Polygonatum odoratum lectin promotes BECN1 expression and induces autophagy in malignant melanoma by regulation of miR1290

Abstract: Autophagy is not only a survival response to growth-factor or nutrient deprivation but also an important mechanism for tumor-cell suicide, including melanoma. Polygonatum odoratum lectin (POL) displays apoptosis- and autophagy-inducing effects in many human tumors. POL also inhibits the growth of melanoma cells, but its role and molecular mechanism in malignant melanoma remain unclear. In this study, we found that POL suppressed proliferation and induced autophagy in melanoma cells. miR1290 was upregulated and inhibited autophagy in melanoma. BECN1 is the direct functional effector of miR1290. Furthermore, we found that POL promoted BECN1 expression though inhibition of miR1290, thus inducing melanoma-cell autophagy. This finding elucidates a new role and mechanism for POL in melanoma, and provides a potential antineoplastic agent for melanoma treatment.

Keywords: autophagy, Polygonatum odoratum lectin, miR1290, BECN1, melanoma

Introduction
Malignant melanoma, the most aggressive skin tumor, is the primary cause of death of skin tumors.1–3 The global incidence of malignant melanoma has been increasing in recent years.4,5 Melanoma is developing rapidly with early metastasis, and there is no effective treatment for patients with advanced malignant melanoma.1 Therefore, it is important to develop new treatments for melanoma.

Cancer is associated with programmed cell death (PCD), which plays important roles in the development and homeostasis of multicellular organisms.6 Apoptosis and autophagy are two main types of PCD. Apoptosis is an intrinsic mechanism for cell suicide that is regulated by numerous molecular signaling pathways.7 Autophagy is independent of phagocytes and a conserved eukaryotic cellular degradative process that clears damaged or superfluous macrocomplexes and organelles.8

Plant lectins are a class of non-immune-origin carbohydrate-binding proteins, and they can agglutinate cells or precipitate polysaccharides and glycoconjugates.9,10 Several major plant-lectin families have been reported to possess antitumor activities via inducing apoptosis and autophagy in many human malignant tumor cells, including melanoma.11,12 Polygonatum odoratum lectin (POL), isolated from traditional Chinese medicine herb P. odoratum (Mill.), Druce, is a mannose binding-specific Galanthus nivalis agglutinin-related family lectin.13 POL displays remarkable apoptosis- and autophagy-inducing effects in human lung adenocarcinoma cells and murine fibrosarcoma cells.14,15 POL also inhibits the proliferation of melanoma cells.13 However, the effect and molecular mechanism of POL in melanoma remains unclear.
In this study, we demonstrated that POL suppressed the proliferation of melanoma cells. We also found that POL induced autophagy in melanoma cells through upregulated expression of BECN1. BECN1 is involved in autophagy at every major step in the autophagic pathway.\(^{16}\) We found that miR1290 was upregulated and inhibited autophagy by targeting BECN1 in melanoma. Moreover, we found that POL regulated BECN1 by inhibiting the expression of miR1290. These results suggest that POL may serve as a potential antineoplastic agent in future melanoma treatment.

Materials and methods

Human tissue samples

Thirty primary malignant melanoma-tissue and adjacent normal tissue samples were collected from melanoma patients undergoing surgery at the Affiliated People’s Hospital of Jiangsu University. Two professional pathologists independently diagnosed the clinical and pathological features of the melanoma patients. Every sample was preserved in liquid nitrogen immediately after collection. All patients provided informed consent for the use of their tissue in this research. The study was approved by the Human Research Ethics Committee of the Affiliated People’s Hospital of Jiangsu University.

Cell lines and cell culture

The human malignant melanoma cell line A375 was obtained from the Chinese Academy of Sciences cell bank (Shanghai, China). A375 cells were cultured in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) with 10% FBS (Thermo Fisher Scientific). Human epidermal melanocytes (HEMa-LP) were purchased from Thermo Fisher Scientific. A375 cells were cultured in DMEM containing 5% carbon dioxide. The cell line was maintained in an atmosphere of 37°C con

Oligonucleotides, plasmids, and reagents

POL was purified as described previously.\(^{13}\) Oligonucleotides were purchased from GenePharma (Shanghai, China). Sequences were: Hsa-miR1290 mimic, 5′-UUGAUAUUUGGAUCAGGGA-3′; BECN1 siRNA (siBECN1), 5′-CCAGCCAGGAUGAUCACAGGA-3′, and 5′-GCUCACUCAGGAGGACAUCUUAUA-3′; negative control, 5′-UUCUCCGAAAGUGUCACGGT-3′; Has-miR1290 inhibitor, 5′-UUCUGUAUCAAAAAUCCCA-3′; inhibitor negative control, 5′-CAGUACUUUUGUGUA GUACAA-3′.

Extraction of RNA and quantitative RT-PCR

We used Trizol (Thermo Fisher Scientific) to extract total RNA from cells and tissue. Fermentas reverse-transcription (RT) reagents and the Applied Biosystems TaqMan microRNA RT kit was used to conduct RT polymerase chain reaction (PCR). The amplification reaction was performed with an ABI StepOne Plus system according to predetermined conditions. The specific primer for miR1290 was chemically synthesized in RiboBio (Guangzhou, China). The following primers were used: BECN1 (forward) 5′-AACCAGATGCGTATGGCCCA-3′, (reverse) 5′-CTGTCCACTGTGGCAGATGT-3′. Relevant data were analyzed using 2\(^{-\Delta\Delta CT}\).

CCK-8 assay

CCK-8 (Beyotime, Haimen, China) assay was used to assess melanoma-cell viability. A375 cells (5×10\(^4\)) were inoculated on 96-well plates. Cells were then treated with different concentrations of POL. Fresh culture media (100 μL) containing 10% CCK-8 was added to A375 cells at different times. A microplate reader (Multiskan FC; Thermo Fisher Scientific) was used to measure absorbance at 450 nm wavelength.

Transmission electron microscopy

A375 cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 hour. After being washed three times using 0.1 M PBS, cells were fixed with 1% osmium tetroxide in 0.1 M PBS. Samples were dehydrated with increasing ethanol concentrations and embedded in epoxy resin. Transmission electron microscopy was used to observe the ultrastructure of A375 cells.

Luciferase reporter assay

A BECN1 promoter luciferase reporter plasmid (contains 949 bp human BECN1 promoter) was obtained from SwitchGear Genomics (Menlo Park, CA, USA). The BECN1 3′-UTR fragment containing the miR1290-binding sequences was inserted into pMiR-Reporter vectors. Mutated plasmid was used as a control. A375 cells were transfected with the related reporter plasmids and Hsa-miR1290 mimic. After transfection for 48 hours, a dual luciferase reporter assay system (Promega, Fitchburg, WI, USA) was used to detect luciferase activity.

Western blot

We used radioimmunoprecipitation-assay buffer (KenGen, Nairobi, Kenya) to extract total protein from cells, and a
bicinchoninic acid protein-assay kit (Beyotime) was used to determine protein concentrations. Equal amounts of protein were separated by sodium dodecyl sulfate polyacrylamide-gel electrophoresis and transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA), and membranes were closed with milk and incubated overnight with diluted antibodies against BECN1 (1:1,000; Abcam, Cambridge, UK), LC3B (1:3,000; Abcam), and p62 (1:1,000; Abcam). Membranes were incubated with HRP-conjugated secondary antibody (1:2,500; Santa Cruz Biotechnology, Dallas, TX, USA), with GAPDH used as a control (1:2,500; Abcam).

**Statistical analysis**
Data are presented as means ± standard error, and SPSS 13.0 was used to analyze the data. Data were analyzed with independent t-tests (two-sided) and one-way analysis of variance. *P* < 0.05 was considered statistically significant. Pearson correlation analysis was performed using MatLab. All experiments were repeated in triplicate.

**Results**

**POL suppressed proliferation and induced autophagy in melanoma cells**
We initially explore the role of POL in melanoma cells. CCK-8 assays revealed that POL significantly inhibited the growth of human melanoma A375 cells in a dose- and time-dependent manner, and the inhibitory rate reached nearly 50% after treatment with 25 μg/mL POL for 24 hours (Figure 1A). However, only a small percentage of CD was found in the human epidermal melanocytes (HEMa-LP) after treatment with POL (Figure 1A). We next used transmission electron microscopy to observe the effect of POL on autophagy. Formation of autophagosome-related structures was readily observed in the POL-treatment group (Figure 1B). LC3I transforming into LC3II is characteristic of autophagy; p62, also known as Sqstm1, bound directly to LC3, and p62 degradation is a result of autophagy. As shown in Figure 1C and D, POL induced the accumulation of LC3II and the degradation of p62 in A375 cells in a dose- and time-dependent manner. These results demonstrated that POL was effective in inducing autophagy in melanoma cells.

**POL increased levels of BECN1 mRNA and protein in melanoma cells**
BECN1 is a key factor in autophagosome formation and involved in autophagy at every major step.15 BECN1 was markedly decreased in primary melanoma tissues compared to adjacent normal tissues (Figure 2A). A375 cells were treated with various concentrations of POL for 24 hours and 25 μg/mL POL for different times. BECN1 mRNA and protein expression increased progressively in response to 0–50 μg/mL POL (Figure 2B and D). Additionally, 25 μg/mL POL led to an increase in expression of BECN1 mRNA and protein in a time-dependent manner in A375 cells (Figure 2C and E). Importantly, siBECN1 rescued the effect of POL on autophagy (Figure 2F). These results suggested that POL induced autophagy in melanoma cells through increasing BECN1 expression.

**POL may regulate BECN1 expression through miR1290**
We further explored the mechanism by which POL increased BECN1 levels. We first generated the BECN1 promoter luciferase reporter construct and transfected plasmid into A375 cells. After 24 hours’ transfection, the cells were treated with different concentrations of POL. No significant change was detected in luciferase activity between the negative-control group and the POL-treatment group (Figure 3A). This result suggested that POL may have regulated BECN1 expression at the posttranscriptional level. miRNAs have been shown to influence gene expression by cleaving or inhibiting the translation of target mRNA.17,18 We next explored whether POL regulated BECN1 expression via targeting specific miRNAs. miR1290 was predicted to bind to the BECN1 3′-UTR with TargetScan (Figure 3B). Real-time PCR showed that miR1290 levels were reduced in POL-treated cells (Figure 3C and D). Moreover, miR1290 mimics abolished the effect of POL on the expression of Becn1 (Figure 3E and F). These findings indicated that POL may have regulated BECN1 though miR1290.

**miR1290 inhibited autophagy in melanoma cells by targeting BECN1**
We further investigated the role and mechanism of miR1290 in malignant melanoma. miR1290 levels were markedly increased in primary melanoma tissues compared to adjacent normal tissues (Figure 4A). Analysis indicated negative Pearson correlations between miR1290 and BECN1 (Figure 4B). The miR1290 inhibitor was transfected into A375 cells (Figure 4C). Transmission electron microscopy showed that miR1290 inhibitor was effective in inducing autophagy (Figure 4D). Western blotting showed that miR1290 inhibitor led to an increase in LC3II levels and p62 clearance (Figure 4G). As mentioned before, miR1290 was predicted to bind to BECN1 3′-UTR. We constructed
the luciferase reporter plasmids containing the wild-type and mutant-binding sites of the 3'-UTR of BECN1 (Figure 3B). Luciferase activity in cells with wild-type plasmid was decreased in the miR1290-mimic transfection group, but no significant change was detected in the mutant plasmid group (Figure 4E). Meanwhile, miR1290 downregulation caused an increase in endogenous BECN1 levels (Figure 4F and G). The effect of miR1290 inhibitor on A375 melanoma-cell autophagy was rescued by siBECN1 (Figure 4D and G). These results showed that miR1290 inhibited autophagy by targeting BECN1 in melanoma cells.

**POL induced autophagy though regulating miR1290 in melanoma cells**

We further investigated whether melanoma-cell autophagy induced by POL could be affected by miR1290. After treatment with 25 μg/mL POL, A375 cells were transfected with miR1290 mimics or inhibitor. Overexpression of
miR1290 repressed the melanoma CD induced by POL, while knockdown of miR1290 led to the reverse effect (Figure 5A). As shown in Figure 5B, overexpression of miR1290 reversed the A375-cell autophagy induced by POL, while knockdown of miR1290 amplified the POL effect. Meanwhile, the effect of POL on Becn1, LC3II, and p62 expression was reversed by the miR1290 mimic and amplified by the miR1290 inhibitor (Figure 5C and D). These results suggested that POL induced autophagy in melanoma via regulating miR1290.

Discussion

Autophagy, type II PCD, is distinguished from apoptosis by the presence of autophagosomes and autolysosomes and an intact nucleus. Autophagy is a eukaryotic cell-degradation process that clears damaged or superfluous...
Figure 3 POL may regulate BECN1 expression though miR1290.

Notes: (A) No significant change was detected in luciferase activity between the control group and the POL-treatment group. (B) Binding sites of miR1290 within the 3′-UTR of BECN1 were predicted by TargetScan. (C, D) Real-time PCR showed expression of miR1290 in POL-treated cells. (E) Real-time PCR showed that miR1290 mimics abolished the effect of POL on BECN1-mRNA expression. (F) Western blot showed that miR1290 mimics abolished the effect of POL on Becn1-protein expression.

**P<0.05; ***P<0.01; ****P<0.001.

Abbreviations: POL, Polygonatum odoratum lectin; PCR, polymerase chain reaction; NC, negative control.

macrocomplexes and helps in maintaining cellular metabolism and homeostasis. Autophagy also plays an important role in tumor-cell suicide. In recent years, research has effectively inhibited melanoma-cell growth via inducing autophagic CD. POL is a mannose-binding G. nivalis agglutinin-related family lectin. POL can induce apoptosis in murine fibrosarcoma cells through death-receptor and mitochondrial pathways. It also can induce autophagy in human lung adenocarcinoma cells by regulation of special miRNAs. POL also inhibits melanoma-cell growth, but its specific role and molecular biological mechanism in malignant melanoma remain to be confirmed. Here, we found that POL significantly inhibited growth and induced autophagy in melanoma A375 cells. We further explore the mechanisms thereof.
Accumulating evidence indicates that \textit{BECN1} is involved in autophagy at every major step in the autophagic pathway.\textsuperscript{16} We demonstrated that POL induced autophagy by increasing \textit{BECN1} expression in melanoma cells. \textit{BECN1} promoter luciferase reporter assays showed that POL may regulate \textit{BECN1} expression at the posttranscriptional level. miRNA, an sncRNA with a length of about 22 nucleotides, plays an important role in regulating gene expression at the posttranscriptional level by binding to the 3′-UTR of its target miRNAs.\textsuperscript{24} In recent years, the role of miRNA in tumorigenesis

\textbf{Figure 4} \textit{miR1290} inhibited autophagy in melanoma cells by targeting \textit{BECN1}.

\textbf{Notes:} (A) \textit{miR1290} levels were analyzed in 30 primary malignant melanoma tissues and adjacent normal tissues. (B) Pearson correlations of \textit{miR1290} and \textit{BECN1} expression in 30 malignant melanoma tissues were negative. (C) Transfection efficiency of \textit{miR1290} inhibitor was determined by PCR. (D) Representative electron microscopy of A375 cells after treatment with \textit{miR1290} inhibitor or in combination with \textit{siBECN1}. Arrows indicate autophagosomes. (E) Overexpression of \textit{miR290} suppressed luciferase activity in A375 cells with the \textit{pMir-BECN1-WT}. (F) Real-time PCR showed that \textit{miR1290} downregulation led to an increase in the expression of endogenous \textit{BECN1}. (G) Western blot identified \textit{Becn1}, \textit{LC3I}, \textit{LC3II}, and \textit{P62} protein-expression changes following transfection with \textit{miR1290} alone or in combination with \textit{siBECN1}. **\textit{P}, 0.01; ***\textit{P}, 0.001.

\textbf{Abbreviations:} PCR, polymerase chain reaction; NC, negative control; WT, wild type; Mut, mutant.
miR1290 functions as an oncoMiR in diverse human tumors.

miR1290 promotes gastric tumor-cell proliferation and metastasis by targeting FoxA1. miR1290 also acts as a tumor oncogene in the progression of esophageal squamous-cell carcinoma by targeting NFIX. However, the role of miR1290 in melanoma has rarely been studied. We found that miR1290 levels were significantly increased in primary melanoma tissues and induced autophagy in melanoma cells. BECN1 is the direct functional effector of miR1290. Moreover, we revealed that POL induced autophagy and regulated BECN1 though miR1290 in melanoma cells.

Conclusion
POL promoted the expression of BECN1 by inhibiting miR1290 levels, thus inducing melanoma-cell autophagy.

Understanding the effect and regulatory mechanism of POL in melanoma could lead to the identification of novel potential antineoplastic agents for melanoma. Future studies to assess the role of POL in a clinical context are warranted.

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Disclosure
The authors report no conflicts of interest in this work.

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