

# Idebenone Maintains Survival of Mutant Myocilin Cells by Inhibiting Apoptosis

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**Key words:** Apoptosis; *CHOP*; Glaucoma; Idebenone; Myocilin

## INTRODUCTION

Glaucoma is a chronic progressive neurodegenerative disease and exhibits heterogeneity, polygenic inheritance, and incomplete penetrance.<sup>[1]</sup> Primary open-angle glaucoma (POAG) is the most common form of glaucoma. Although the underlying etiology of POAG is unknown, there is evidence that genetic mutations are closely associated with this disease.<sup>[2]</sup> Among myocilin (*MYOC*) mutations, Pro370Leu (P370L) is responsible for one of the most severe glaucoma phenotypes.<sup>[3]</sup> However, the function of *MYOC* protein is still not well understood.

The *MYOC* mutation, P370L, is associated with features of mitochondrial dysfunction in trabecular meshwork cells and can cause impaired function and even cell death. These studies suggested that preventive mitochondrial protection might delay the onset of POAG in patients carrying the *MYOC* mutation, P370L.<sup>[4]</sup> *CHOP* (also called *GADD153* or *DDIT3*) encodes a member of the CCAAT/enhancer-binding proteins, which is a family of transcription factors.<sup>[5]</sup> The *CHOP* protein is activated by endoplasmic reticulum (ER) stress and the mitochondrial unfolded protein response and can promote apoptosis.

## METHODS

### Small interfering RNA transfection

According to our previous procedure, the P370L mutant *MYOC* gene was cloned into pDsRed2-N1 generating the pDsRed2-N1-mMYOC construct and transfected into COS7 cells.<sup>[3]</sup> *CHOP*/*GADD153* protein levels were reduced using small interfering RNA (siRNA). The synthesized, scrambled siRNA oligonucleotides and siRNA targeting *CHOP* were

purchased from QIAGEN (Shanghai, China). The siRNA target sequences for *CHOP* were as follows: GAA UCU AAU ACG UCG AUC AdTdT (dT is deoxythymidine). For transfection, Lipofectamine™ 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) was used, and cells were seeded into 6-well plates for 24 h before the experiment. After 48 h transfection, the targeted gene expression levels were determined by reverse transcription polymerase chain reaction (RT-PCR) and/or Western blotting analysis.

### Semiquantitative reverse transcription polymerase chain reaction analysis

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions and purified using an RNeasy Mini Kit (Qiagen, Germany). RT-PCR was performed using FastStart DNA Master SYBR Green I Reagent (Toyobo Co., Osaka, Japan) by means of a Roche LightCycler™ (Roche, Basel, Switzerland). A melting curve analysis was performed to verify the specificity of the products, and expression was quantified by the  $\Delta\Delta C_t$  ( $C_t$  [target gene] –  $C_t$  [ $\beta$ -actin]) method. Primers were purchased from Invitrogen. The primer sequences are listed in Table 1.

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**Table 1: Primer sequences used for qPCR in this study**

Name	Locus	Sequences
<i>MYOC</i>	NM_000261.1	Forward: 5'-AGACACCGGATATGGAGAAC-3' Reverse: 5'-CTCGCATCCACACACCATAAC-3'
<i>CHOP</i>	NM_001195057.1	Forward: 5'-CAACTGCAGAGATGGCAGCTA-3' Reverse: 5'-CTGATGCTCCCAATTGTTTCAT-3'
<i>BCL-2</i>	NM_000657.2	Forward: 5'-ATGTGTGTGGAGAGCGTCAA-3' Reverse: 5'-GCCGTACAGTTCACAAAAGG-3'
<i>Caspase-3</i>	NM_032991.2	Forward: 5'-AGGTATCCATGGAGAACAACACTG-3' Reverse: 5'-ACCAGACCGAGATGTCATTCC-3'
<i>β-actin</i>	XM_006715764.1	Forward: 5'-CTGGCACACACCTTCTACAATG-3' Reverse: 5'-CCTCGTAGATGGGCACAGTGTG-3'

*MYOC*: Myocilin; qPCR: Quantitative polymerase chain reaction.

### Western blotting analysis

For Western blotting analysis, rabbit polyclonal antibodies were used in all cases. Cellular proteins (20 μg) were separated on denaturing 10% polyacrylamide gels. The membrane was incubated with antibodies against MYOC (1:500; Abcam, polyclonal, UK), CHOP (1:500; Cell Signaling, L63F7, USA), BCL2 (1:500; Abcam, bcl2/100, UK), and caspase-3 (1:1000; Abcam, 3G2, UK) in blocking buffer overnight at 4°C. Secondary antibodies (peroxidase-conjugated streptavidin) were diluted 1:1000 in blocking buffer and incubated for 1 h at room temperature. As a loading control to ensure equal protein loading, the same blot was subsequently incubated with a GAPDH monoclonal antibody (1:1000; Abcam, 6C5, UK).

### Apoptosis assay

Apoptosis was assessed in transfectants treated with CHOP-siRNA or scramble control. The cells were fixed for 30 min in 4% paraformaldehyde in PBS, followed by incubation with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, USA) for 30 min, and analyzed by flow cytometry (Becton Dickinson FACS Aria, San Diego, USA). Cell lines were gated by light scatter characteristics. Data were analyzed using FlowJo 7.6.1 software (FlowJo, Palo Alto, USA).

### Drug treatment assay

The mutant MYOC (mMYOC) cells were maintained in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Idebenone was purchased from Sigma-Aldrich (I5659, Sigma, USA). The various concentrations of idebenone were used for the treatment of mMYOC cells, including 0, 1, 5, 10, 20, and 50 μmol/L (repeated 3 times). Cells were seeded into 6-well dishes and incubated at 37°C with 5% CO<sub>2</sub>; the medium was changed at the 3<sup>rd</sup> day combined with the same concentrations of idebenone, and the cells were collected for the further detection at the 5<sup>th</sup> day.

### Statistical analysis

The gene expression levels were plotted using the error bar, and the difference between groups was compared using paired samples *t*-test. All analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). A *P* < 0.05 was considered statistically significant.

## RESULTS

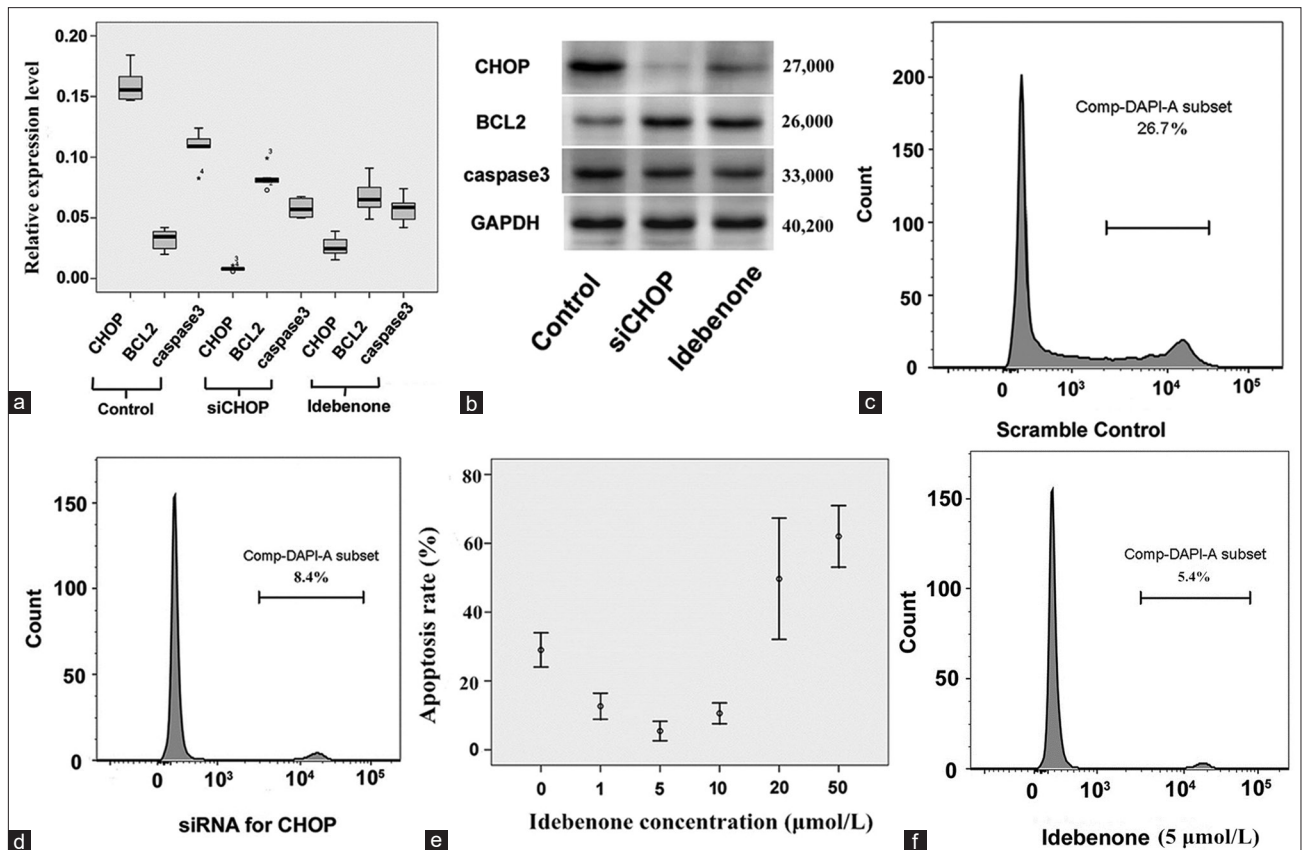
### Apoptosis detection and expression of apoptosis-associated genes

For investigating the pathogenesis of glaucoma induced by mutant *MYOC* gene, we established an *in vitro* model in which COS7 cells stably expressed either wild-type *MYOC* (*wtMYOC*) or *mMYOC* according to our previous procedure.<sup>[3]</sup> For evaluating the extent of apoptosis, DAPI was used for detecting dead cells. In *wtMYOC* cells, reduced apoptosis (<5%) was observed. However, the extent of apoptosis in *mMYOC* cells increased over time, and approximately, 30% of *mMYOC* cells were apoptotic at day 5, which was significantly greater than that of apoptotic *wtMYOC* cells (<3%). In addition, the expression levels of CHOP and caspase-3 were upregulated, and the expression of BCL2 was downregulated in *mMYOC* cells relative to *wtMYOC* cells.

### Treatment of mutant myocilin cells with CHOP-small interfering RNA or idebenone drug

Because the expression of *CHOP* gene changed more significant in apoptosis situation, for inhibiting apoptosis, we tried to silence the expression of *CHOP* gene in *mMYOC* cells using CHOP-siRNA. Compared with the scrambled control [Figure 1a], the specific CHOP-siRNA decreased the RNA expression of *CHOP* (*t* = 12.35, *P* = 0.006). Accompanied with the downregulated *CHOP*, the RNA expression of *caspase-3* was also decreased (*t* = 5.15, *P* = 0.026), but the RNA expression level of *BCL2* was conversely without statistical significance (*t* = -2.87, *P* = 0.108). We also performed the protein detection of those genes using Western blotting; the similar results with the RNA expression were observed [Figure 1b]. Furthermore, apoptosis status of the *mMYOC* cells interfered by siRNA for *CHOP* was detected using flow cytometry; we found that the fraction of apoptotic *mMYOC* cells was reduced to 8.4% at the 5<sup>th</sup> day after *CHOP* silenced [Figure 1c and 1d].

The drug idebenone can release ER stress and has been reported to reduce apoptosis. Therefore, we tried to determine whether idebenone could reduce apoptosis induced by mutant *MYOC* gene. First, we assessed apoptosis in *mMYOC*



**Figure 1:** The mutant myocilin cells were transfected with scrambled control or CHOP-small interfering RNA, reverse transcription polymerase chain reaction detected the RNA expression level of apoptosis-associated genes (a), the protein expression level was detected using Western blotting (b), and flow cytometry analysis showed their apoptosis status (c and d). The mutant myocilin cells were treated with different concentrations of idebenone, the apoptosis status was detected using flow cytometry (e and f), and the expression level of protein and RNA was also shown in a and b, separately.

cells treated with various concentrations of idebenone (1–50  $\mu\text{mol/L}$ ). We found that mMYOC cells treated with 5  $\mu\text{mol/L}$  idebenone exhibited the least apoptosis (5.4%), and when the concentration was increased higher than 10  $\mu\text{mol/L}$ , apoptotic cells number was increased gradually [Figure 1e and 1f]. After that, we detected the expression of RNA and protein of those genes, consistent with reduced apoptosis, we found that the mRNA expression level of *CHOP* ( $t = 11.72$ ,  $P = 0.006$ ) and *caspase-3* ( $t = 9.25$ ,  $P = 0.017$ ) were downregulated, but the mRNA expression level of *BCL2* was increased without statistical significance ( $t = -1.39$ ,  $P = 0.130$ ). The expression levels of CHOP and caspase-3 in mMYOC cells treated by idebenone were slightly higher than those in mMYOC cells treated with CHOP-siRNA, without statistical significance ( $t = -3.05$ ,  $P = 0.065$  and  $t = -0.62$ ,  $P = 0.731$ , respectively) [Figure 1a and 1b].

## DISCUSSION

The product of the *MYOC* gene plays an important role in protein metabolism and secretion pathways. One of the risk factors for glaucoma is the elevation of intraocular pressure, which can cause damage to the optic nerve. In the current study, we tried to use idebenone and siRNA for *CHOP* gene to inhibit apoptosis induced by the

*MYOC* protein. We observed upregulation of the *CHOP* gene and increased apoptosis in mMYOC cells, and the increased apoptosis situation was successfully decreased by inhibiting CHOP expression. The previous study has shown that the expression of CHOP correlated with ER stress and the mitochondrial unfolded protein response.<sup>[5]</sup> Our findings suggested that targeting the *CHOP* gene might provide an avenue for treating ER stress-derived apoptosis caused by mMYOC cells. However, the CHOP pathway may be not the only pathway for the apoptosis, JNK kinase might also be involved in the apoptosis procedure.

Studies have revealed that idebenone has antioxidant and antiapoptotic properties.<sup>[6]</sup> In this study, idebenone was an effective inhibitor of apoptosis induced by *MYOC* mutation with a dose-dependent manner. As well, we observed reduced *CHOP* and *caspase-3* expression at both protein and mRNA levels when idebenone played its role to inhibit apoptosis. It is difficult to confirm which one of them was the target gene of idebenone. However, CHOP has been regarded as a key mediator of cell death in response to ER stress,<sup>[7]</sup> and siRNA for *CHOP* was able to inhibit apoptosis of mMYOC cells in this study, which maybe imply that idebenone might rescue P370L mutant MYOC cells and might release ER stress by inhibiting apoptosis via the CHOP pathway. However, the

further study should be designed to clarify that the *CHOP* gene is the target gene or only is the downstream gene changed following the target gene.

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### Conflicts of interest

There are no conflicts of interest.

### REFERENCES

1. Quigley HA, Broman AT. The number of people with glaucoma worldwide in 2010 and 2020. *Br J Ophthalmol* 2006;90:262-7. doi: 10.1136/bjo.2005.081224.
2. Zhang X, Zhou M. Neovascular glaucoma: Challenges we have to face. *Chin Med J* 2014;127:1407-9. doi: 10.3760/cma.j.issn.0366-6999.20140284.
3. Wang JW, Sun HM, Ji J, Li XR, Zheng JQ. Comparative study of eukaryotic cell expression of wild-type and Pro370Leu mutation type myocilin gene (in Chinese). *Nati Med J China* 2006;86:700-4. doi: 10.3760/j.issn:0376-2491.2006.10.012.
4. Haynes CM, Ron D. The mitochondrial UPR-protecting organelle protein homeostasis. *J Cell Sci* 2010;123(Pt 22):3849-55. doi: 10.1242/jcs.075119.
5. Oyadomari S, Mori M. Roles of CHOP/GADD153 in endoplasmic reticulum stress. *Cell Death Differ* 2004;11:381-9. doi: 10.1038/sj.cdd.4401373.
6. Kernt M, Arend N, Buerger A, Mann T, Haritoglou C, Ulbig MW, *et al.* Idefenone prevents human optic nerve head astrocytes from oxidative stress, apoptosis, and senescence by stabilizing BAX/Bcl-2 ratio. *J Glaucoma* 2013;22:404-12. doi: 10.1097/IJG.0b013e31824caf90.
7. Tabas I, Ron D. Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. *Nat Cell Biol* 2011;13:184-90. doi: 10.1038/ncb0311-184.