa-miR-10a-5p-RT, 5¢-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACCACAAAT-3¢; hsa-miR-10a-5p F, 5¢-GGTACCCTGTAGATCCGAATT-3¢; hsa-miR-10a-5p R, 5¢-CAGTGCGTGCGTGGAGT-3¢; PIK3CA F, 5¢-ACTATCCCCGAAATTCTACCCAA-3¢; and PIK3CA R, 5¢-AGGCCAATCTTTTACCAAGCAA-3¢. We repeated these reactions 3 times and calculated the test result using the $2^{-\Delta\Delta}$CT value.

Immunohistochemistry

Fresh cholesteatoma and skin samples collected during surgery were dehydrated with alcohol, embedded in paraffin, and serially sectioned at a thickness of 3 μm. The sections were stained with DAB and hematoxylin and then blocked. The samples were incubated with 100 μL of *PIK3CA* primary antibody diluted at 1 : 50 ratio at 4°C and then incubated with 100 μL of biotinylated goat antimouse/rabbit immunoglobulin G polymer (secondary antibody) at 20-25°C. We used known positive sections as positive controls and normal external auditory canal skin samples as negative controls. The primary antibody was replaced with Tris-buffered saline containing 0.020% Triton X-100 to exclude nonspecific staining.

Western Blot Detection

We powdered 20 mg of tissues and added 200 μL of WB Super RIPA Lysis Buffer to extract the total protein in each group. The BCA method was used to detect protein concentration.[13] Samples were denatured at 100°C for 10 minutes, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and were subsequently transferred onto a polyvinylidene difluoride membrane. The polyvinylidene difluoride membrane was blocked in 5% skim milk for 2 hours. We added a 1 : 500-fold dilution of the primary antibody and incubated the samples at 4°C overnight. The secondary antibody was incubated for 2 hours at 20-25°C, the film was washed, and the color was allowed to develop by chemiluminescence. Grayscale analysis was performed using ImageJ software (National Institutes of Health, Bethesda, Md, USA). The ratio of the grayscale value of the target band (*PIK3CA*) to that of the internal reference *GAPDH* band denoted the relative protein expression content.

Statistical Analyses

For data analysis, Statistical Package for Social Sciences version 26.0 (IBM SPSS Corp., Armonk, NY, USA) and GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA) software packages were used. Normally distributed data are presented as means and standard deviations. Unpaired *t*-tests were used to compare 2 independent samples. We used Pearson correlation analysis to compare the relative expression of 2 genes. *P* values of $<.05$ were considered statistically significant.

RESULTS

Bioinformatics Methods for Predicting the Target Genes of miR-10a-5p

Target gene prediction results showed that the 3′ untranslated regions of *PIK3CA* had conserved binding sites to miR-10a-5p, suggesting that *PIK3CA* might be a downstream target gene of miR-10a-5p (Supplementary Figures 1 and 2).

Results of qRT-PCR Analysis for miR-10a-5p and PIK3CA mRNA

This experiment was strictly included in the qRT-PCR analysis standard following the Starter Kit instructions. After obtaining samples with total RNA purity (A260/A280) ranging from 1.8 to 2.0, 17 middle ear cholesteatoma tissue samples and 13 normal posterior ear skin tissue samples were analyzed using qRT-PCR. The results displayed the typical S-type fluorescence quantitative amplification curve, and the dissolution curve was unimodal. No other abnormal waveforms were noted, and the shape of the wave crest was sharp. These results indicated that the product was well amplified; moreover, no nonspecific amplification was observed (Supplementary Figures 3 and 4).

Both miR-10a-5p and *PIK3CA* mRNA were expressed in the cholesteatoma and normal posterior ear skin tissues. The miR-10a-5p expression level in cholesteatoma was significantly lower than that in the posterior skin ($t = 2.208$, $P = .036$) (Table 1, Supplementary Figure 5). In contrast, the *PIK3CA* mRNA expression level was significantly higher in the cholesteatoma than in the posterior skin ($t = 3.951$, $P < .001$) (Table 2, Supplementary Figure 6).

Immunohistochemical Analysis of PIK3CA

In middle ear cholesteatoma, *PIK3CA* was primarily located in the cytoplasm and cell membrane; its expression may have been accompanied by partial nuclear staining. It was expressed from the basal layer to the upper basal layer. The staining degree was mostly moderate to strong, and the positive expression of *PIK3CA* was indicated by yellow or brownish-yellow findings (Figure 1). Conversely, in normal skin tissues, *PIK3CA* was primarily located in the nucleus and cell membrane. Its expression might be accompanied by partial cytoplasmic staining, which was characterized by the staining of cells in the basal layer and a few suprabasal layers. In contrast to the amount of staining observed in cholesteatoma tissues, the amount of staining noted in normal skin tissues was mainly weakly positive. The positive expression of *PIK3CA* was indicated by yellow or brownish-yellow findings (Figure 2).

Western Blot Analysis of PIK3CA

Protein blots of *PIK3CA* were present in both the experimental and control specimens, with significantly higher *PIK3CA* expression in cholesteatoma tissue samples than in normal posterior ear skin tissue samples ($P < .05$) (Figure 3). These results were also consistent with the qRT-PCR detection results.

Table 1. miR-10a-5p Expression in Patients with Middle Ear Cholesteatoma and Normal Skin Tissues

Group	n	miR-10a-5p	*t*	*P*
Middle ear cholesteatoma tissue	17	0.99 ± 0.03	2.208	.036
Normal posterior ear skin tissue	13	1.61 ± 0.18		

Data are presented as mean±standard deviation

Table 2. Relative Expression of *PIK3CA* mRNA in Patients with Middle Ear Cholesteatoma and Normal Skin Tissues

Group	n	*PIK3CA*	*t*	*P*
Middle ear cholesteatoma tissue	17	2.06±0.23	3.951	<.001
Normal posterior ear skin tissue	13	1.02±0.05		

Data are presented as mean ± standard deviation
PIK3CA, phosphatidylinositol-4,5-bisphosphonate 3-kinase catalytic subunit α.

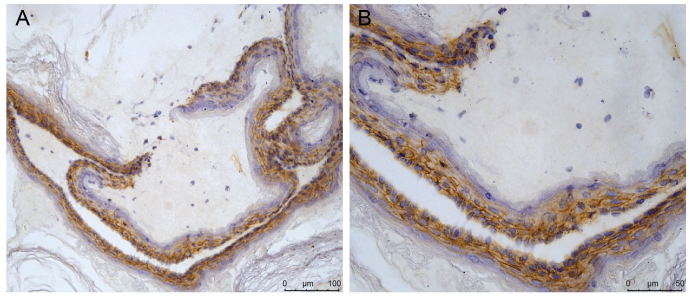

Figure 1. Expression of phosphatidylinositol-4,5-bisphosphonate 3-kinase catalytic subunit α (*PIK3CA*) in middle ear cholesteatoma (A: ×200; B: ×400).

Pearson Correlation Analysis of miR-10a-5p and PIK3CA mRNA Expression in Middle Ear Cholesteatoma

Pearson correlation analysis was used to determine the correlation between miR-10a-5p and *PIK3CA* mRNA expression in middle ear cholesteatoma, and the results indicated a significant negative correlation ($r = -0.926$, $P < .001$) (Figure 4).

DISCUSSION

Middle ear cholesteatoma may be harmful and may require surgical treatment, which has associated risks. The development of a new treatment for middle ear cholesteatoma would have substantial clinical benefits. The current research focus is on gene therapy.

Researchers have conducted several studies on the role of miRNA in tumors. Li et al[14] showed that miRNA-21 was significantly upregulated in cancer cells from various sources and that miRNA-21 regulated the initiation, transformation, invasion, and metastasis of cancer. Yoshikawa et al[15] intraperitoneally injected synthetic miR-214 into mice with canine angiosarcoma and observed that it produced antitumor effects by inducing apoptosis and inhibiting cell proliferation. In addition, miRNA expression has been shown to affect the occurrence, development, and prognosis of various malignancies, including lung cancer,[16] prostate cancer,[17] and liver cancer.[18] Since the critical role of miRNAs in cancer has been demonstrated, gene therapy based on miRNA has attracted considerable attention.[19,20]

Since Friedland et al[21] first reported the expression of miRNA-21 and its downstream target genes *PTEN* and *PDCD4* in middle ear cholesteatoma and normal skin tissues in 2009, researchers have reported that miRNA is involved in regulating the proliferation, apoptosis,

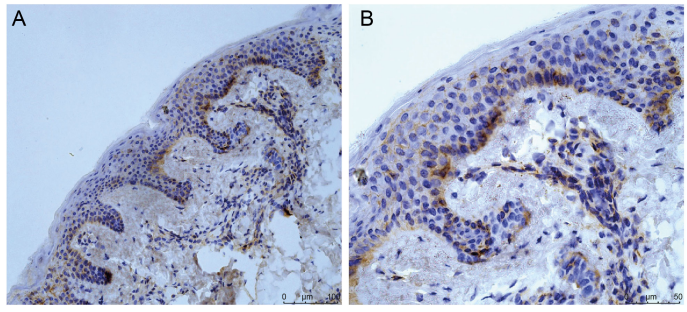

Figure 2. Expression of phosphatidylinositol-4,5-bisphosphonate 3-kinase catalytic subunit α (*PIK3CA*) in normal posterior ear skin (A: ×200; B: ×400).

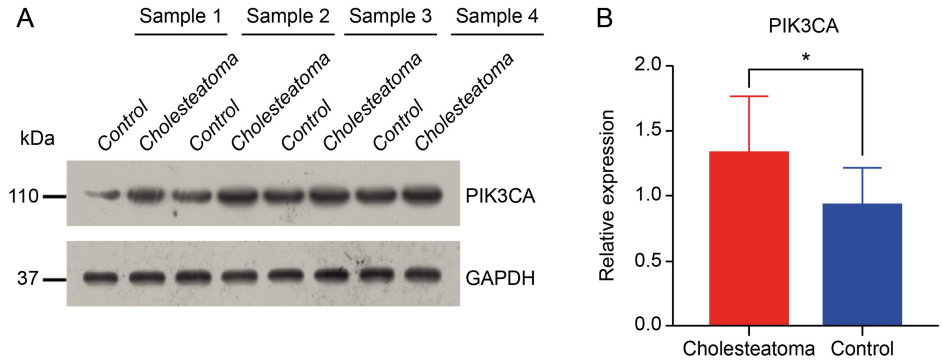

Figure 3. Expression of phosphatidylinositol-4,5-bisphosphonate 3-kinase catalytic subunit α (*PIK3CA*) protein in middle ear cholesteatoma and retroauricular normal skin tissues (*$P < 0.0$).

invasion, bone destruction, angiogenesis, and other pathophysiological processes of cholesteatoma cells.[22-24] Moreover, miR-10a-5p belongs to the miR-10 family and is downregulated in various malignant tumors, such as cervical cancer,[25] renal cell carcinoma,[26] and liver cancer.[27]

Double luciferase reporter gene testing in breast cancer MCF-7 cells by Zhang et al[28] showed that miR-10a-5p could directly target the 3′ untranslated region of *PIK3CA*. *PIK3CA* mutations play an important part in cell proliferation, survival, and tumorigenesis by activating the PI3K-Akt signaling pathway.[29,30] Liu et al[7] confirmed that the EGFR-PI3K-Akt-cyclin D1 signaling pathway is active in cholesteatoma and may play a vital role in cholesteatoma epithelial hyperproliferation.

The qRT-PCR findings in the present study indicate that the miR-10a-5p expression level in middle ear cholesteatoma tissues was lower than that in normal skin tissues. Our results are consistent with those of Xie et al.[6] who conducted microarray analysis and qRT-PCR verification. Vaher et al[31] showed that miR-10a-5p could inhibit keratinocyte proliferation and regulate cell cycle. Therefore, the results of this experiment are consistent with those on miR-10a-5p inhibition in the growth and proliferation of middle ear cholesteatoma.

In this study, the expression level of *PIK3CA* mRNA was significantly higher in middle ear cholesteatoma tissues than that in normal skin tissues according to qRT-PCR and Western blot findings.

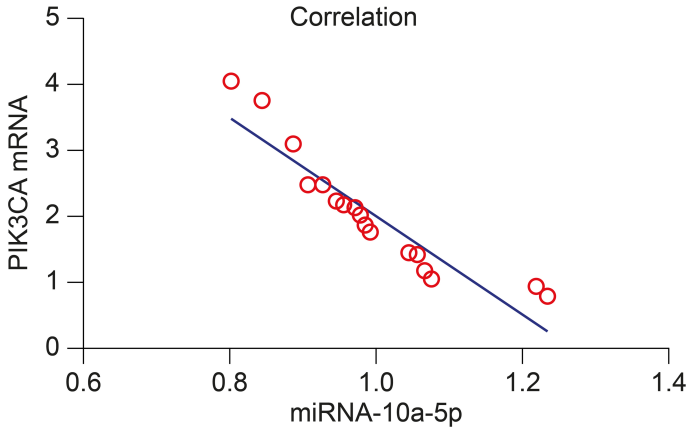

Figure 4. Correlation analysis of the expression of miR-10a-5p and phosphatidylinositol-4,5-bisphosphonate 3-kinase catalytic subunit α (*PIK3CA*) mRNA in middle ear cholesteatoma.

Immunohistochemistry showed that PIK3CA staining was mostly moderate to strongly positive in middle ear cholesteatoma but was mostly weakly positive in normal skin tissues sampled from behind the ear. While we did not retrieve reports regarding the expression of *PIK3CA* in cholesteatoma, we found that *PIK3CA* was highly expressed in various malignancies, such as gastric cancer,[32] breast cancer,[33] and cervical cancer,[34] and promoted the growth of these tumors. We hypothesized that *PIK3CA* also promotes the proliferation of cholesteatoma based on the many malignant characteristics of cholesteatoma. The high expression of *PIK3CA* in cholesteatoma detected in this study is in line with the logic that *PIK3CA* promotes the proliferation of cholesteatoma.

Our results demonstrated that miR-10a-5p expression was negatively correlated with *PIK3CA* expression in patients with middle ear cholesteatoma, suggesting that downregulated miR-10a-5p in middle ear cholesteatoma may negatively regulate the function of its target gene *PIK3CA* and further activate the PI3K-AKT signaling pathway to accelerate the proliferation and differentiation of cholesteatoma cells. Zhang et al[28] reported that the expression of *PIK3CA* could be downregulated by increasing the expression of miR-10a-5p in breast cancer cells. Downregulation inhibits activation of the PI3K-AKT signaling pathway, ultimately suppressing cell proliferation and promoting cell apoptosis.

This study has some limitations. We only used a small number of clinical samples, and we did not examine the regulatory mechanisms. Future studies should replicate these experiments with a larger sample size. In addition, further studies should prove whether miR-10a-5p can reduce the activation of *PIK3CA* and can inhibit the proliferation of cholesteatoma and evaluate whether the underlying mechanism of miR-10a-5p and *PIK3CA* regulates the proliferation and differentiation of middle ear cholesteatoma.

CONCLUSION

This study confirmed that low miR-10a-5p expression and high *PIK3CA* expression in middle ear cholesteatoma were negatively correlated. miR-10a-5p may affect the proliferation and differentiation of cholesteatoma by negatively regulating *PIK3CA*. Our study provides a novel approach to nonsurgical treatment options for middle ear cholesteatoma.

Ethics Committee Approval: Ethical committee approval was received from the Ethics Committee of Zhuhai People's Hospital (No. 69 of 2020).

Informed Consent: Written informed consent was obtained from all patients or their guardians. .

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – Q.L., J.Y.; Design – J.Y., S.T.; Supervision – Q.L., W.Y.; Funding – W.Y., S.T.; Materials – S.T., Z.H.; Data Collection and/or Processing – Z.H., M.Y.; Analysis and/or Interpretation – M.Y., Z.L.; Literature Review – J.Y., Z.L.; Writing – J.Y.; Critical Review –Q.L.

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Declaration of Interests: The authors declare that they have no competing interest.

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Conserved

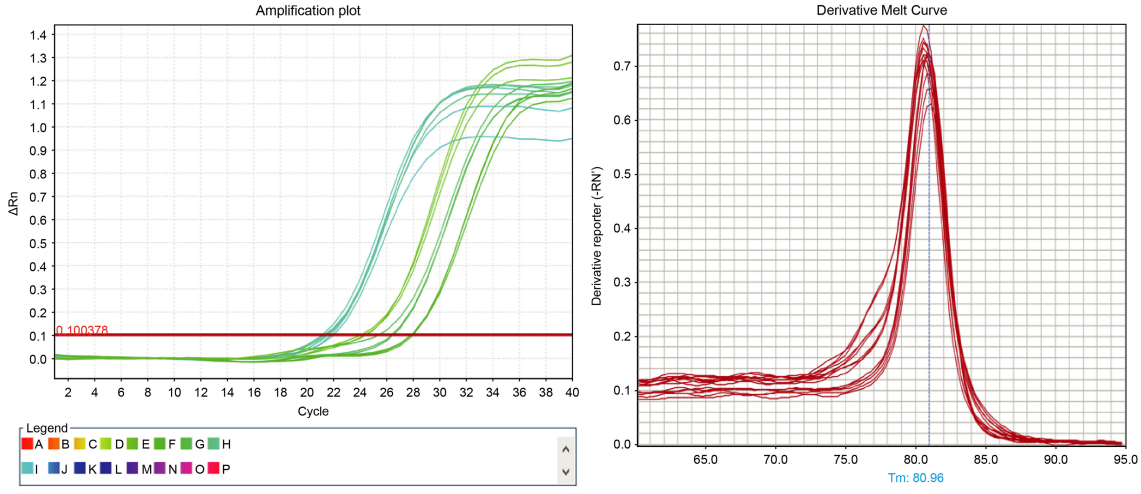
	Predicted consequential pairing (top) target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	P_{CT}
Position 170-176 of PIK3CA3' UTR hsa-miR-10b-5p	5' ...AUAAUGUAAACGCAAACAGGGUU... 3' GUGUUUAAGCCAAGAUGUCCCAU	7mer-m8	-0.35	98	-0.35	6.096	0.64
Position 170-176 of PIK3CA3' UTR hsa-miR-10a-5p	5' ...AUAAUGUAAACGCAAACAGGGUU... 3' GUGUUUAAGCCAAGAUGUCCCAU	7mer-m8	-0.35	98	-0.35	6.096	0.64

Supplementary Figure 1. The TargetScan software predicts phosphatidylinositol-4,5-bisphosphonate 3-kinase catalytic subunit α (*PIK3CA*) as a target gene for miR-10a-5p.

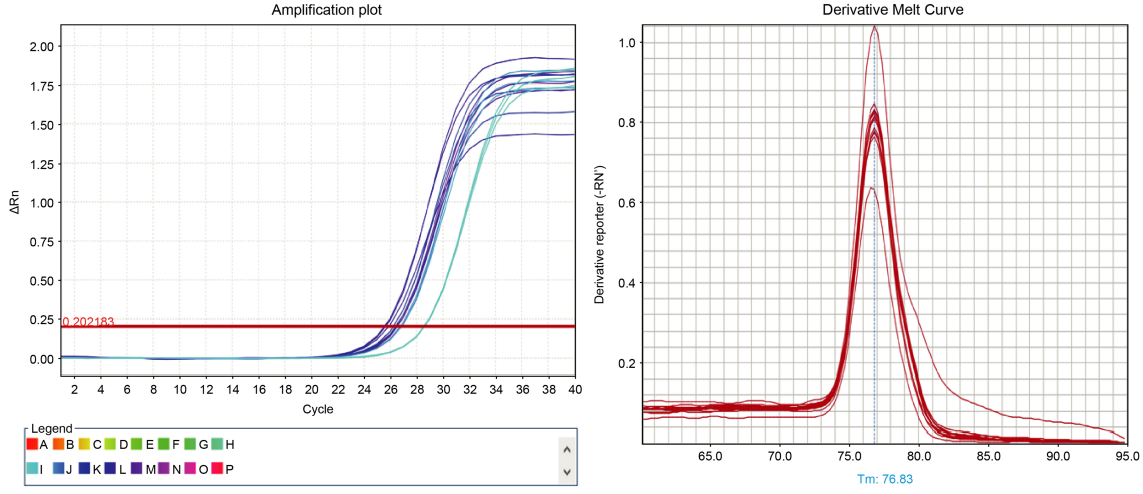
MicroRNA and Target Gene Description:

miRNA Name	hsa-miR-10a-5p	miRNA sequence	UACCCUGUAGAUCCGAAUUUGUG
Previous Name	hsa-miR-10a		
Target score	72	Seed location	170
NCBI gene ID	5290	GenBank Accession	NM_006218
Gene symbol	PIK3CA	3' UTR length	5740
Gene description	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subnit alpha		

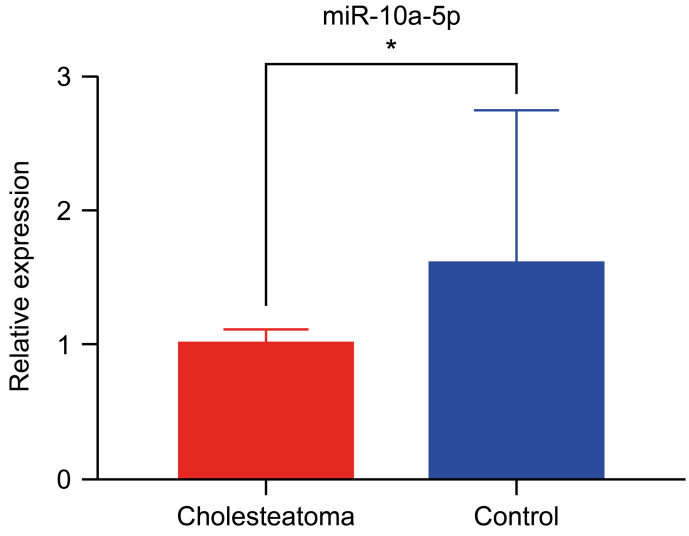
Supplementary Figure 2. The miRBase software predicts phosphatidylinositol-4,5-bisphosphonate 3-kinase catalytic subunit α (*PIK3CA*) as a target gene for miR-10a-5p.



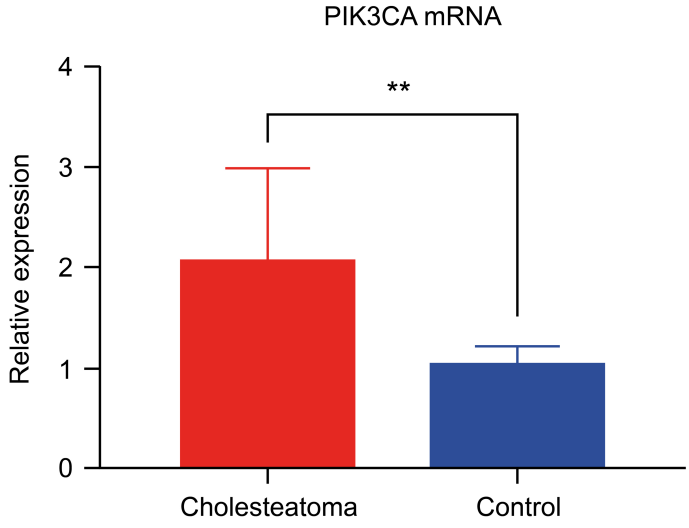
Supplementary Figure 3. Amplification and dissolution curves of miR-10a-5p.



Supplementary Figure 4. Amplification and dissolution curves of phosphatidylinositol-4,5-bisphosphonate 3-kinase catalytic subunit α (*PIK3CA*) mRNA.



Supplementary Figure 5. Expression of miR-10a-5p in middle ear cholesteatoma and normal posterior ear skin tissues (*$P < .05$).



Supplementary Figure 6. Expression of phosphatidylinositol-4,5-bisphosphonate 3-kinase catalytic subunit α (*PIK3CA*) mRNA in middle ear cholesteatoma and normal posterior ear skin tissues (**$P < .001$).