Neoplasma 2022; 69(5): 1070–1078

doi:10.4149/neo_2022_220428N462

BRAF inhibition promotes ER stress-mediated cell death in uveal melanoma

Yinu ZHAO[1], Yue WANG[2], Li ZHANG[1], Weibin WANG[2], Thomas J. FAHEY 3rd[3], Ke YAO[1,*]

[1]Eye Center, Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China; [2]Department of Surgical Oncology, First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China; [3]Department of Surgery, New York Presbyterian Hospital and Weill Medical College of Cornell University, New York, United States

**Correspondence: xlren@zju.edu.cn*

Received April 28, 2022 / Accepted June 13, 2022

Melanoma with a BRAF mutation is more common to develop into a fatal disease. BRAF mutation inhibitor-induced autophagy affects the drug efficacy in many cancer types. The role of autophagy during BRAF inhibition in uveal melanoma (UM) remains unclear. In this study, we examined the autophagic flux and compared the number of autophagic vacuoles during the BRAF inhibition in UM. The PKR-like endoplasmic reticulum (ER) kinase (PERK) arm was studied to test whether the ER stress was involved. The effects of downregulation of ER stress by targeting the PERK arm (pharmacologically and genetically) were also assessed. We found a dose-dependent increase of autophagic flux in OCM1A cells during the BRAF inhibition. This phenomenon was further verified by an enhanced number of GFP-LC3 puncta and was finally confirmed by raised autophagic index examined by transmission electron microscopy. Pathway analysis revealed that the vemurafenib (the BRAF inhibitor)-induced autophagy was independent of the MAPK signaling pathway. Instead, it was possibly regulated via the enhanced ER stress response. We further found that the inhibition of ER stress response rescued cell death. Therefore, our results suggest BRAF inhibition promotes ER stress response-induced autophagy in UM. Targeting ER stress response can partially revert autophagy and rescue cell death, which may impair the anti-tumor effect of BRAF inhibitor in UM.

Key words: autophagy, uveal melanoma, BRAF gene, V600E point mutation, ER stress

Uveal melanoma (UM) is a rare form of melanoma, with its incidence in wide variation from 0.1 to 8.6 per million in the world [1, 2]. However, it is the most frequently diagnosed primary intraocular malignancy, especially for those patients between 40 and 60 years old [3, 4]. Even though the primary tumor is successfully treated with radiotherapy or surgical resection, nearly half of patients would develop metastases to the liver (89%), lungs (29%), and bones (17%) (via hematogenous spread) [5, 6]. Once metastases developed, patients become resistant to chemotherapy and typically would die within 1 year [7]. Long-term survival for these patients is rare. Thus, a better understanding of the biological behavior and the development of new therapies are urgently required for this disease.

Somatic mutations, especially point mutations, play an important role in tumorigenesis in UM. Mutations in GNA family genes (such as GNA11 and GNAQ) are carried in about 80% of UM patients [8] but no specific inhibitor is available so far. The B-Raf proto-oncogene (BRAF) encodes a serine/threonine kinase that monitors the mitogen-activated protein kinase pathway (MAPK). A missense mutation in codon 600 of exon 15 (V600E) of the BRAF gene has been reported as a driver mutation in a proportion of neoplasms, including cutaneous melanoma [9]. BRAF mutant melanomas are much more likely to progress to advanced disease, such as metastasizing to the brain, than BRAF wild-type cancers [10, 11]. Previous studies suggest that BRAF inhibitors can play a therapeutic role in terms of inhibiting tumor proliferation, promoting cell apoptosis, and reducing distant metastasis [12, 13]. The rate of BRAF mutation in UM patients is not high and remains controversial. Tumor heterogeneity and sensitivity of detecting techniques may account for this disparity [14, 15]. By using a very sensitive technology, Maat et al. found that 6 (13%) of 45 primary UV tumors were positive for BRAF V600E mutation, of which, all were negative by direct sequencing [15]. Their findings pave the road for treating UM patients with BRAF inhibitors.

Autophagy, known as basic cellular homeostasis, is believed to help cells to degrade portions of cytoplasmic contents [16]. Increasing data suggest that autophagy may

play a dual role in tumors. It can act as a cancer suppressive player during tumorigenesis by eliminating damaged organelles producing genotoxic stresses, which may facilitate tumor initiation [17, 18]. During tumor invasion, however, autophagy can serve as a protective effect against various diverse conditions [19]. Melanomas carrying BRAF mutations are often characterized by a strong resistance to autophagy and apoptosis [20].

The endoplasmic reticulum (ER) is the biggest membrane-bound intracellular compartment in eukaryotic cells. It functions in many processes including synthesis of protein and lipid, storage of calcium ions, and detoxification of compounds [21]. Hypoxia, nutrient deprivation, and alternations in calcium ions can induce ER stress, which will consequently trigger a range of responses in cancer cells, including autophagy [22, 23]. Targeting ER stress-induced autophagy has been reported to overcome BRAF inhibitor resistance in melanoma [22]. However, the possible role of autophagy during BRAF inhibition in UM is rarely investigated. In our previously published study, we initially found that starvation-trigged autophagy was probably BRAF V600E dependent in UM cells [24]. Here we further looked to determine the change of autophagy during BRAF inhibition and to explore whether the ER stress is responsible for the induction of autophagy in UM cells. We found the ER stress response mediated autophagy was induced during BRAF inhibition in UM. Targeting ER stress response can partially revert autophagy and rescue cell death, which may impair the antitumor effect of BRAF inhibitor in UM.

Materials and methods

Reagents. We used the primary antibodies including: anti-LC3 (#48394, Abcam), BRAF (#109312, Santa Cruz), anti-GAPDH (#32233, Santa Cruz), anti-p62/SQSTM1 (#56416, Abcam), eIF2α and p-eIF2α (#5324 and #3398 Cell Signaling), ATF4 (#11815, Cell Signaling), CHOP (#7351, Santa Cruz), p-mTOR (#137133, Abcam), p-AMPK (#4184, Cell Signaling), p-ULK1 (#37762, Cell Signaling), p-AKT (#9271T, Cell Signaling), p-P70S6K (#9234, Cell Signaling), anti-phospho-ERK (#4370, Cell Signaling), anti-ERK1/2 (#4695, Cell Signaling). The following reagents were applied to treat the cells: PLX4032 (#1267, Selleck Chemicals), bafilomycin A1 (#120497, Abcam), GSK2606414 (#1337531-89-1, Sigma-Aldrich) and U0126 (#1102, Selleck Chemicals).

Cell culture. Dr. Gary K. Schwartz from Columbia University Medical Center nicely provided uveal melanoma cell line OCM1A. We maintained the cells as previously described [22].

Western blot analysis. After treatment, cells were washed with PBS, harvested, and lysed by using the RIPA Buffer Kit (Santa Cruz Biotechnology) according to the manufacturer's protocols. An equal amount of protein was firstly loaded and then size-fractionated by SDS-PAGE. After that, the protein was transferred to the membrane and blocked by 5% powdered milk in a TBS-T solution. After blocking, we incubated the membranes with certain primary antibodies and probed them with secondary antibodies that were polyclonal HRP-conjugated the next day. Targeted bands were then visualized with ECL detection reagent and exposed to the film, and finally were quantified by using the software named ImageJ from NIH. All the experiments were performed and repeated at least 3 times.

GFP-LC3 cells stabilization and GFP-LC3 punctate detection. We generated OCM1A cells that stably express GFP-LC3 as we previously described [21]. GFP-LC3-OCM1A cells were cultured on coverslips coated with gelatin and then treated with different drug combinations for 24 h. After that, the cells were fixed and mounted on microscopic glass slides. Finally, we measured the GFP-LC3 punctate by a laser scanning fluorescence confocal microscope (Nikon Instruments Inc).

Transmission electron microscopy. After drug treatment for 24 h, OCM1A cells were then washed with PBS, fixed with glutaraldehyde, and processed as we previously described [25]. We finally examined the number of autophagic vacuoles with the Tecnai-G2 electron microscope. All the experiments were performed and repeated at least 3 times.

RNA interference. BRAF small interfering RNA (#8935, Cell Signaling), EIF2α small interfering RNA (#35272, Santa Cruz), and negative-control mismatch RNA (#37007, Santa Cruz) were transfected into OCM1A cells by using HiPerFect (Qiagen) according to the manufacturer's guider. After 48 hours of transfection, cells were then treated with the BRAF inhibitor or DMSO and further cultured for additional 24 h. After that, we performed western blot to confirm the efficacy of interference and did cell proliferation and apoptosis assay to compare the difference under treatments.

Cell proliferation assay. To measure the cell proliferation after treatment, the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was applied. We seeded OCM1A cells into the 96-well plates incubation with DMSO or vemurafenib. After 24 hours, we added the MTT solution and measured the absorbance with Micro Plate Reader (Flexstation, MD, USA) at 586 nm wavelength. All the experiments were performed and repeated at least 3 times.

Apoptosis assay. Cell apoptosis after drug treatment was determined by Annexin V-FITC cell kit. After transfection, OCM1A cells were exposed to DMSO or vemurafenib for 24 hours. Cells were then harvested the next day and washed with ice-cold PBS. They were further stained with Annexin V-FITC and detected by a flow cytometer (BD Biosciences). We considered cells, which were scored as Annexin V+/PI− as apoptotic cells. All the experiments were performed and repeated at least 3 times.

Statistical analysis. To assess statistical significance in different groups, one-way ANOVA was performed. We considered it significantly different if the p-value was less than 0.05.

Results

BRAF inhibitor increases autophagic flux in UM cells. To measure whether the BRAF inhibitor (vemurafenib) could induce autophagy in BRAF mutant UM cells, we firstly examined LC3 (microtubule associated protein 1 light chain 3) and p62 expression during BRAF inhibition in OCM1A cells. LC3 is a crucial marker to detect autophagy since it is the only protein that remains attached to the autophagosome. When autophagy happens, the cytosolic form of LC3 (LC3 I) will be conjugated to phosphatidylethanolamine (LC3 II) and recruited to autophagosomal membranes. Hence, the rate of LC3 II to LC3 I can serve as an indicator of the change in the autophagy level [26]. The p62 protein is another marker of autophagosome formation since it binds to LC3 and is degraded during the autophagic progress [27]. As shown in Figure 1A, vemurafenib treatment triggered an increase in the value of LC3 II to LC I and a decrease of p62 in OCM1A cells in a dose-dependent manner. A higher dose of vemurafenib (5 μM) significantly triggered a greater increase in the percentage of LC3 II to LC3 I and a more pronounced decrease of p62 (Supplementary Figure S1A). The ERK phosphorylation was inhibited by vemurafenib at the same time, which was as expected.

Considering the accumulation of LC3 II can be caused by either enhanced autophagosome formation (autophagic flux) or decreased autophagosome degradation, we further conducted our analyses by combining lysosomal degradation inhibitor bafilomycin A1 (BAF). As indicated in Figure 1A, the value of LC3 II to LC3 I increased in the presence of BAF, which suggests that autophagosome degradation is inhibited. The finding that accumulation of LC3 II to LC3 I was further significantly enhanced in a higher dose of vemurafenib treatment indicates the formation of autophagosome was augmented (Supplementary Figure S1A).

To confirm these results, we generated the OCM1A cells with stable expression of GFP-LC3 (OCM1A-GFP-LC3) and monitored the compartmentalization of endogenous LC3 II by examining the GFP positive puncta. When compared to the control group (DMSO, Figure 1B), an increased number of puncta was detected in the vemurafenib-treated group (PLX). When in the presence of BAF, much more puncta were noticed again in vemurafenib-treated cells ($p<0.05$, Figure 1B; Supplementary Figure S1B). These results suggest that vemurafenib treatment increases autophagic flux in BRAF mutant UM cells.

Furthermore, we used transition electron microscopy (TEM) to examine the formation of autophagic vacuoles (AVs) in vemurafenib-treated OCM1A cells. As shown in Figure 1C, we found that more vacuoles with double layer composite membrane were detected in the BRAF-inhibited group, whereas only a few nanoparticles were noticed in the control cells. We also found the number of AVs per cell, which represents the autophagic index, was much higher in vemurafenib-treated cells (Supplemental Figure S1B). Taken all the above experiments together, we demonstrated that autophagic flux was induced by BRAF inhibitor in BRAF mutant UM cells.

BRAF inhibitor-induced autophagy was via targeting the BRAF. To ensure that autophagy was induced by BRAF inhibition rather than the off-target effect of vemurafenib, we used siRNA against BRAF (siBRAF) in OCM1A cells. The

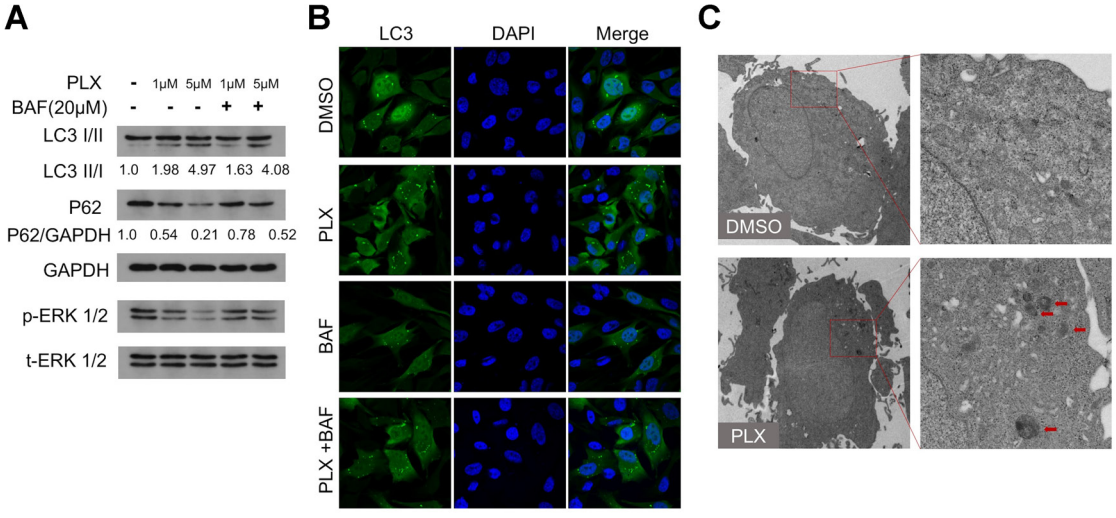

Figure 1. Vemurafenib treatment increased autophagosome formation in BRAF mutant UM cells. A) OCM1A cells were treated with DMSO, 1 μM PLX (vemurafenib), 5 μM PLX, and with or without a combination of 20 μM BAF (bafilomycin A1) for 24 h. Total protein lysates were harvested and then incubated with the indicated primary antibodies. Abbreviations: p-phospho; t-total B) Representative confocal microscopic pictures of OCM1A-GFP-LC3 cells incubated with DMSO or PLX (vemurafenib) for 24 h, with/without the combination of BAF. Notes: Green: FITC-labeled LC3; Blue: DAPI-labeled nucleus C) Representative pictures of OCM1A cells treated with BRAF inhibitor (PLX) or DMSO for 24 h under the transmission electron microscopy. Typical vacuoles of autophagosome were detected in BRAF inhibitor-treated cells but were rarely seen in the control group (indicated by arrows)

extent of autophagy induction, as evidenced by elevated LC3 II/LC3 I rate and dropped P62, was significantly decreased in siBRAF-treated cells as compared to control cells (siRNA-NC) (Figure 2A; Supplementary Figure S2A).

Then we confirmed these results in the OCM1A-GFP-LC3 cells. In the control group (siRNA-NC), more GFP-positive puncta were observed when cells were treated with vemurafenib (Figure 2B). While in the siRNA-BRAF group, the number of GFP-positive puncta decreased, although still more than that in the DMSO group (Supplementary Figure S2B). This phenomenon was finally verified by the TEM results. siRNA-BRAF weakened the induction effect of vemurafenib in terms of the autophagic index (Figure 2C; Supplementary Figure S2C). Therefore, we suggest that

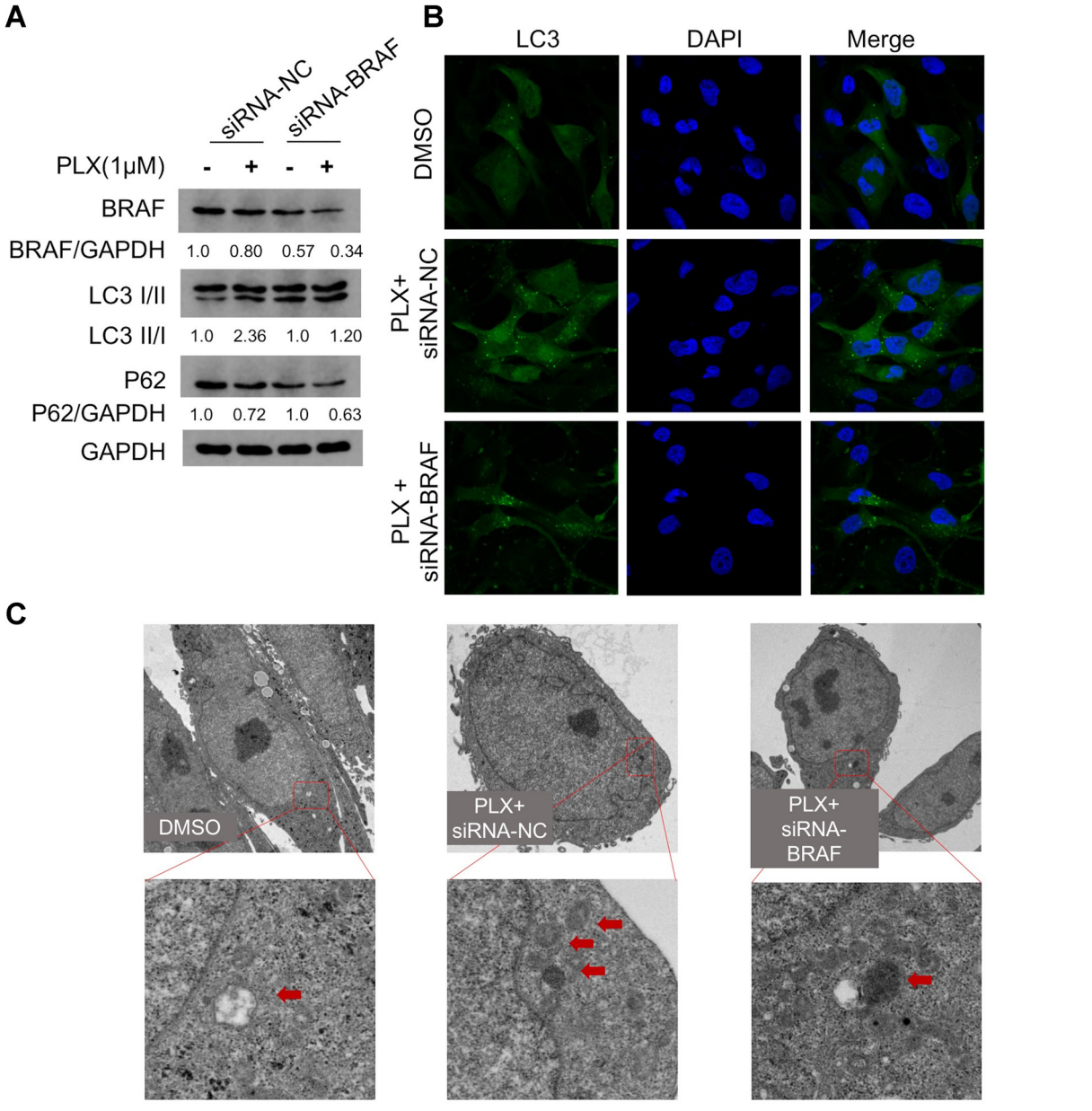

Figure 2. BRAF inhibitor-induced autophagy was via targeting the BRAF. A) Immunoblotting against autophagy markers in OCM1A cells under combined treatment of PLX (vemurafenib) with siRNA-BRAF or siRNA-NC (negative control). Protein gel blot was analyzed by using the primary antibodies as indicated. We calculated the relative quantity of BRAF/GAPDH and LC3 II/LC3 I and normalized them to control (DMSO-siRNA-NC or DMSO-siRNA-BRAF). B) Representative confocal microscopic pictures of OCM1A-GFP-LC3 cells incubating siRNA-BRAF or siRNA-NC for 48 hours and treating with DMSO or PLX (vemurafenib) for additional 24 h. Notes: Green: FITC-labeled LC3; Blue: DAPI-labeled nucleus. C) Representative pictures of OCM1A cells treated with siRNA against BRAF (siBRAF), or the control (siRNA-NC), in the presence of BRAF inhibitor (PLX) for 24 h and examined under the transmission electron microscope. Typical vacuoles of autophagosomes are indicated by arrows.

BRAF inhibitor-induced autophagy is via the inhibition of BRAF rather than the off-target effect of vemurafenib.

BRAF inhibitor-induced autophagy was independent of the MAPK signaling pathway.

BRAF is one of the key components of the MAPK signaling pathway, which could also adjust the level of autophagy [28]. We explored the possibility that BRAF inhibitor-induced autophagy was due to suppression of the MAPK pathway by comparing BRAF inhibition with a specific inhibitor of downstream of BRAF (MEK). Firstly, we found that phosphorylation of ERK was suppressed by either vemurafenib or MEK inhibitor (U0126) after 24 h treatment (Figure 3A). Furthermore, in the U0126 treated cells, we found the downregulation in LC3 II and upregulation in p62, both suggesting that the autophagy was inhibited. In contrast, in vemurafenib-treated cells, autophagy was activated instead. Collectively, these data suggest that inhibition of the MAPK pathway will suppress autophagy. Thus, the MAPK pathway is not responsible for vemurafenib-induced autophagy in UM cells and another pathway must be involved.

ER stress response mediates the autophagy in BRAF mutant UM cells. To explore an alternative mechanism that promotes autophagy during BRAF inhibition in UM cells, we further focused on ER stress, since it is another possible regulator of autophagy. ER stress occurs when proteins are not properly folded and the misfolded proteins accumulate in the ER [29, 30]. PERK arm is one of the most well-studied connections between ER stress and autophagy. Once the PERK arm is activated, it can phosphorylate eukaryotic initiation factor 2-alpha (eIF2α). As a result, the transcription factors including ATF4 (activating transcription factor 4) and CHOP (C/EBP homologous protein) [31] will be upregulated at the same time. Here in our study, upregulation of eIF2α, ATF4, and CHOP was observed under treatment with vemurafenib. With a higher dose of vemurafenib, we noticed that the ER stress response was obviously activated in terms of the raised levels of phosphorylated eIF2α, ATF4, and CHOP (Figure 3B).

Under irreversible ER stress responses, the mTOR-AMPK-ULK1 pathway may be involved to promote autophagic cell death [32, 33]. Whether vemurafenib treatment moderated the mTOR/AMPK/ULK1 pathway was examined by western blot. As shown in Figure 3C, in UM cells, vemurafenib treatment decreased the levels of p-mTOR, p-AKT, and p-P70S6K, and meanwhile enhanced the levels of p-AMPK and p-ULK1 in a dose-dependent manner. These findings suggest that vemurafenib suppresses the mTOR pathway activation and triggers the AMPK/ULK1 pathway in UM cells.

Then we used a specific PERK inhibitor (GSK2606414) to explore whether the PERK arm-dependent ER stress response is necessary for BRAF inhibitor-induced autophagy in UM. As shown in Figure 4A, the PERK inhibitor alone inactivated the PERK arm and downregulated the autophagy level as well. This was evidenced by decreased ATF4, CHOP, and the value of LC3 II/LC3 I. Furthermore, as compared to vemurafenib solo treated group, the combination of PERK inhibitor with vemurafenib reduced autophagy (evidenced by the decreased percentage of LC3 II/LC3 I and increase of p62), indicating that vemurafenib-induced autophagy was partially reversed when ER stress response was inhibited. (Figure 4A).

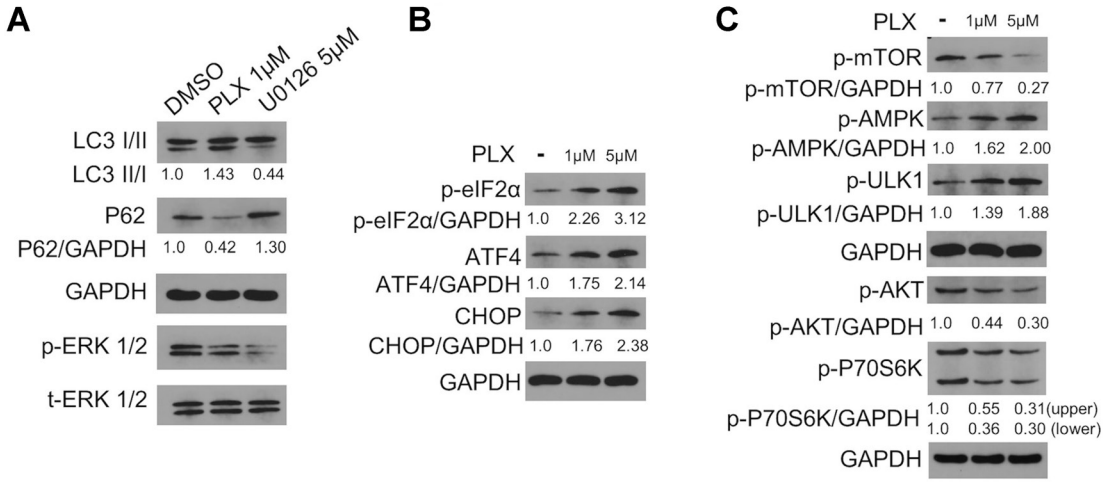

Figure 3. BRAF inhibitor-induced autophagy was independent of the MAPK signaling pathway and possibly through the ER stress response. A) OCM1A cells were exposed to 1 μM BRAF inhibitor (PLX) and 5 μM MEK inhibitor (U0126) for 24 h. Protein gel blot was then analyzed by using the primary antibodies as indicated. We calculated the quantity of each band and normalized it to control (DMSO). Abbreviations: p-phospho; t-total B) OCM1A cells were exposed to BRAF inhibitor (PLX) at the indicated doses for 24 h. Protein gel blot was analyzed by using the primary antibodies targeting ER stress response. We calculated the quantity of each band and normalized it to control (DMSO). Abbreviations: p-phospho C) Cells were treated with BRAF inhibitor (PLX) at the indicated doses for 24 h. The protein gel blot was analyzed by using the primary antibodies. We analyzed the protein gel blot targeting the mTOR/AMPK/ULK1 pathway and calculated the quantity of each band and normalized it to control (DMSO). Abbreviations: p-phospho

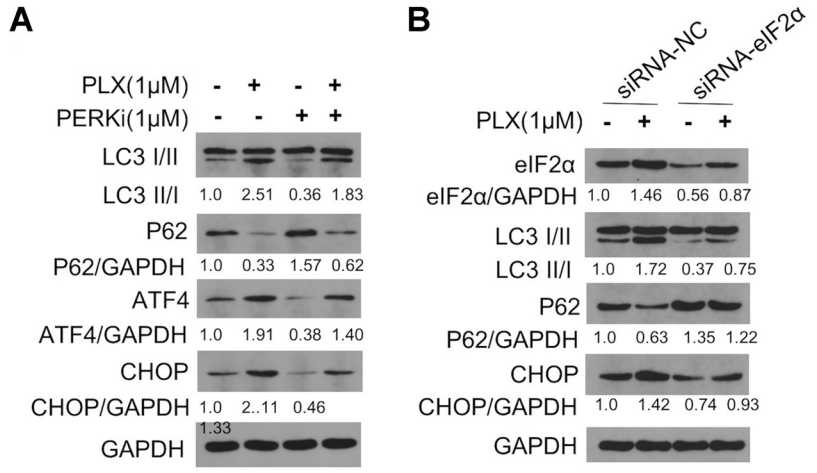

Figure 4. Targeting ER stress response partially reverses BRAF inhibitor-induced autophagy. A) OCM1A cells were exposed to 1 μM BRAF inhibitor (PLX) and/or 1 μM PERK inhibitor (PERKi) for 24 h. Total protein lysates were then subjected to analysis by the primary antibodies as indicated. The intensity of each band was calculated and normalized. B) Immunoblotting against autophagy markers in OCM1A cells under combined treatment of BRAF inhibitor (PLX) with siRNA- eIF2α or siRNA-NC (negative control). Protein gel blot was analyzed by using the indicated primary antibodies. We calculated the quantity of each band and normalized it to control.

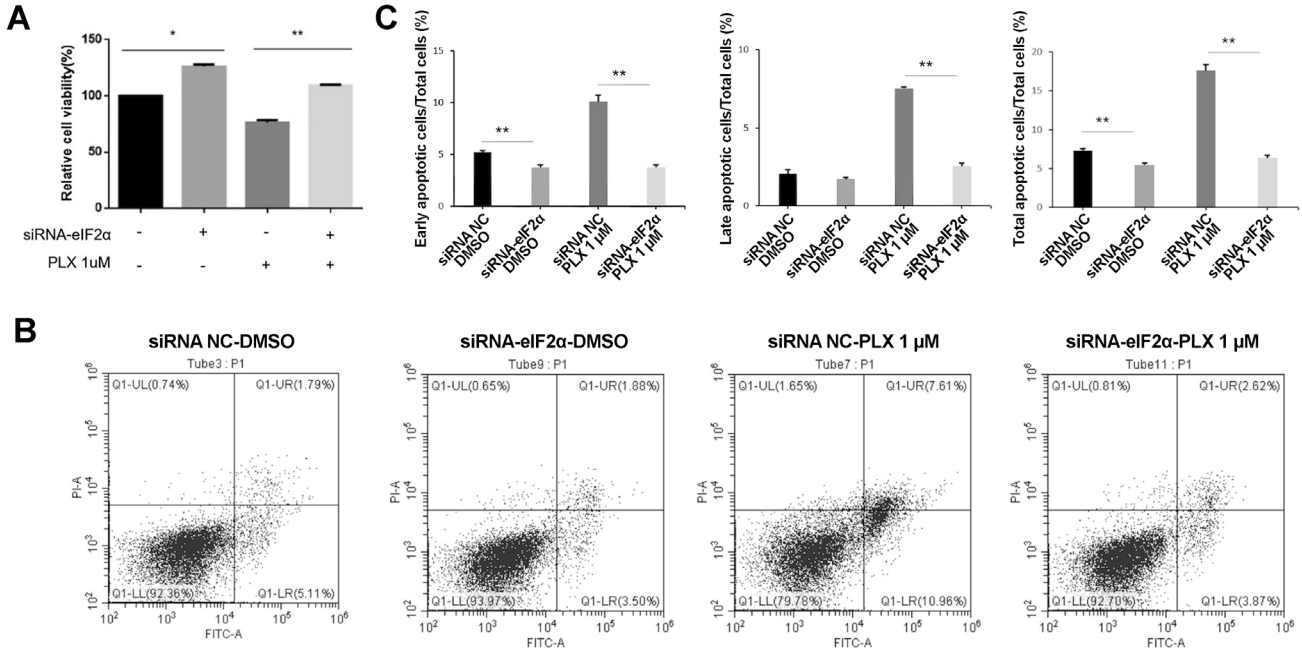

Figure 5. Inhibition of ER stress response partially rescues cell death in UM. A) OCM1A cells were exposed to BRAF inhibitor (PLX) in combination with siRNA targeting eIF2α or non-target siRNA for 24 h. MTT assays were then applied to analyze the cell proliferation. *p<0.05, **p<0.01 B) OCM1A cells were treated with BRAF inhibitor (PLX) alone or in a combination of combination with siRNA targeting eIF2α or non-target siRNA for 24 h and stained with annexin V-FITC (FITC) and propidium iodide (PI) followed by flow cytometry. Representative pictures of flow cytometry results in OCM1A cells exposed to DMSO, 1 μM BRAF inhibitor (PLX), and a combination of siRNA targeting eIF2α or non-target siRNA, respectively. C) We grouped cells stained with both PI and annexin V-FITC as later stage apoptosis, and cells stained with annexin V-FITC as early-stage apoptosis. The apoptosis results are presented by mean ± SEM. *p<0.05, **p<0.01

To suppress the PERK arm more specifically, we genetically inhibited the PERK arm by small interfering RNA against eIF2α, one of the essential components for ER stress regulation. Once eIF2α siRNA suppressed eIF2α expression (Figure 4B), ER stress was inhibited as evidenced by decreased level of CHOP. Autophagy was also inhibited, as shown by the reduced value of LC3 II/LC3 I and enhanced expression of P62. Furthermore, siRNA targeting eIF2α significantly decreased the extent of vemurafenib-induced autophagy in OCM1A cells compared to vemurafenib solo treated cells, as

shown by reduced LC3 II/LC3 I and accumulated p62. Therefore, we conclude that BRAF inhibitor-induced autophagy is possibly through the enhanced PERK arm-dependent ER stress in UM cells.

Inhibition of ER stress response partially rescues cell death in BRAF mutant UM cells. In our previous published paper, we demonstrated that an autophagy inhibitor (hydroxychloroquine) could significantly increase the cell viability and impair the treatment efficacy of vemurafenib in UM cells [24]. Here we further examined the cell growth impact of inhibiting ER stress response by using siRNA targeting eIF2α. As shown in Figure 5A, cell viability was increased significantly in OCM1A cells treated with the BRAF inhibitor (vemurafenib) and siRNA targeting eIF2α, compared to the BRAF inhibitor only treated group. This suggests that ER stress response inhibition partially rescues cell death induced by vemurafenib in BRAF V600E mutant UM cells.

To explore whether the cell growth impact of the combination (vemurafenib plus siRNA targeting eIF2α) is via cell apoptosis, the PE Annexin V Apoptosis Detection Kit was used to compare the ratio of apoptotic cells in different groups. As shown in Figures 5B and 5C, cells treated with BRAF inhibitor and siRNA targeting eIF2α had a much lower rate of apoptosis, including early and late apoptosis, than the vemurafenib alone treated group, which indicates that the rescue of cell death induced by inhibition of ER stress response is possibly via decreased cell apoptosis.

Discussion

In recent years, the role of autophagy in the development of cancer has been clearer. On the one hand, autophagy was thought to prevent tumorigenesis. On the other hand, once the cancer was formed, increased autophagic flux was documented to promote the growth and survival of tumor cells [31]. However, studies on the possible role of autophagy in UM are limited and controversial. Giatromanolaki et al. found the autophagy-related protein BECN1 was commonly unregulated in UM tissues and was correlated with earlier tumor metastasis and poorer prognosis [34]. High expression of BNIP3, a BH3-containing protein of the BCL-2 family regulating autophagy, was reported to be associated with more pigment, deeper scleral invasion, and a lower overall survival rate in UM patients [35]. In our previous study, we demonstrated the BRAF V600E mutation-dependent role of autophagy in UM cells [24]. Starvation can trigger protective autophagy in BRAF mutant UM cells. We also found that LC3, one of the autophagy markers was upregulated during BRAF inhibition. The present study demonstrated that the BRAF inhibitor (vemurafenib) increased the autophagic flux in a dose-dependent manner. This phenomenon was further verified by a higher level of GFP-LC3 puncta and the autophagic index that was calculated based on the number of autophagic vacuoles under transmission electron microscopy.

Mammalian target of rapamycin complex 1 (mTORC1) and AMP-activated Protein Kinase (AMPK) are two main signaling pathways that can regulate autophagy [36]. The activation of the MAPK pathway is another possible factor to induce autophagy in some cancer cells [37]. However, our current study found that BRAF inhibition-induced autophagy was independent of the MAPK signaling pathway, which is consistent with another report on cutaneous melanoma [22].

ER stress may be the alternative mechanism for how BRAF inhibition promotes autophagy. Upon ER stress, a complementary adaptive set of mechanisms termed unfolding protein response (UPR) will be activated to deal with various protein-folding alterations. The primary goal of the UPR is to protect cells from the accumulation of unfolded/misfolded proteins. Once the stress is over its capacity, then a cell death response like autophagy will occur. In BRAF mutant cutaneous melanoma, it has been reported that oncogenic BRAF induces ER stress response resulting in an increased basal level of autophagy [38]. BRAF inhibitor appears to promote ER stress response that could subsequently activate cytoprotective autophagy [22]. However, whether ER stress is also involved during BRAF inhibition in UM cells remains unclear. Here we found that the PERK-dependent ER stress was activated when there was clear evidence of BRAF inhibitor-induced autophagy. This vemurafenib-induced autophagy can be partially reversed by pharmacological and genetic inhibition of ER stress response. Therefore, we infer that the ER stress response is an important inducer of autophagy when BRAF is inhibited in UM cells. Mechanistically, mutant BRAF is identified to bind to GRP78, the gatekeeper controlling the ER stress response [22, 32]. BRAF inhibitor is able to promote further binding of mutant BRAF and GRP78, which indicates a possible mechanism as to how vemurafenib activates ER stress during BRAF inhibition [22].

ER stress-induced autophagy usually has dual roles. It usually acts as a potential pro-survival mechanism contributing to therapy resistance. Ma et al. found that inhibition of ER stress-induced autophagy could overcome BRAF inhibitor resistance in cutaneous melanoma [22]. Tomasz et al. reported that miR-410-3p, which was induced by vemurafenib in melanoma cells via ER stress, contributes to the resistance to BRAF inhibitor [39]. In thyroid cancer, HMGB1-mediated autophagy may account for vemurafenib resistance [40]. Our team also demonstrated that targeting autophagy could sensitize BRAF-mutant thyroid cancer to vemurafenib [25]. On the other hand, potent and prolonged ER stress may lead to apoptosis. Upregulation of ER stress sensors could trigger CHOP accumulation, which subsequently mediates multiple autophagic genes and eventually result in cell death in cutaneous melanoma [41]. Here we demonstrated that inhibition of ER stress either by PERK inhibitor or genetically targeting eIF2α rescued some of the cell death and decreased the rate of apoptosis, indicating the anti-tumor effect of BRAF inhibitor was impaired in

BRAF mutant UM cells. This is in line with what we found in our previous study. When we inhibited the autophagy by hydroxychloroquine, the treatment efficacy of vemurafenib in UM cells was weakened [24]. Other than apoptosis, a recent study on pancreatic cancer provides a new term called autophagy-dependent ferroptotic death [42]. They reported that zalcitabine, an antiviral drug for human immunodeficiency virus infection, can suppress the growth of pancreatic cancer cells through the induction of ferroptosis, an iron-dependent form of regulated cell death. Whether autophagy-dependent ferroptotic death also occurred during BRAF inhibition in UM cells needs to be further investigated.

In conclusion, we provide initial evidence that BRAF inhibition promotes ER stress response-mediated autophagy in UM cells. Inhibition of ER stress response can partially revert autophagy and rescue cell death, which might impair the anti-tumor effect of BRAF inhibitor in UM.

Supplementary information is available in the online version of the paper.

Acknowledgments: This study was supported by the Natural Science Foundation of Zhejiang Province under Grant LQ18H120002. We want to thank Dr. Gary K. Schwartz from Columbia University Medical Center to provide us with OCM1A cells.

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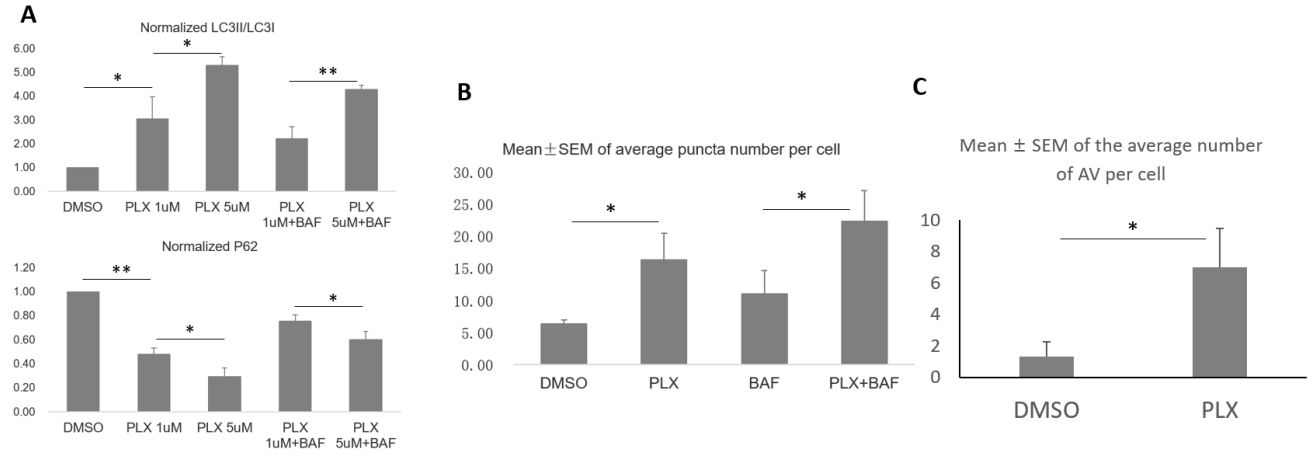
https://doi.org/10.4149/neo_2022_220428N462

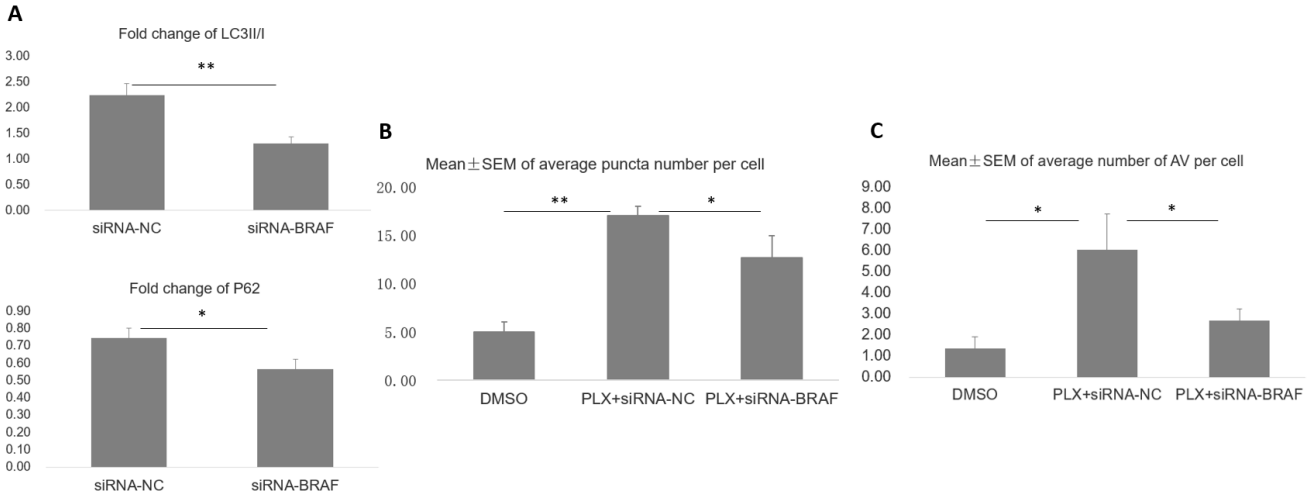
BRAF inhibition promotes ER stress-mediated cell death in uveal melanoma

Yinu ZHAO[1], Yue WANG[2], Li ZHANG[1], Weibin WANG[2], Thomas J. FAHEY 3rd[3], Ke YAO[1,*]

Supplementary Information



Supplementary Figure S1. Vemurafenib treatment increased autophagosome formation in BRAF mutant uveal melanoma cells. A) We calculated the quantity of each band from Figure 1A and presented the ratios of LC3II/LC3I and P62 in the histogram. *p<0.05, **p<0.01 Notes: Error bars=SEM from three independent experiments B) The average puncta number in each group from Figure 1B was presented in the histogram. *p<0.05 Notes: Error bars=SEM from three independent experiments C) Autophagic index from Figure 1C was calculated and compared between control (DMSO) and vemurafenib (PLX) treated groups. *p<0.05



Supplementary Figure S2. BRAF inhibitor-induced autophagy was via targeting the BRAF. A) The fold change of LC3II/LC3I and P62 appeared in Figure 2A were further presented in the histogram. *p<0.05, **p<0.01 Notes: Error bars=SEM from three independent experiments B) The average puncta number in each group from Figure 2B was presented in the histogram. *p<0.05, **p<0.01, Notes: Error bars=SEM from three independent experiments C) Autophagic index from Figure 2C was calculated and compared among different groups that treated with siRNA against BRAF (siBRAF), or the control (siRNA-NC), in the presence of BRAF inhibitor (PLX). *p<0.05