The Polysaccharide Extracted from Umbilicaria esculenta Inhibits Proliferation of Melanoma Cells through ROS-Activated Mitochondrial Apoptosis Pathway

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Melanoma is one of the most aggressive skin cancers with an increasing rate of morbidity. Umbilicaria esculenta is an edible lichen and its main component of extracts—polysaccharide (PUE) has shown significant antitumor effects in a variety of cancer types such as stomach adenocarcinoma. However, whether it has an anti-melanoma effect and the underlying mechanism has not been revealed. In this article, we showed that PUE extracted from Umbilicaria esculenta could inhibit the growth of A875 and A375 melanoma cells but without obvious toxicity to normal vascular endothelial cells. The generation of reactive oxygen species (ROS) in A875 cells was significantly elevated when treated with PUE for 24h. In addition, the expression of caspase-3 and -9 also increased as compared to the controlled group which resulted in the apoptosis of A875 melanoma cells. In the meantime, when pre-treated with N-acetylcysteine (NAC), the ROS scavenger, PUE induced apoptosis and cell death could be reversed via suppression of elevated generation of ROS and ROS-mediated caspase-9 expression. In summary, our study demonstrated that PUE extracts from Umbilicaria esculenta have a potent anti-melanoma effect through the induction of ROS and caspases-3 and -9. It could provide a promising strategy of melanoma therapy with the components from the extracts of natural and edible plants such as lichen Umbilicaria esculenta.

Key words Umbilicaria esculenta; extract; melanoma; reactive oxygen species; caspase

Melanoma is a malignancy notorious for its tendency of rapid metastasis and high mortality. It has been reported that over 76000 newly diagnosed cases of melanoma and nearly 9710 melanoma-related deaths were estimated in the United States in 2014.1 The incidence worldwide of melanoma is 15-25 per 100000 individuals. Although the proportion of melanoma in skin tumors is less than 5%, melanoma accounts for up to 75% of the deaths caused by all skin cancers.2 Chemo-therapeutics such as Dacarbazine (DTIC) and immunotherapy (high-dose interleukin-2 and interferon-α) have been approved by the Food and Drug Administration (FDA) for melanoma treatment. Unfortunately, low response rates (ca. 15%), severe adverse effects as well as no improvement on overall survival (OS) limited their clinical application.3,4 The emergence of targeted therapy such as BRAF and mitogen-activated protein extracellular kinase (MEK) inhibitors, and immune checkpoint blockade such as anti-CTLA-4 and anti-PD-1 monoclonal antibodies marked great breakthrough in the management of patients with advanced melanoma.5,6 However, it is also not satisfied that, although these small targeted molecules have been proved to improve patients’ QOL to some extent, adverse events such as pyrexia, photosensitivity and secondary cutaneous squamous-cell carcinomas and drug resistance which developed in about half of patients within several months treatment limited their effects.7-9

Due to the characteristics of good efficacy, safety, and less toxicity to normal body tissues and organs, the natural medicine have attracted growing attention. It has been demonstrated that extracts of Umbilicaria esculenta, a kind of Chinese lichen and usually found on mountain rocks at high altitude in East Asia, have numerous functions, including antithrombotic activity, antioxidant activity, glucosidase inhibition, melanogenesis inhibition, inhibitory effect on the replication of human immunodeficiency virus (HIV), antidementia acetycholinesterase inhibition and so on.10-17 Additionally, the water extracts of Umbilicaria esculenta was reported to possess strong anticancer effects against human stomach adenocarcinoma cells SNU-1 through inhibiting telomerase activity.18 Besides, the polysaccharide (PUE), which is one of the main components extracted from Umbilicaria esculenta, has been proved to have the ability to activate antitumor immunity via stimulating immune cells.19 However, whether the PUE has an anti-tumor effect on melanoma and its underlying mechanism has not been revealed.

The goal of this study is to investigate the anti-melanoma effect of PUE and reveal the mechanism of its tumor inhibitory activity. We tested the viability of A875 melanoma cells after treated with different concentrations of PUE, and evaluated the expression levels of reactive oxygen species (ROS) and caspase family of A875 cells. The results indicated that PUE showed a strong anti-melanoma effect by inducing the generation of ROS, which further upregulated expression of caspase-9 and -3 and finally led to the apoptosis of melanoma cells. According to its anti-proliferative effect on melanoma and the characteristic of natural and edible, lichen Umbilicaria esculenta may be used potentially as a safe and effective anti-melanoma clinical agent.

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MATERIALS AND METHODS

Collection of Umbilicaria esculenta Umbilicaria esculenta was collected from Mt. Huang (30 N, 1864 m altitude in Anhui Province, China). They were identified according to its morphological characteristics by Professor Keli Chen, who was working in Hubei University of Chinese Medicine. And all the specimens were deposited in Tongji Medical College.

Isolation of Polysaccharide from Umbilicaria esculenta (PUE) Dried Umbilicaria esculenta was finely ground into powder and made it defatted with dichloromethane for three times. The residue was collected after centrifugation, and then extracted twice with deionized water at 100°C for 3h. Three volumes of absolute ethyl alcohol were added to the aqueous extract to form a precipitate. The precipitate was concentrated to small crude polysaccharide under reduced pressure. The crude polysaccharide was collected by centrifugation and washed with absolute ethyl alcohol for twice time. At last, suspend the precipitate in water and then lyophilize it.

Cell Counting Kit-8 (CCK-8) Viability Assay A875 and A375 melanoma cell and human umbilical vein endothelial cell (HUVEC) survival was determined in vitro by a CCK-8 detection kit (Dojindo Molecular Technologies Inc., Kumamoto, Japan). Melanoma cells or HUVECs were inoculated in 96-well plate for 24h, treated with PUE in different concentrations respectively, and further incubated for 24h. Then 10µL CCK-8 reagent was added into each well, and incubated for 1.5h at 37°C. The absorbance at 450 nm was measured using an enzyme-linked immunosorbent assay (ELISA) plate reader (Infinite F50, Tecxan Austria, Austria).

Detection of Apoptosis by Annexin-V/Propidium Iodide (PI) Staining and Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nick-End Labeling (TUNEL) Assay A875 melanoma cells were treated with PUE in different concentrations for 12h and apoptosis was then evaluated using Annexin-V/PI staining kit (KeyGEN BioTECH, China). Four hundred and eighty-eight nanometre excitation and 530nm emissions for fluorescein isothiocyanate (FITC) and 600nm for PI fluorescence by FACS Canto II flow cytometer (BD Biosciences, U.S.A.). Meanwhile, apoptosis of A875 cells were further ascertained by TUNEL assay using an in situ cell apoptosis detection kit conjugated with Cy3 (Boster, China) followed by counterstaining with 4’6-diamidino-2-phenylindole (DAPI) according to the manufacturer’s instructions.

Measurement of Intracellular ROS The generation of intracytoplasmic ROS with or without pretreatment of PUE was determined with 2’,7’-dichlorofluorescein diacetate. A875 cells or HUVECs were diluted to 3.0×10^5 per mL, seeded into six-well plate with 2mL in each well. After incubation with various concentrations of PUE for 24h or pre-incubated with N-acetylcysteine (NAC), thienoyl trifluoracetone (TTFA, a specific inhibitor of mitochondrial electron transport complex II) or diphenyle iodonum (DPI, inhibitor of nicotinamide adenine dinucleotide (NAD)(P)H oxidase) for 2h, the cells were collected and washed with phosphate buffered saline (PBS) for three times. Then the cells were suspended in PBS to which 10µm DCFH-DA was added and incubated in dark for 30min at 37°C. After washing with ice cold PBS, cells were centrifuged and re-suspended in PBS. Data were collected using a FACS Canto II flow cytometer (BD Biosciences) and analyzed by FlowJo software (Tree Star). All the reagents mentioned above were obtained from Sigma (U.S.A.).

Caspase Activity Assay The intracytoplasmic levels of caspase family were detected by Fluorometric Assay Kit (Biovision, Milpitas, CA, U.S.A.). To evaluate the activity of caspase-3, -8 and -9, the cell lysates were harvested after the A875 cells had been treated with various concentrations of PUE. Each well of the 96-well plates was added 10µL protein of cell lysate, 10µL caspase-3 substrate (2mmol/L Ac-DEVDP-pNA) or caspase-9 substrate (2mmol/L Ac-LEHD-pNA) or caspase-8 substrate (2mmol/L Ac-IETD-pNA), and 80µL reaction buffer. They were incubated for 4h at 37°C. The samples were measured with an enzyme-linked immunosorbent assay (ELISA) reader at an absorbance of 450nm.

Cell Culture A875 and A375 melanoma cells and HUVECs were cultivated in plastic flasks in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, U.S.A.) containing 10% heat-inactivated fetal bovine serum (FBS, Gibco), penicillin (100IU/mL), and streptomycin (100mg/mL), and maintained at 37°C with 5% CO₂. Cultures were provided with fresh medium three times every week.

Statistical Analysis All experiments were repeated independently at least three times. The analysis of all data was performed using SPSS18.0 software (SPSS, Chicago, IL, U.S.A.) and the results were expressed as means±standard error of the mean (S.E.M). The differences among groups were estimated by Student’s t-test. p<0.05 was regarded as statistically significant.

RESULTS

PUE Induced the Apoptosis of A875 Melanoma Cells in Vitro To investigate whether PUE has an anti-melanoma effect, we treated A375 and A875 melanoma cells with different concentrations of PUE varying from 0.8 to 4mg/mL for 24h and its inhibitory effects on melanoma cells were evaluated by CCK8 cell proliferation assay. As shown in Fig. 1A, under different PUE concentrations ranging from 0.8 to 4mg/mL, the survival rates of A875 melanoma cells were 64.9, 42.8, 25.7 and 16.4% as compared to phosphate buffered saline (PBS) control, respectively. In addition, the frequency of apoptotic A875 cells in different concentrations of PUE were also measured. But the cell viabilities of HUVECs under different concentrations of PUE were also measured. The cell viabilities of HUVECs between PUE and PBS treated groups showed no statistical difference, suggesting that PUE is non-toxic to normal vascular endothelial cells.

We used Annexin-V/PI flow cytometric analysis to determine the apoptosis and necrosis of A875 cells. As shown in Fig. 1B, the percentage of Annexin-V+ A875 cells increased gradually when the concentration of PUE elevated from 0.8 to 4mg/mL. In addition, the frequency of apoptotic A875 cells in PUE treated groups was much higher than that of PBS control, which indicated that PUE could induce the apoptosis of A875 melanoma cells in a concentration-dependent manner. To further ascertain these effects, DNA fragmentation by TUNEL assay was analyzed followed by DAPI staining. As shown in Fig. 1C, the percentage of TUNEL positive A875 cells was significantly increased after PUE treatment, which was also in accordance with the flow cytometric analysis.
PUE Increased the Generation of ROS in A875 Melanoma Cells

To further elucidate the underlying mechanisms of the inhibitory effect of PUE on melanoma cells, we analyzed the intracellular levels of ROS in A875 and HUVEC cells after PUE incubation for 24h with DCFH-DA staining. Flow cytometry analysis showed the percentages of DCFH-DA

Fig. 1. PUE Could Induce the Apoptosis of Melanoma Cells

(A) Cell viabilities of A875 melanoma cells, A375 melanoma cells and HUVECs after incubation with different concentrations of PUE for 24h. (B) A875 melanoma cells were treated with PUE for 12h and apoptosis was quantified by Annexin V-tagged FITC-PI flowcytometry. (C) Apoptosis of PUE-treated A875 cells was assessed by TUNEL assay. Cy3-stained nuclei of A875 cells appeared red, which represented apoptotic cells; DAPI-stained nuclei appeared blue. Results represent at least three independent experiments. Error bars indicate mean±S.E.M. *p<0.05; **p<0.01; NS, None significance.
positive A875 cells substantially increased after PUE treatment in a concentration-dependent manner (Fig. 2A), and the mean intensity also elevated when the concentration of PUE gradually increased from 0.8 to 4 mg/mL (Fig. 2B). Although PUE could increase the production of ROS in A875 melanoma cells remarkably, there is no significant difference in the percentages of DCFH-DA positive HUVECs between PBS control and PUE treatment (Fig. 2C), which suggested that PUE could not promote the generation of ROS in HUVECs. That is, PUE could induce ROS-dependent apoptosis in melanoma cells rather than in normal HUVECs. These results, from the other perspective, revealed the excellent safety profiles of the natural and edible lichen *Umbilicaria esculenta*.

**NAC Pre-treatment Could Decrease the PUE-Induced Elevated Intracellular ROS Levels in A875 Melanoma Cells** However, when pre-treated with NAC, the scavenger of ROS, the inhibitory effects of PUE on the survival of A875 melanoma cells could be remarkably reversed. As shown in Figs. 3A and B, there were significantly statistical difference in the cell viabilities of A875 melanoma cells between the groups pre-treated with or without NAC under different concentrations of 0.8, 1.6 and 3.2 mg/mL of PUE, respectively (*p* < 0.05). Notably, no statistical significance could be observed between the groups pre-treated with or without NAC with PUE concentration of 4 mg/mL, which probably because under such a PUE concentration most A875 melanoma cells have proceeded to necrosis even though NAC pre-treatment could decrease the PUE-induced elevated intracellular ROS levels (Fig. 3C). To further investigate the main source of ROS, A875 cells were first pretreated with NAC, TTFA and DPI for 2 h, respectively. Then these cells were incubated with 3.2 mg/mL PUE for 24 h. The production levels of cytosolic

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**Fig. 2.** The Production Levels of Intracellular ROS in A875 Cells and HUVECs Induced by PUE and Stained with DCFH-DA

(A) Flow cytometry analysis of the intracellular ROS levels of different groups incubated with different concentrations of PUE for 24 h. (B) The fluorescence intensity of intracellular DCFH-DA of different groups. (C) Flow cytometry analysis of the intracellular ROS levels of HUVECs incubated different concentrations of PUE for 24 h. Results represent at least three independent experiments. Error bars indicate mean±S.E.M. *p* <0.05; **p** <0.01; NS, None significance.
ROS in both NAC (p<0.05) and TTFA (p<0.05) pretreated groups were inhibited significantly, whereas no inhibitory effect of ROS generation was observed in cells pretreated with DPI (Figs. 3D–F). Since TTFA is a specific inhibitor of mitochondrial electron transport complex II whereas DPI is an NAD(P)H oxidase inhibitor, these results suggested that the...
mitochondria were the major source of ROS generation and played a vital role in polysaccharide-induced apoptosis.

**PUE Induces the Generation of Caspase-9 and -3 Partly Dependent on Increased ROS** Caspase family plays a significant role in apoptosis signaling pathway, among which caspase-8 is a key initiator of the death-receptor pathway while mitochondrial apoptosis pathway requires the activation of caspase-9, and the level of caspase-3 activation converges both of the two pathways.\(^{20}\) To explore which pathway and its activation, indeed, contribute to the PUE-induced apoptosis, we analyzed the intracytoplasmic levels of caspase-8, -9 and -3 in treated A875 cells using fluorometric assay kit. The results showed that PUE treatment significantly increased the levels of caspase-3 and -9 in A875 melanoma cells (Figs. 4A, B). But no significant change of caspase-8 was observed after PUE treatment (Fig. 4C). Accordingly, PUE leads to cell apoptosis possibly through mitochondria mediated apoptosis pathway.

It is universally acknowledged that ROS simulates intrinsic mitochondrial apoptotic pathway, which further triggers caspase-dependent or independent signaling events.\(^{20}\) In order to determine whether ROS associates with caspase family in melanoma cell apoptosis, we pretreated A875 cells with NAC.

As shown in Fig. 4D, the elevated expression of caspase-9 could be remarkably reversed by NAC, which indicated that it is the increased ROS level induced an elevated production of caspase-9 and finally led to melanoma cell apoptosis.

**DISCUSSION**

*Umbilicaria esculenta*, an edible lichen chief found in East Asian countries, has been reported to possess strong antitumor effect in a variety of cancers including stomach adenocarcinoma.\(^{18}\) However, researches examining the anti-melanoma properties of *Umbilicaria esculenta* are limited. In this study, we investigated whether *Umbilicaria esculenta* extracted glucan (PUE) has an anti-tumor effect on melanoma. Our study demonstrated that PUE could induce the apoptosis of A875 and A375 cells, but it has no significantly inhibiting effect on HUVECs (Fig. 1), which suggested that PUE had little effect on the induction of ROS generation in HUVECs. This may be partly due to the differences of cellular redox state between melanoma cells and normal HUVECs. However, the mechanism of its apoptosis-inducing effect is still a puzzle, which drives us to conduct further study.
Aberrant regulation of proliferation and apoptosis is essential in tumorigenesis. As a key factor in regulating cellular homeostasis, ROS also plays a significant role in various pathological processes such as tumor development, apoptosis and metastasis. Previous research has shown that elevated intracellular ROS levels in a certain range could promote melanoma metastasis, whereas excessive ROS could induce tumor cell damage and apoptosis through a wide variety of mechanisms. In this study, we tested the level of ROS in A875 melanoma cells after treatment of PUE, and the result that the ROS level in A875 melanoma cells detected by flow cytometry significantly increased in a dose-dependent manner, which demonstrated that high level of ROS in treated melanoma cells could be induced by PUE. Furthermore, the ROS induced cell apoptosis could be reversed by the ROS scavenger NAC and mitochondrial electron transport complex II inhibitor TTFA rather than NAD(P)H oxidase inhibitor DPI, which illustrated the PUE-induced intracellular ROS was mainly derived from mitochondria and played a significant role in this process of apoptosis.

Studies performed over the past decades have confirmed that there are two major pathways inducing apoptosis, one that induces mitochondrial release of cytochrome c and another that begins with activation of cell surface death receptors. In death receptors pathway, Fas/FasL was considered as the most important death receptors, which was activated followed by caspase-8 activation. In mitochondrial pathway, cytochrome c release from mitochondria and bind to Apaf-1 (apoptosis protease activating factor-1) due to dATP/ATP hydrolysis, which activates caspase-9. Besides, caspase-8 and -9 finally activate caspase-3 on both terminal apoptotic pathways.

Melanoma, with a naturally high rate of genetic mutation, is one of the most immunogenic tumors. Though multiple small targeted molecules have certain therapeutic effects, drug resistance limits their efficacy not long after the start of treatment. Therefore, that whether Umbilicaria esculenta extracts could inhibit the proliferation of drug-tolerance melanoma via changing cell metabolism and regulating cell apoptosis is expected for further investigation.

CONCLUSION

In conclusion, we find that Umbilicaria esculenta extracts have a strong anti-proliferation effect on melanoma in vitro.
The cancer inhibitory activity could be induced by excessive production of ROS in melanoma cells, which leads to elevated expressions of caspase-9 and -3, finally activating the cell apoptosis and cell death. On the basis of these results, lichen Umbilicaria esculenta may be used as a potential natural and safe anti-melanoma agent.

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Conflict of Interest The authors declare no conflict of interest.

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