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Lithium Chloride Induces Apoptosis in Human Choroidal Melanoma Cells via the NOXA/Mcl-1 Axis and Induction of ER Stress

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Abstract

Background: Choroidal melanoma is the most common primary intraocular malignancy that occurs in adults. Lithium chloride (LiCl) has been safely used in the clinic to treat psychiatric disorders for several decades. In this study, we aimed to understand whether LiCl exerts anticancer effects on choroidal melanoma cells and elucidate the underlying molecular mechanisms.

Methods: Human choroidal melanoma cells were treated with LiCl, and cell survival was assessed with MTT assays. Cell proliferation was measured by plate colony formation assays. Cell apoptosis was evaluated using flow cytometry, and proteins were detected using western blotting. A human choroidal melanoma xenograft model was established to demonstrate the effect of LiCl on human choroidal melanoma in vivo. An unpaired t-test was used to compare differences between two groups, and one-way ANOVA was used to compare differences among more than two groups.

Results: We found that LiCl inhibited cell survival and clonogenic potential and induced apoptosis in human choroidal melanoma cells. LiCl also reduced the proliferation of choroidal melanoma cells in vivo. Moreover, the upregulation of NOXA and downregulation of Mcl-1 were responsible for LiCl-induced apoptosis. Mcl-1 overexpression obviously impaired LiCl-induced apoptosis and cleavage of casp8, casp9, casp3 and PARP. Moreover, the protein expression of endoplasmic reticulum stress markers, including IRE1α, Bip, p-eIF2α, ATF4 and CHOP, was upregulated following treatment with LiCl.

Conclusions: In summary, LiCl induced an endoplasmic reticulum stress response while activating intrinsic apoptosis. Furthermore, the NOXA/Mcl-1 axis contributed to LiCl-induced apoptosis both in vitro and in vivo. The present study provides important mechanistic insight into potential cancer treatments involving LiCl and enhances the understanding of human choroidal melanoma.

Background

Choroidal melanoma is the most common primary intraocular malignancy that occurs in adults. The incidence of malignant melanoma is increasing worldwide[1]. Due to its latency and metastatic potential, the lethality of choroidal melanoma is approximately 50%[2]. Once choroidal melanoma metastasizes, the median survival time after detection of the first metastasis is 8 months[3]. Poor prognosis is related to various molecular factors[4], but the mechanisms of choroidal melanoma development remain to be elucidated.

Enucleation, phototherapy, and various forms of radiotherapy are currently common treatments for choroidal melanoma[5]. However, present treatments offer only temporary relief and have several shortcomings, such as neovascular glaucoma, retinal detachment and tumour recurrence[6]. Therefore, exploring new treatment strategies for early detection and improved prognosis of choroidal melanoma patients is a matter of great urgency[7].

Lithium compounds have been used in the clinic to treat the acute symptoms of bipolar diseases for several decades[8]. Lithium chloride (LiCl), an inhibitor of GSK3β that promotes GSK3β phosphorylation and inactivation, has been safely used in the clinic for the treatment of psychiatric disorders for many years[9, 10]. It has also been reported that LiCl can induce apoptosis in pancreatic ductal malignant gland cells and G2/M cell cycle arrest in liver cancer and non-small cell lung cancer[11]. In addition, lithium also plays a role as an adjuvant for radiation and chemotherapy, while increasing evidence has confirmed the value of combination treatment with lithium for cancers[12]. However, the molecular mechanism underlying the anticancer effect of LiCl is still unclear. Determining whether LiCl exhibits an anticancer effect on choroidal melanoma and understanding the underlying molecular mechanism are important for developing better chemotherapeutics for this condition.

In this study, we investigated whether LiCl exerts apoptotic effects on choroidal melanoma cells. We also explored the role of c-FLIP and NOXA as regulators of this effect. Our findings suggest that LiCl is a promising candidate therapy for the treatment of choroidal melanoma and enriches our understanding of the underlying molecular mechanisms for LiCl-induced choroidal melanoma cell apoptosis.

Materials And Methods

Antibodies and reagents

LiCl was purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against caspase8 (cat no. 9746), caspase9 (cat no. 9502), Bip (cat no. 3183), poly(ADP-ribose) polymerase (PARP; cat no. 9542), phospho-eIF2α (Ser51) (D9G8) (cat no. 3398S), eIF2α (cat no. 9722S), IRE1α (cat no. 3294), ATF-4 (D4B8) (cat no. 11815S) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies targeting caspase3 (cat no. NB100-56708) were purchased from Imgenex (Novus Biologicals, LLC, Littleton, CO, USA). The NOXA antibody (cat no. OP180) was obtained from Calbiochem (Merck KGaA, Darmstadt, Germany). Antibodies against CHOP (cat no. sc-7351) and Mcl-1 (cat no. sc-12756) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Cell lines and cell culture

The human choroidal melanoma lines OCM1 and M619 were obtained from the China Centre for Type Culture Collection (Wuhan, China) and were grown in monolayer cultures at 37 °C in a humidified atmosphere consisting of 5% CO_2 and 95% air. OCM1 cells were cultured in Dulbecco's modified Eagle's medium containing 5% foetal bovine serum, and M619 cells were cultured in RPMI-1640 medium containing 5% foetal bovine serum (both Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA).

Cell viability assay

Cells were seeded in 96-well plates at a density of 5.0×10^3 cells/well and were then treated with the indicated concentrations of LiCl on the second day. The cells were cultured with chemotherapeutics for 24, 36 or 48 h then subjected to the MTT assay. Each sample was incubated with 20 μl of (5 mg/ml) MTT (SigmaAldrich; Merck KGaA) at 37 °C for 4 h. Then, the solution was discarded, and 100 μl of dimethyl

sulfoxide was added. The absorbance at 495 nm due to formazan was measured by an ELISA Multiskan reader (Thermo Fisher Scientific, Inc.).

Colony formation assay

The cells were seeded into 6-well plates at a density of 1×10^4 cells per well. After the cells were incubated overnight, the cells were treated with 0, 2.5, 5, or 10 mM LiCl and incubated for approximately 2 weeks. During this period, the indicated concentrations of LiCl were added to the wells every 72 h. When the cell colonies were visible to the naked eye, the cells were subjected to the colony formation assay. The culture solution was discarded, and the cells were washed twice with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 20 min. After that, the cells were washed with PBS 3 times, stained with 1% crystal violet for 20 min, washed out slowly with water, and dried in air. The number of cell colonies (> 50 cells) was counted under a microscope.

Apoptosis assay

Apoptosis was evaluated according to a previously described protocol[13]. The Annexin V-FITC/ propidium iodide (PI) apoptosis detection kit was purchased from BIO-BOX Biotech (Nanjing, China). The cells were treated with various concentrations of LiCl for 36 h, and then 2×10^6 cells were collected, washed with prechilled PBS and resuspended in 500 µl of binding buffer. Then, each sample was incubated with 5 µl of Annexin V-FITC and 5 µl of PI for 15 min in the dark at room temperature. Then, the cells were analysed in a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). Data analysis was performed using FlowJo software (version 7.2.2; Tree Star, Inc. San Carlos, CA, USA).

Western blotting analysis

Whole-cell protein lysates were prepared and analysed by western blotting according to a previously described protocol[13]. After being harvested and rinsed with prechilled PBS, the cells were lysed, and the extract was centrifuged at 12,000 x g at 4 °C for 15 min. Wholecell protein lysates (40 µg) were electrophoresed on 12% denaturing polyacrylamide slab gels and transferred to Hybond-enhanced chemiluminescence (ECL) membranes through electroblotting. The membranes were blocked with 5% nonfat milk for 1 h at room temperature and then probed with specific primary antibodies and subsequently with secondary antibodies. Antibody binding was detected using an ECL system (EMD Millipore, Billerica, MA, USA) according to the manufacturer's protocol. The protein expression levels were quantified using ImageJ software (version 1.6.0_24; National Institute of Health, Bethesda, MD, USA).

Plasmid transient transfection

The pcDNA3.1-Mcl-1 plasmid was obtained from Addgene (Cambridge, MA, USA). OCM1 and M619 cells were seeded in 6-well plates and transfected with pcDNA3.1 and pcDNA3.1-Mcl-1 plasmids using X-treme GENE HP DNA Transfection Reagent (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer’s protocol. Then, the cells were treated with the indicated concentration of LiCl for 24 h and subjected to western blotting and apoptosis analysis.

In vivo tumorigenesis analysis

Five-week-old BALB/c nude male mice were obtained from Beijing Vital River Laboratory Animal Technology (Co., Ltd./Charles River Laboratories, Beijing, China). The BALB/c nude male mice were randomly divided into a control group and a LiCl group with 5 mice per group. M619 cells were subcutaneously injected into the right flank region of each mouse (3×10^6 cells in 100 μl of PBS). The tumour size was recorded every 3 days beginning on the day the tumours were first visible. The LiCl group was treated with LiCl (141.3 mg/kg; i.p., daily) for 2 weeks, while the control group received an equal volume of normal saline. Finally, the mice were sacrificed on the 15th day, and the tumour tissues were collected for western blotting and immunohistochemical analysis. The study was approved by the Shandong University Second Hospital Ethics Committee.

Immunohistochemical analysis

Immunohistochemical (IHC) analysis was performed according to a previously described protocol[14]. The tumour tissues were fixed in 10% formalin. Following proper dehydration, the tumours were embedded in paraffin and then cut into 5-μm-thick sections. After deparaffinization and rehydration, the sections were submerged in sodium citrate antigen retrieval solution (pH 6.0) and microwaved for 815 min for antigen retrieval. Endogenous peroxidase was deactivated by H_2O_2. Then, the slides were blocked using 10% goat serum and incubated in the corresponding primary antibodies overnight at 4 °C. After being washed, the sections were incubated with HRP-conjugated secondary antibody for 50 min at room temperature, followed by incubation with 3,3-diaminobenzidine (DAB) solution and counterstaining with haematoxylin. The anti-Ki67 rabbit mAb (cat no. GB 13030-2) was purchased from Wuhan Servicebio Technology Co., Ltd. (China).

Statistical analysis

All experiments were repeated at least three times. All statistical analyses were performed using SPSS statistical software (version 20.0; IBM Corp., Armonk, NY, USA). The data are represented as the mean ± S.D. of at least three independent assays performed in duplicate or triplicate. An unpaired t-test was used to compare differences between two groups, and one-way ANOVA was used to compare differences among more than two groups. A value of $P < 0.05$ was considered statistically significant.

Results

LiCl inhibits the survival and clonogenic potential of human choroidal melanoma cells

The cytotoxicity of LiCl in choroidal melanoma cells was investigated by treating OCM1 and M619 cells with various concentrations of LiCl for different times. We analysed the effect of LiCl on cell viability by performing the MTT assay. As shown in Fig. 1a and b, LiCl inhibited the survival of human choroidal melanoma cells in a dose- and time-dependent manner. With increased concentrations and times, the cell viability decreased gradually. Next, choroidal melanoma cells were incubated with LiCl at various concentrations, and a colony formation assay was performed. As shown in Fig. 1c and d, the number and

the size of colonies formed by the choroidal melanoma cells were not obviously decreased in the presence of 2.5 mM LiCl compared with that of the control cells. Statistical analysis showed that the number of colonies were significantly decreased by 5 mM and 10 mM LiCl (Fig. 1e). These findings suggest that LiCl effectively suppressed the survival and clonogenic potential of human choroidal melanoma cells.

LiCl induces apoptosis in human choroidal melanoma cells

The present study also sought to determine whether apoptosis was involved in LiCl-induced inhibition of survival. The western blotting results showed that the expression levels of the apoptotic proteins casp8, casp9, casp3 and PARP were increased in a concentration- and time-dependent manner in choroidal melanoma cells after LiCl treatment (Fig. 2a, b). Annexin V/PI staining was performed to evaluate the effect of LiCl on apoptosis (Fig. 2c, d). Flow cytometry analysis revealed that LiCl induced apoptosis in human choroidal melanoma cells in a concentration-dependent manner. When treated with LiCl (040 mM), the frequency of apoptosis increased from 4.30–37.60% in OCM1 cells and 8.10–39.40% in M619 cells. These findings indicate that treatment with LiCl triggers apoptosis in human choroidal melanoma cells.

Contribution of the NOXA/Mcl-1 axis to LiClinduced apoptosis

The NOXA/Mcl-1 axis has been reported to contribute to chemotherapeutically induced apoptosis In many types of tumour cells[15]. To discover the molecular mechanism of LiClinduced apoptosis in human choroidal melanoma cells, we analysed the expression of marker proteins in the intrinsic apoptotic signalling pathway after LiCl treatment. The western blotting results showed that NOXA expression was upregulated in both a concentration- and time-dependent manner following treatment with LiCl, while Mcl-1 expression was downregulated (Fig. 3a and b). In addition, the pcDNA3.1-Mcl-1 plasmid was transfected into OCM1 and M619 cells to confirm whether Mcl-1 downregulation accounted for LiClinduced apoptosis. As shown in Fig. 3c, Mcl-1 overexpression obviously restrained LiCl-induced cleavage of casp8, casp9, casp3 and PARP. Flow cytometry analysis showed that Mcl-1 overexpression reduced LiClinduced apoptosis in human choroidal melanoma cells (Fig. 3d). In summary, the data demonstrated that the NOXA/Mcl-1 axis is involved in the anticancer effect of LiCl in choroidal melanoma cells.

LiCl triggers endoplasmic reticulum stress in choroidal melanoma cells

It has been documented that the endoplasmic reticulum (ER) response is activated when chemotherapeutics induce apoptosis in cancer cells[16]. We examined relevant proteins in the ER stress pathway to confirm whether LiCl triggers ER stress in choroidal melanoma cells. The western blotting results demonstrated that expression of the marker proteins IRE1α, Bip, p-eIF2α, ATF4 and CHOP were

upregulated in a concentration-dependent manner after LiCl treatment (Fig. 4). These data indicate that LiCl triggers ER stress in choroidal melanoma cells.

LiCl inhibits choroidal melanoma cell tumorigenesis in vivo

We transplanted M619 cells into nude mice to determine the inhibitory effect of LiCl on tumorigenesis. The results showed that the tumour size in the LiCl group was significantly smaller than that in the control group (Fig. 5a, b). Moreover, the tumour weight (Fig. 5c) and growth (Fig. 5d) were noticeably decreased in the LiCl group compared with the control group at 15 days after inoculation. Western blotting analysis revealed that tumour tissues from the LiCl group displayed higher levels of NOXA and lower levels of Mcl-1 than those from the control group (Fig. 6a, b). These results indicate that LiCl inhibits choroidal melanoma cell tumorigenesis in vivo and that the NOXA/Mcl-1 axis contributes to this inhibitory effect. To further investigate the mechanism by which LiCl inhibits tumour growth, the expression levels of a proliferative marker (Ki67) were determined by immunohistochemical analysis. The results showed a decrease in Ki67 expression in tumours from mice treated with LiCl compared to control mice (Fig. 6c). Overall, these results were consistent with the in vitro results and showed that LiCl was an efficacious antitumour drug in the xenograft model. The mouse weights and tumour volumes are shown in Table 1.

Table 1
The body weights (a) and the tumour volumes (b) of the nude mice.

a Mice Weight(g)							
Group	Date		1/10	1/13	1/16	1/19	1/22	1/25
Control	1		14.57	15.46	16.68	17.18	16.88	18.00
	2		17.75	17.10	19.80	20.00	20.01	21.00
	3		15.62	15.48	16.91	17.37	17.39	18.23
	4		14.42	14.16	16.47	16.94	16.39	18.78
	5		13.68	15.40	16.03	16.53	15.53	17.63
LiCl	1		16.86	17.43	19.08	18.75	17.44	19.28
	2		16.90	16.23	17.85	18.33	17.90	19.47
	3		13.11	15.26	16.66	17.36	17.03	18.14
	4		16.88	17.61	19.19	19.35	17.89	18.05
	5		13.53	15.30	16.90	17.11	16.32	17.24
b Tumor Volume(mm3)								
Group	Date		1/10	1/13	1/16	1/19	1/22	1/25
Control	1	L	6.86	8.04	9.11	11.03	12.26	13.14
		W	4.73	5.62	7.70	9.47	9.82	10.15
		V	76.74	126.50	270.07	494.59	591.13	676.86
	2	L	6.49	7.86	8.70	10.02	11.97	12.19
		W	4.62	5.67	7.61	10.84	10.43	11.54
		V	69.26	126.35	251.92	544.17	651.08	811.68
	3	L	6.00	8.27	9.95	11.59	13.06	13.60
		W	5.97	6.33	8.14	8.77	9.39	9.91
		V	106.92	165.68	329.64	445.71	575.76	667.82
	4	L	6.11	9.88	11.35	13.82	14.89	16.90
		W	4.59	7.30	8.91	9.55	10.86	10.91
		V	64.36	263.25	450.53	630.21	878.06	1005.79
	5	L	6.48	5.40	8.00	10.35	11.45	12.45
		W	4.53	5.58	6.62	8.05	9.58	10.02

a Mice Weight(g)								
		V	66.49	81.36	175.30	335.35	525.42	624.99
LiCl	1	L	6.48	7.98	9.74	9.82	11.01	11.22
		W	5.61	6.63	8.28	9.15	9.35	9.99
		V	101.97	175.39	333.88	411.08	481.26	559.87
	2	L	5.45	5.60	5.93	5.58	6.52	6.62
		W	5.45	5.40	5.36	5.57	5.13	5.09
		V	80.94	81.65	85.18	86.56	85.79	85.76
	3	L	6.14	7.23	8.04	10.34	11.59	12.20
		W	5.21	5.23	7.76	8.27	8.53	8.90
		V	83.33	98.88	242.07	353.59	421.65	483.18
	4	L	5.09	5.87	6.13	7.21	7.33	8.05
		W	5.09	5.62	6.12	6.45	8.00	8.21
		V	65.94	92.70	114.80	149.98	214.92	266.01
	5	L	5.82	6.58	8.72	8.94	9.18	10.14
		W	5.82	7.07	9.35	9.58	9.83	10.44
		V	98.57	153.05	355.48	382.83	414.19	536.71

Discussion

Choroidal melanoma is a serious metastatic malignant melanoma with poor prognosis. Common treatments for choroidal melanoma offer only temporary relief and are ineffective in inhibiting tumour metastasis or improving the survival rate[17]. Thus, exploring new treatment strategies is important for improving the prognosis of choroidal melanoma patients[7].

LiCl has been safely used in the clinic for the treatment of psychiatric disorders for several decades[18]. LiCl has also been reported to exhibit antitumour effects in various cancers[11]. As a GSK3β inhibitor, the most robust antineoplastic effect of lithium is related to GSK3β[19]. However, one study showed that LiCl exerts antitumour effects that are independent of GSK3β[12]. The present study investigated whether LiCl exhibited antitumour effects in choroidal melanoma cells and demonstrated the underlying molecular mechanism.

In this study, we evaluated the effect of LiCl on choroidal melanoma cell survival and colony formation and provided a potential mechanism. We analysed the cytotoxicity of LiCl against choroidal melanoma

cells and found that LiCl displayed a concentration- and time-dependent inhibitory effect on the survival of choroidal melanoma cells. Furthermore, the number of colonies formed notably declined with increasing concentrations of LiCl. Flow cytometry analysis revealed that LiCl induced apoptosis in choroidal melanoma cells in a concentration-dependent manner. Subsequently, it was demonstrated that the levels of the cleaved forms of apoptosis-associated proteins were distinctly increased both in a dose- and time-dependent manner following LiCl treatment of OCM1 and M619 cells. These results revealed that LiCl exerts anticancer effects by decreasing cell viability, inhibiting colony formation, and inducing caspase-dependent apoptosis in choroidal melanoma cells.

Apoptosis, known as programmed cell death, suppresses carcinogenesis in normal cells due to various genes[20]. As an anti-apoptotic protein, Mcl-1 belongs to the BCL-2 family, which is closely connected to inhibitions of mitochondrial apoptosis[21]. Blocking Mcl-1 makes tumour cells more susceptible to anticancer agents[22]. NOXA, a BH3-only protein in the Bcl-2 family, has been reported to participate in chemotherapy-induced apoptosis in melanoma[23]. Interactions between Mcl-1 and NOXA regulate the mitochondrial apoptotic pathway[24]. In the present study, we found that after LiCl treatment, the level of Mcl-1 was obviously decreased, while the protein NOXA was upregulated in a concentration- and time-dependent manner. We found that Mcl-1 overexpression dramatically weakened LiCl-induced cleavage of apoptosis-associated proteins and impaired apoptosis after drug treatment. These results demonstrated that the NOXA/Mcl-1 axis may contribute to LiCl-induced intrinsic mitochondrial apoptosis in choroidal melanoma cells.

GSK3β plays multiple roles in different cancers, but its importance is still controversial[25]. Promotion of GSK3β inactivation by LiCl sensitizes both hepatoma and prostate cancer to TRAIL-induced apoptosis[26]. However, the study indicated that LiCl significantly enhanced cell apoptosis in non-small cell lung cancer by upregulating the death receptors DR4 and DR5, and LiCl sensitized cells to TRAIL-induced apoptosis independent of GSK3β[12]. Therefore, further studies are required to understand whether GSK3β participates in LiCl-induced ER stress and intrinsic apoptosis also needs further research.

To further investigate whether LiCl could affect tumorigenesis, we transplanted M619 cells into nude mice and found that xenograft tumour growth in the LiCl group was significantly slower than that in the control group. At 15 days after inoculation, tumour size and weight were dramatically decreased in the LiCl group. Ki-67, a nuclear protein, is widely used as a tumour proliferation marker. Our study showed that the number of Ki67-positive cells from the xenograft tumours also declined in the LiCl group. Furthermore, LiCl treatment significantly induced Mcl-1 expression and upregulated the NOXA level in M619 cell-based xenografts. In summary, LiCl prevents xenograft tumour growth in mice, and the NOXA/Mcl-1 axis is associated with this effect.

A previous study showed that cell apoptosis may be induced by ER stress and mitochondrial membrane permeability[27, 28]. CHOP, a key protein of ER stress-mediated cell death, also known as a bZIP-containing transcription factor, targets many apoptotic genes, including NOXA, modifying their expression and ultimately resulting in apoptosis[29]. Therefore, to evaluate whether the PERK/eIF2α/ATF4/CHOP

signalling pathway was involved in LiCl-induced apoptosis, we examined p-eIF2α, eIF2α, ATF4 and CHOP levels, and the results showed that p-eIF2α, ATF4 and CHOP were upregulated in a concentration-dependent manner following treatment with LiCl. Additionally, we examined the expression of IRE1α and Bip, two other molecules involved in ER stress, and the results showed that both were upregulated by LiCl in a concentration-dependent manner. These data indicate that the anticancer effect of LiCl in choroidal melanoma cells was mediated by activation of the ER stress pathway, but further investigation is required to determine whether ER stress participates in LiCl-induced apoptosis and whether CHOP is involved in LiCl-induced NOXA upregulation.

Conclusion

In summary, we demonstrate that LiCl exerts apoptotic effects on choroidal melanoma cells. The NOXA/Mcl-1 axis is involved in LiCl-induced intrinsic apoptosis both in vitro and in vivo. Furthermore, LiCl triggers ER stress in choroidal melanoma cells to induce intrinsic apoptosis. LiCl inhibits choroidal melanoma cell tumorigenesis in vivo. This study may provide an important theoretical basis for promoting LiCl as a potential clinical therapeutic strategy to treat choroidal melanoma.

Abbreviations

LiCl
lithium chloride;PARP□poly(ADP-ribose) polymerase□PBS□phosphate-buffered saline□PI□propidium iodide□ECL:enhanced chemiluminescence; IHC:immunohistochemical; DAB:diaminobenzidine; ER:endoplasmic reticulum; L:length; W:width; V:volume; NS:not significant; CF:cleaved form; CFs:plural of cleaved form; Casp:caspase.

Declarations

Ethics approval and consent to participate

The animal experiments in this study was approved by the Animal Ethics Committee of the Second Hospital of Shandong University Cheeloo College of Medicine, in accordance with the National Institutes of Health (NIH) Guide for the animal treatments of Laboratory Animals.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

The subject design was completed by ZXF and ZH. The operation of the experiment was carried out by ZQQ, LHY and ZQW. Data analysis by ZQQ, LHY and ZQW. Article written by ZQQ and ZXF completed. The final article was reviewed by ZXF and ZH. All authors read and approved the final manuscript.

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Figures

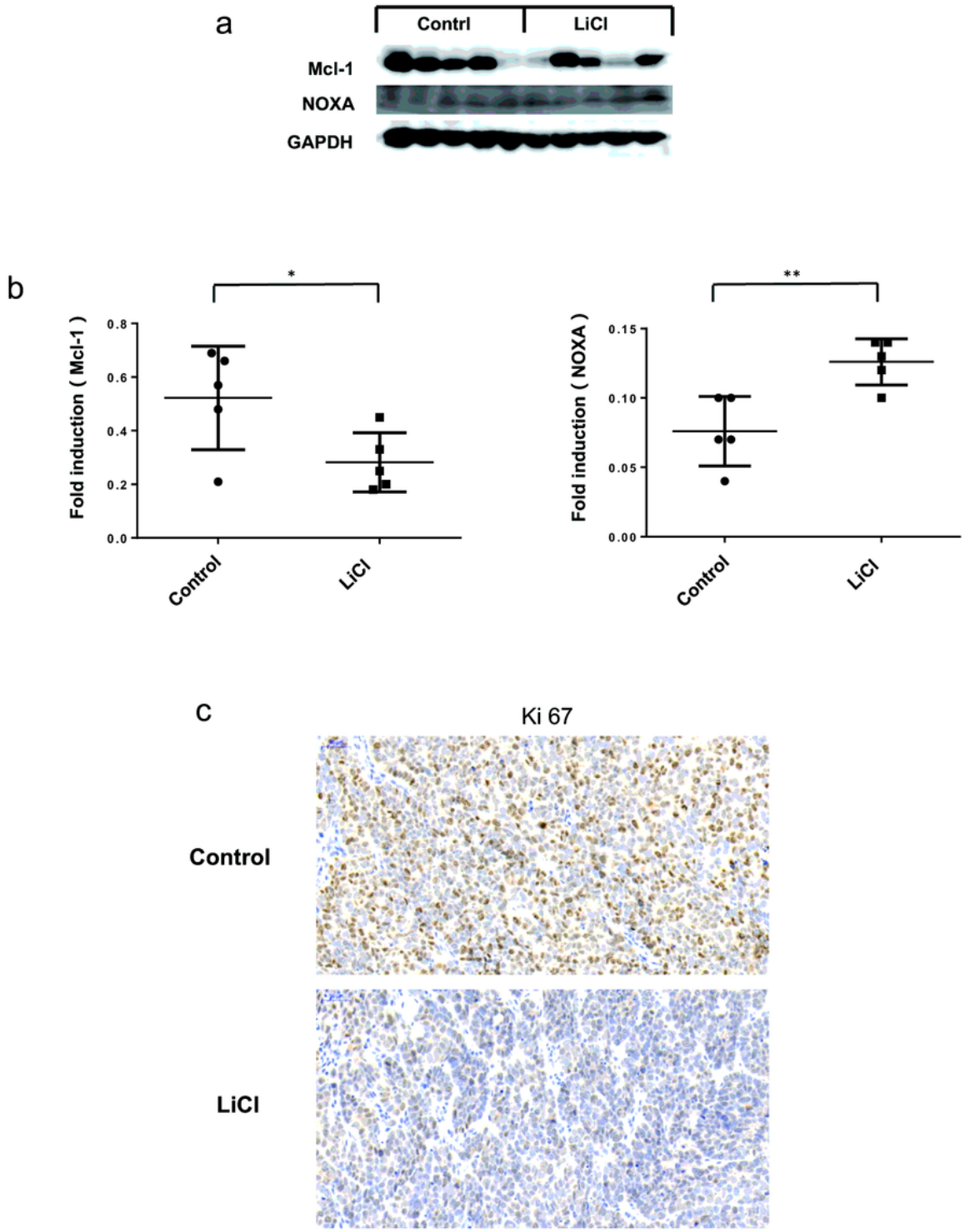

Figure 1

LiCl inhibits the survival and clonogenic potential of choroidal melanoma cells. (a) OCM1 and (b) M619 cells were seeded in 96-well plates, treated with 0, 2.5, 5, 10, 20 or 40 mM LiCl and incubated for 36 h. Cell survival was examined using the MTT assay. The survival rate at each drug concentration was compared with that of the control group and analysed using SPSS software. All data are presented as the mean ± S.D. *P<0.05, **P<0.01, ***P<0.001. NS: not significant. c OCM1 and M619 cells were seeded in 6-well

plates at a concentration of 1× 104 cells/well and cultured for approximately 2 weeks. Cells were treated with 0, 2.5, 5, or 10 mM LiCl, and various concentrations of LiCl were added to the wells every 72 h. When the cell colonies were visible to the naked eye, the culture was terminated, and the cells were stained with 1% crystal violet. d The size of the colonies. Magnification: ×100. Corresponding scale bars are depicted in the lower right corner of each image. Scale bars=100 μm. e The number of colonies (>50 cells) was counted under a microscope. The experiments were repeated three times independently. All data are presented as the mean ± S.D. **P<0.01, ***P<0.001. NS: not significant.

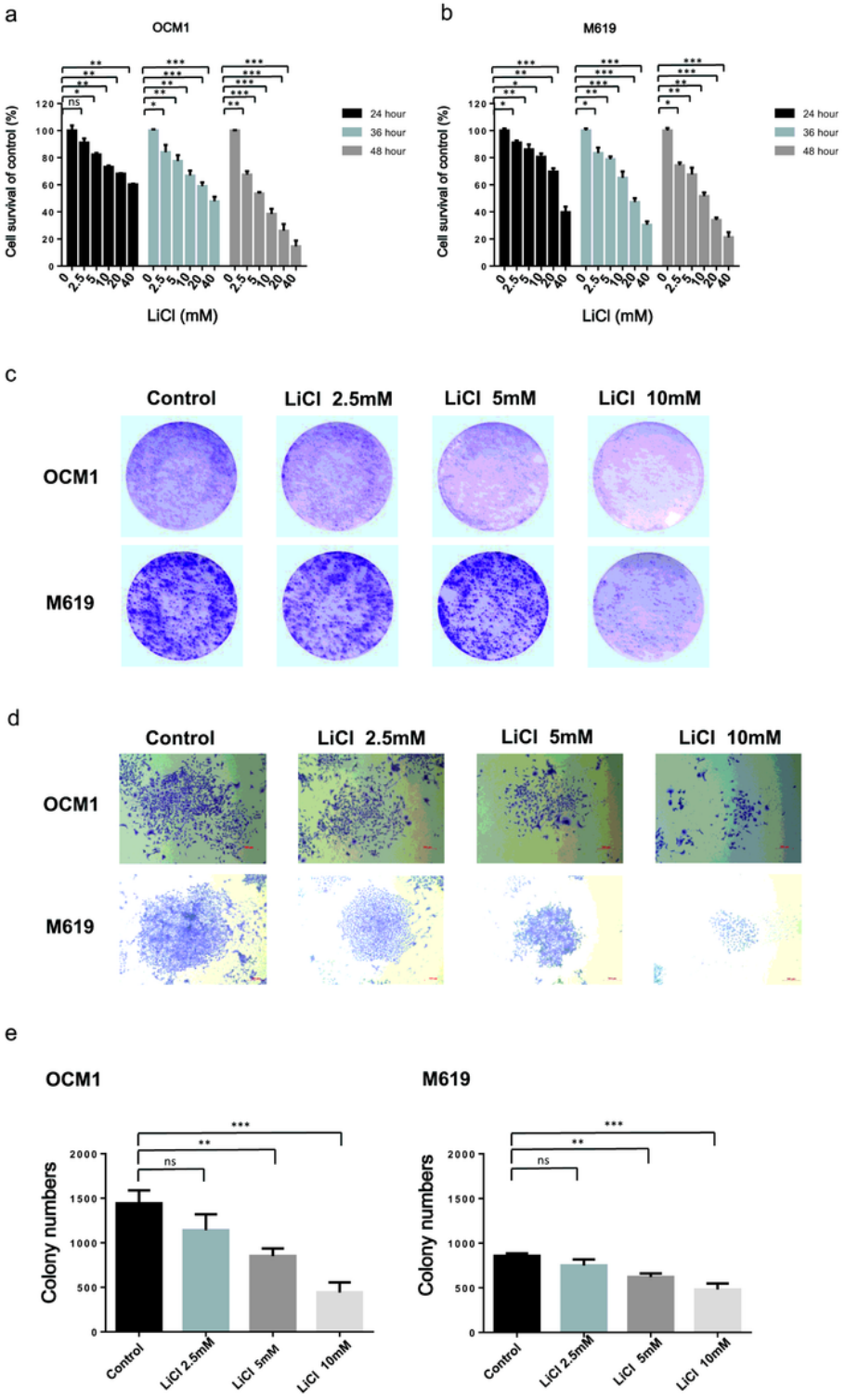

Figure 2

LiCl induces apoptosis through a caspase-dependent pathway in a concentration- and time-dependent manner. a To perform a dose-gradient assay, OCM1 and M619 cells were treated with 0, 2.5, 5, 10, 20 or 40 mM LiCl for 36 h and then harvested for western blotting analysis. b For the time-gradient assay, OCM1 and M619 cells were treated with 20 mM LiCl for 0, 6, 12, 24 and 36 h and then harvested for western blotting analysis. Apoptosis-related protein (casp8 casp9, casp3 and PARP) expression was quantified using ImageJ software and analysed with GraphPad Prism 5.0 software. CF: cleaved form. CFs: plural of cleaved form. PARP: poly (ADP-ribose) polymerase; Casp: caspase. All data are presented as the mean ± S.D. c d Annexin V/PI staining was performed to evaluate the effect of LiCl on apoptosis. (c) OCM1 and (d) M619 cells were treated with 0, 10, 20 or 40 mM LiCl for 24 h and then harvested for apoptosis analysis. Data analysis was performed using FlowJo software and SPSS software. All data are presented as the mean ± S.D. *P<0.05, **P<0.01, ***P<0.001. Q1: (Annexin V- FITC)-/PI+, necrotic cells. Q2: (Annexin V+FITC) +/PI+, late apoptotic cells. Q3: (Annexin V- FITC) +/PI-, early apoptotic cells. Q4: (Annexin V-FITC)-/PI-, normal control cells. PI, propidium iodide; FITC, fluorescein isothiocyanate.

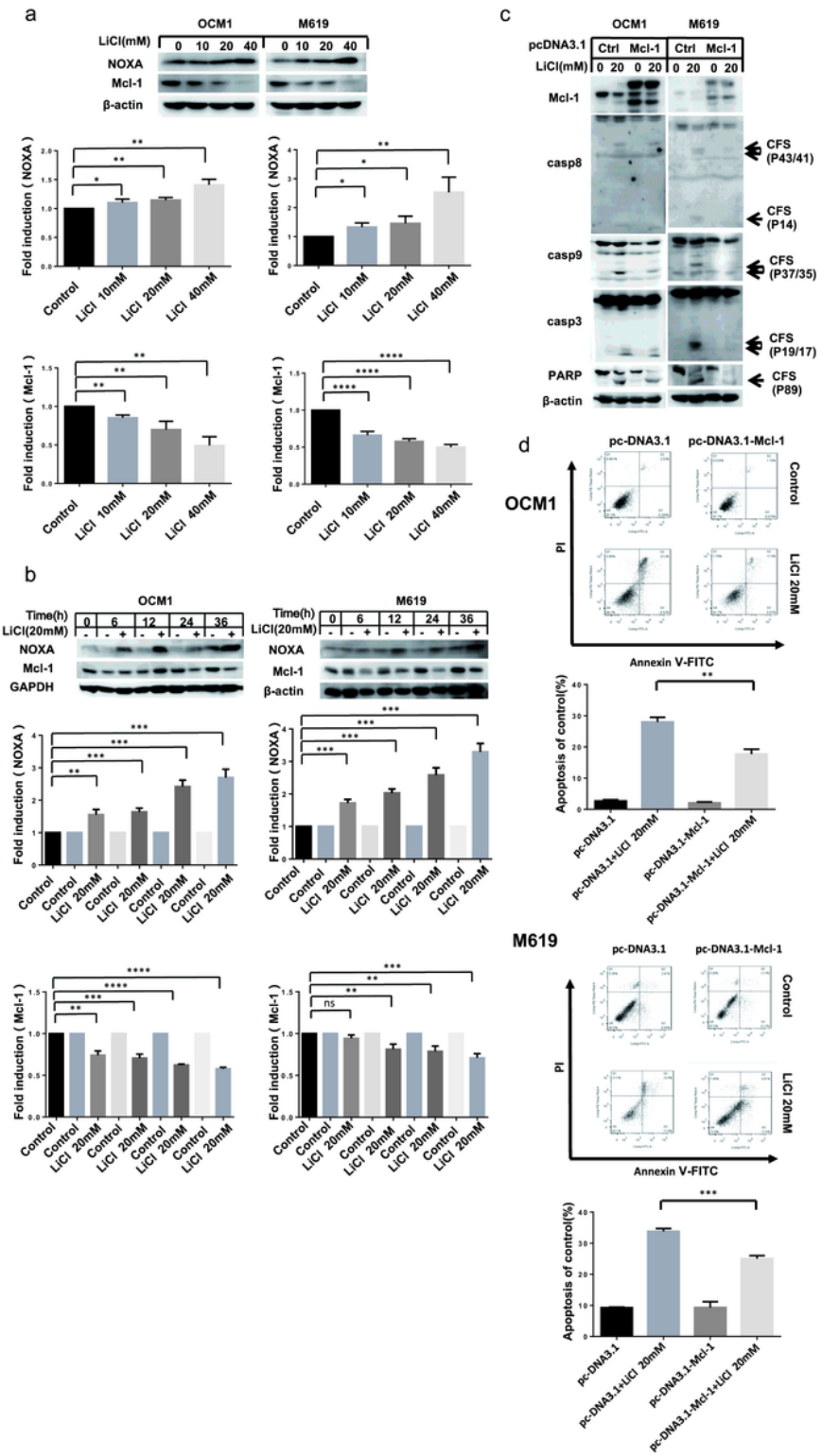

Figure 3

The NOXA/Mcl-1 axis contributes to LiCl-induced apoptosis. a OCM1 and M619 cells were treated with 0, 10, 20, or 40 mM LiCl for 36 h and then harvested for western blotting analysis. b OCM1 and M619 cells were treated with 20 mM LiCl for 0, 6, 12, 24 and 36 h and then harvested for western blotting analysis. NOXA and Mcl-1 expression was quantified using ImageJ software and analysed with GraphPad Prism 5.0 software. c d OCM1 and M619 cells were seeded in 6-well plates and transfected with control or pc-

DNA3.1- Mcl-1 plasmids on the second day. After 48 h of transfection, the cells were exposed to 20 mM LiCl for 24 h and then harvested for western blotting (c) and apoptosis analysis (d). All data are presented as the mean ± S.D. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

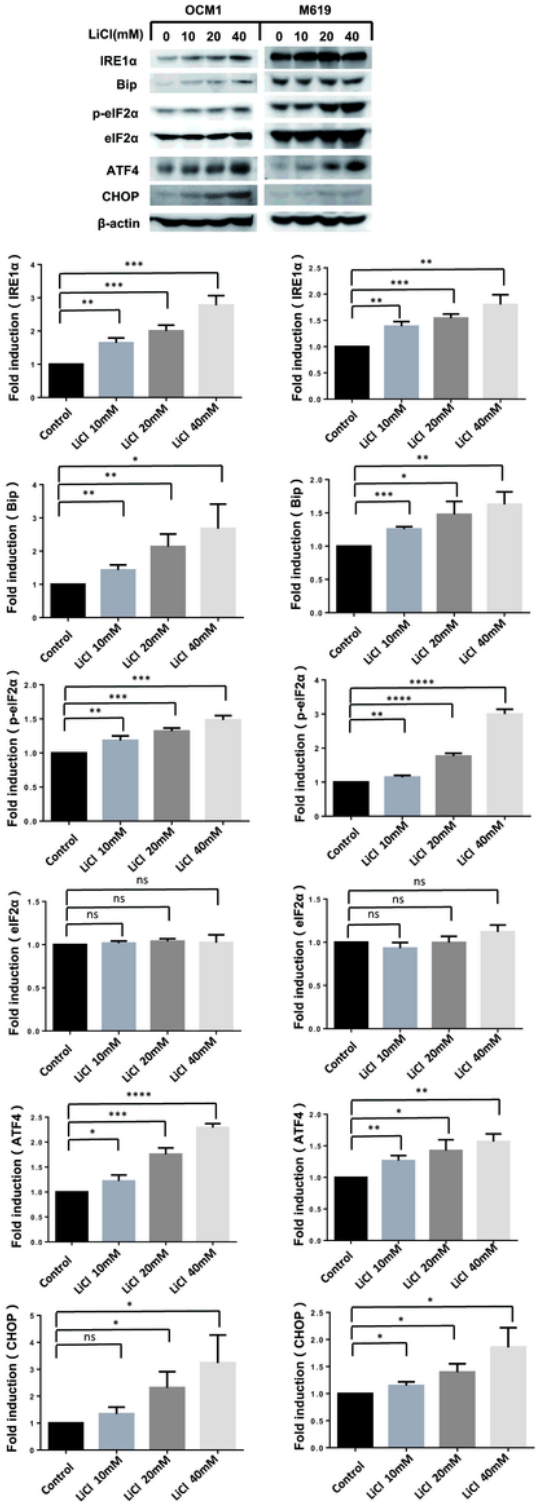

Figure 4

LiCl triggers endoplasmic reticulum stress in human choroidal melanoma cells. OCM1 and M619 cells were treated with 0, 10, 20, or 40 mM LiCl and incubated for 36 h. Following treatment, endoplasmic

reticulum stress-related proteins were quantified by western blotting analysis. Protein expression was quantified using ImageJ software and analysed with GraphPad Prism 5.0 software. All data are presented as the mean ± S.D. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. NS: not significant.

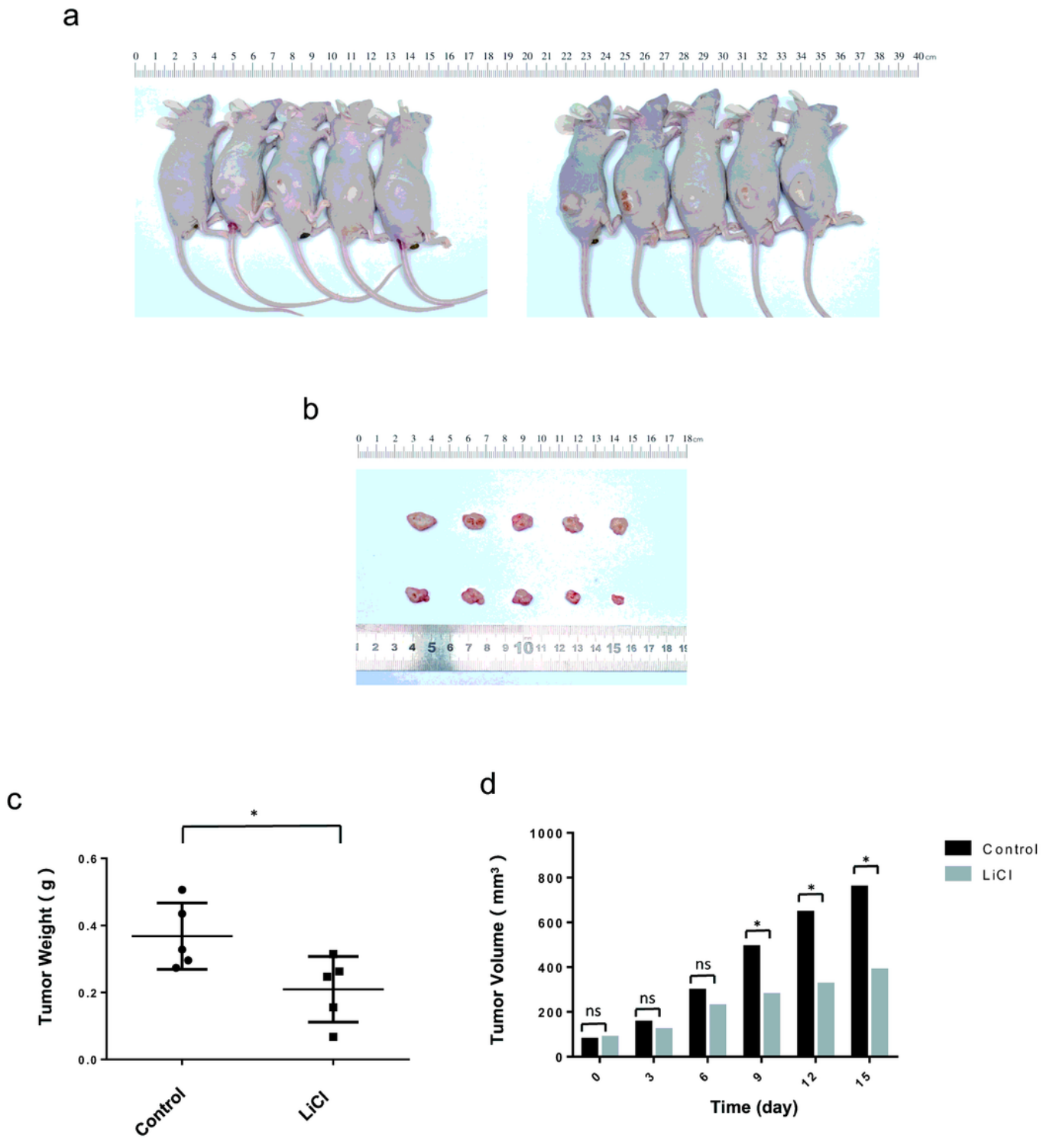

Figure 5

LiCl inhibits choroidal melanoma cell tumorigenesis in vivo. a The mouse experiments were performed once. BALB/c nude male mice were randomly divided into a LiCl group and a control group, with 5 mice

per group. M619 cells (3 × 106 cells in 100 μl of PBS) were subcutaneously injected into the right flank region of each nude mouse, and then tumours were grown in the mice. The LiCl group was treated with LiCl (141.3 mg/kg; i.p., daily), and the control group received an equal volume of normal saline for 2 weeks. b c At 15 days after inoculation, xenograft tumours were recovered and weighed. d The tumour volume was recorded every 3 days beginning on the day at which the tumours were first visible after injection. *P<0.05. NS: not significant.

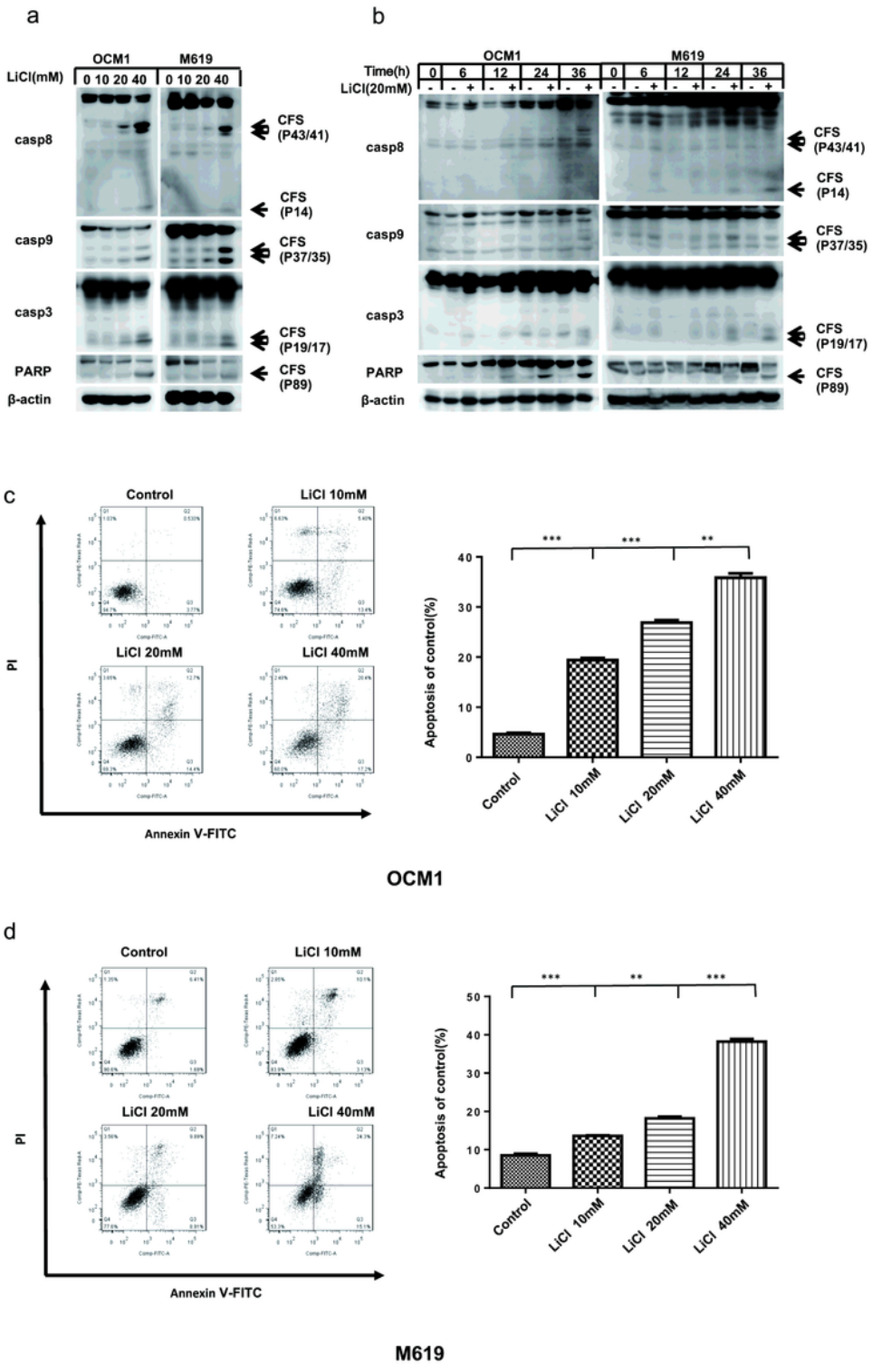

Figure 6

The NOXA/Mcl-1 axis contributes to LiCl-induced apoptosis in vivo. a b The nude mice were sacrificed for western blotting and immunohistochemical (IHC) analysis. c Western blotting was performed to assess the protein levels of NOXA and Mcl-1 in xenograft tumours. The tumour sections were stained for IHC analysis using antibodies against Ki67. Magnification: ×20. Corresponding scale bars are depicted in the upper left corner of each image. Scale bars=50 μm. The percentages of Ki67-positive cells in the control and LiCl groups and representative IHC images are shown in figure . All data are presented as the mean ± S.D. *P<0.05, **P<0.01.