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Original Article

Anthocyanin is involved in the activation of pyroptosis in oral squamous cell carcinoma

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ABSTRACT

Background: The anti-carcinogenic effects of anthocyanin are well documented. Oral squamous cell carcinoma is one of the most common and lethal cancer types due to its high degree of malignancy and poor prognosis. The main purpose of the current study was to investigate the potential inhibitory effects of anthocyanin on oral squamous cell carcinoma and identify effective targets for therapy.

Methods: Cell viability was measured using cell counting kit-8 (CCK8). Cell migration and invasion abilities were determined using scratch-wound and Transwell invasion assays, respectively. mRNA and protein expression patterns of nucleotide-binding oligomerization domain-like receptor pyrin domain containing 3 (NLRP3), caspase-1 and IL-1β were detected using qRT-PCR, immunofluorescence and western blot. The gasdermin D (GSDMD) level was determined via confocal microscopy and western blot.

Results: Anthocyanin reduced the viability of oral squamous cell carcinoma cells and inhibited migration and invasion abilities. Simultaneously, activation of pyroptosis was associated with enhanced expression of NLRP3, caspase-1, and IL-1β. Upon administration of caspase-1 inhibitors, anthocyanin-activated pyroptosis was suppressed and cell viability, migration, and invasion rates concomitantly enhanced.

Conclusion: Anthocyanin promotes the death of oral squamous cell carcinoma cells through activation of pyroptosis and inhibits tumor progression.

Introduction

Oral squamous cell carcinoma (OSCC) is a malignancy particularly prone to metastasis (Petersen, 2009) and the eleventh most prevalent carcinoma worldwide according to the World Health Organization (WHO, 2005). Notably, developing countries have a higher incidence rate with an annual increasing trend (Wang et al., 2018; de Camargo Cancela et al., 2010). OSCC is a complex process involving multiple factors and stages, and therefore, traditional diagnostic methods, such as histopathology, are not historically effective for disease detection in the early stages (Fukuda et al., 2012; Ralhan, 2007). In terms of treatment, surgery remains the best choice, although no success has been reported for advanced metastatic tumors. Despite concerted attempts to treat advanced tumors with combinations of chemotherapy or radiotherapy and surgery, the 5-year survival rate remains low and recurrence rate is high (Ralhan, 2007). Clarification of the molecular mechanisms underlying OSCC and identification of potential novel therapeutic targets are critical for improvement of therapeutic options.

Abbreviations: OSCC, oral squamous cell carcinoma; NLRP3, nucleotide-binding oligomerization domain-like receptor pyrin domain containing 3; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate buffered saline; qRT-PCR, quantitative reverse transcription polymerase chain reaction

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Pyroptosis is a caspase-1-mediated programmed cell death program also known as cell inflammatory necrosis. Unlike other cell death processes, pyroptosis is characterized by swelling of the cell until the membrane dissolves, leading to release of the cell contents and pro-inflammatory cytokines and eventually cell death. Several studies to date have shown that caspase-1 participates in the pathological processes of diabetic cardiomyopathy, hepatitis and hepatic fibrosis (Li et al., 2014; Wree et al., 2014.). At the same time, activation of pyroptosis has positive significance in carcinoma treatment. Pyroptosis is reported to play an important role in several carcinoma subtypes, including breast cancer (Pizato et al., 2018), non-small cell lung cancer (Wang et al., 2018) and gastric cancer (Wang et al., 2018). However, the significance of pyroptosis activation in OSCC remains to be established.

Anthocyanin is a water-soluble natural pigment widely found in plants that belongs to the flavonoid family. Owing to their anti-inflammatory properties, anthocyanin compounds are commonly used to prevent cardiovascular disease, control obesity and reduce diabetic conditions (He and Giusti, 2010). In recent years, accumulating studies have disclosed anti-carcinogenic effects of anthocyanin, although its potential effects on OSCC are currently unknown. In view of the earlier finding by our group that anthocyanin can activate caspase-1, we have conducted a follow-up study to investigate the potential effects of pyroptosis activation by anthocyanin on OSCC and the underlying mechanisms.

Materials and methods

Cell culture

HaCaT cell line was purchased from Fuheng biology (Shanghai, China). Tca8113 and SCC15 cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). HaCaT, Tca8113 and SCC15 cells were maintained in high-glucose DMEM (GE Healthcare HyClone, Logan, UT, USA) supplemented with 10% FBS (BI, Kibbutz Beit Haemek, Israel) and 1% penicillin/streptomycin solution (Beyotime, Biotechnology, Jiangsu, China) at 37 °C in a humidified atmosphere containing 5% CO_2. Anthocyanin was purchased from Lingoberry Biotech co., Ltd. (Daxing'anling, China), the general structure and HPLC were provided in Fig. S1.

Analysis of cell viability by CCK8 assay

The viability of HaCaT, Tca8113 and SCC15 cells was determined by measuring cellular metabolism using 96-well plates. Then, 1×10^4 HaCaT, Tca8113 and SCC15 cells in 200 μl of 10% FBS medium per well were seeded into 96-well plates and incubated overnight (37 °C, 5% CO_2). The next day, medium was replaced by high-glucose DMEM containing different concentrations of anthocyanin (62.5 μg/ml, 125 μg/ml, 250 μg/ml, 500 μg/ml, 1000 μg/ml) or in combination with caspase-1 inhibitor AC-YVAD-CMK (100 μM, Fig. S2). After treatment for various time periods (24, 48, 72 h), the medium of every well was replaced by 100 μl medium containing 10 μl of CCK8 solution, then cells were incubated again for another 4 h at 37 °C, protected from light. Finally, the absorbance was measured at 450 nm in a microplate reader.

Scratch-wound assay

Cells were seeded into a 12-well culture plate until Mono-layer cells were formed, then scratched a straight line on Mono-layer cells with a sterile 20-μl pipette tip. Then, culture medium was removed and wells were washed thrice with the culture medium to remove the debris. Anthocyanin and caspase-1 inhibitor AC-YVAD-CMK were then administrated. The width of the scratch area was measured 48 h later to estimate the migration capacity.

Transwell invasion assay

After pretreatment with anthocyanin or caspase-1 inhibitor AC-YVAD-CMK for 48 h, cells were collected and seeded into Polycarbonate Membrane Transwell® Inserts (Corning Cat# 3422) at 2×10^4 cells per well in 200 μl of serum-free DMEM, and incubated for another 48 h at 37°, 5% CO_2. The bottom chambers were covered with 600 μl DMEM with 10% FBS. After incubation, the cells that didn't migrate on the upper surface of the filter were removed with cotton swabs. The chambers were fixed with 4% paraformaldehyde for 30 min (Biosharp, Anhui, China), stained with crystal violet for 15 min, then washed with PBS for three times. Cell numbers were counted under a light microscope.

Immunofluorescence and confocal staining

Cultured cells were first stained with DAPI for 30 min and then fixed with 4% paraformaldehyde at room temperature for 20 min, afterwards nonspecific binding was blocked by goat serum for 2 h, subsequently the cells were incubated with primary antibody (1:50) at 4 °C overnight. Next day, cells were washed with PBS for three times and incubated with secondary antibody for 1 h. The images were captured with a fluorescence microscope.

RNA isolation, reverse transcription and RT-PCR

Total RNA was extracted fromTca8113 and SCC15 cells after 48-hour-treatment, using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA for 500 ng was reversely transcribed to cDNA by ReverTra Ace® qPCR RT Kit (Toyobo, Osaka, Japan). Real-time PCR analysis was conducted on ABI 7500 fast Real Time PCR system (Applied Biosystems, Carlsbad, CA, USA) using SYBR®Green Realtime PCR Master Mix (Toyobo, Osaka, Japan). GAPDH served as an internal control. Relative quantification of target gene expression was determined by the $2^{-\Delta\Delta CT}$ method. The sequences of primers used are presented in Table 1.

Western blot

Total protein was extracted from cells. The protein samples were subjected to 12% acrylamide gel electrophoresis (SDS-PAGE) based on the manufacturer's instructions followed by electroransfer onto a nitrocellulose membrane. After blocking with 5% (w/v) non-fat milk dissolved in PBS for 2 h, the membranes were incubated at 4 °C overnight with primary antibodies of NLRP3, caspase-1 and IL-1β (Cell Signaling, MA, USA), GSDMD (BIOSS, Beijing, China) and GAPDH (ZSGB-BIO, Beijing, China), followed by incubation with HRB labeled goat anti-mouse IgG or anti-rabbit IgG (1:1000) (ZSGB-BIO, Beijing, China) for 1 h. Western blot bands were analyzed by Quantity One software.

Table 1
PCR primer sequence and interfering RNA sequence.

Primer and interfering		RNA Sequence
GAPDH	Forward	5′-ATCACTGCCACCCAGAAGAC-3′
	Reverse	5′-TTTCTAGACGGCAGGTCAGG-3′
NLRP3	Forward	5′-ATAACCTTGGGCTTGTCTTTCA-3′
	Reverse	5′-GTGGAGATCCTAGGTTTCTCTG-3
caspase-1	Forward	5′-ACACGTCTTGCCCTCATTATCT-3′
	Reverse	5′-ATAACCTTGGGCTTGTCTTTCA-3′
IL-1β	Forward	5′-CCCTGCAGCTGGAGAGTGTGG-3′
	Reverse	5′-TGTGCTCTGCTTGAGAGGTGCT-3′

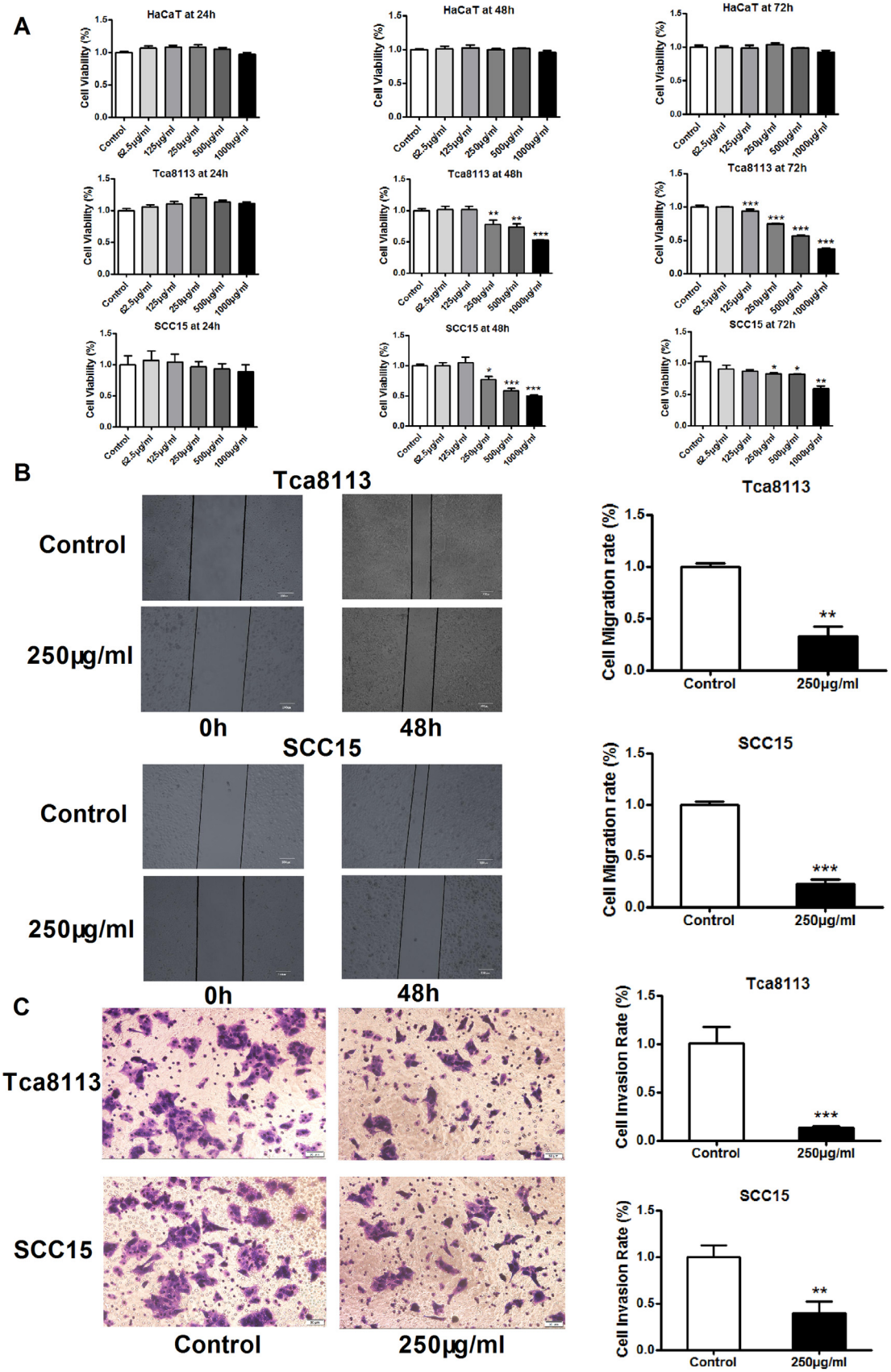

Fig. 1. Anthocyanin suppresses OSCC cells viability, migration and invasion. A. Alterations of viability of HaCaT (first line), Tca8113 (second line) and SCC15 (third line) cells, as detected by CCK8 assay. **B.** Migration rate of Tca8113 and SCC15 cells, as measured by scratch-wound assay. **C.** Invasion rate of Tca8113 and SCC15 cells, as revealed by Transwell invasion assay. *$p < 0.05$ versus Control, **$p < 0.01$ versus Control, ***$p < 0.001$ versus Control; $n = 3$.

Statistical analysis

All graphs were generated using Graphpad Prism 5.0. Data were expressed as mean ± standard error of mean (mean ± SEM) and analyzed with SPSS 13.0 software. Statistical comparisons between two groups were performed using unpaired Student's *t*-test. Statistical comparisons among multiple groups were performed using analysis of variance (ANOVA). A two-tailed $p < 0.05$ was considered statistically significant.

Results

Anthocyanin suppresses OSCC cell viability, migration and invasion

To determine the effects of anthocyanin on Tca8113, SCC15 and HaCaT cells, we examined changes in cell viability in response to treatment with different concentrations of anthocyanin for different time-periods (Fig. 1(A)). Anthocyanin treatment at all the concentrations examined had no effect on HaCaT cells, whereas 250 μg/ml anthocyanin induced a reduction in viability of Tca8113 and SCC15 at 48 h, which were subsequently employed as the dosage concentration and time for further experiments. Simultaneously, scratch-wound and Transwell invasion experiments were conducted using Tca8113 and SCC15, with a view to determining the effects of anthocyanin on migration and invasion of OSCC cells (Fig. 1(B, C)). The collective results clearly indicate that anthocyanin inhibits the viability, migration and invasion of the OSCC cell lines Tca8113 and SCC15.

Anthocyanin is involved in activation of pyroptosis in OSCC

Since cell viability was significantly reduced and cell death evident under a light microscope after administration of anthocyanin, we examined mRNA and protein expression patterns of the pyroptosis-associated factor, caspase-1, in Tca8113 and SCC15 cells, its upstream-related factor, NLRP3, and downstream-related factor, IL-1β. In both OSCC cell lines, levels of NLRP3, caspase-1, and IL-1β in the anthocyanin-treated group were significantly increased, compared to the control group via qRT-PCR (Fig. 2(A)). Immunofluorescence results further revealed elevated protein expression of NLRP3, caspase-1, and IL-1β in both anthocyanin-treated cells (Fig. 2(B, C)). Western blot results were consistent with immunofluorescence findings (Fig. 2(D)), supporting the theory that anthocyanin participates in activation of pyroptosis in OSCC.

Caspase-1 inhibition attenuates the effects of anthocyanin on Tca8113 and SCC15 cells

To further illustrate the association between anthocyanin and pyroptosis, we treated cells with the caspase-1 inhibitor, AC-YVAD-CMK, along with anthocyanin. Data from cell viability, scratch-wound and Transwell invasion assays revealed that after administration of AC-YVAD-CMK, cell viability, migration, and invasiveness were increased relative to the anthocyanin-treated groups (Fig. 3(A–C)). Simultaneously, mRNA and protein levels of NLRP3, caspase-1, and IL-1β were assessed. Notably, mRNA levels of these three factors were significantly increased after treatment with AC-YVAD-CMK, compared to the anthocyanin-only group (Fig. 3(D)). Data obtained from both immunofluorescence and western blot experiments indicate that the elevated expression of NLRP3, caspase-1, and IL-1β proteins induced by anthocyanin is reversed in the presence of AC-YVAD-CMK (Fig. 3(E–G)). Based on these findings, we propose that the effects of anthocyanin on OSCC are exerted, at least in part, through its involvement in activation of pyroptosis.

Validation of the involvement of anthocyanin on pyroptosis in OSCC

To further clarify the mechanism underlying anthocyanin-induced activation of pyroptosis, we examined the expression of the pyroptosis-associated factor Gasdermin D. Immunofluorescence experiments showed that in both cell lines, expression of GSDMD was enhanced upon treatment with anthocyanin, consistent with activation of the caspase-1 inflammatory pathway (Fig. 4(A, B)). Simultaneous western blot analyses consistently showed elevated GSDMD protein expression in the presence of anthocyanin (Fig. 4(C)). Our results suggest that anthocyanin upregulates GSDMD, which subsequently participates in activation of pyroptosis.

Discussion

Oral cancer is a malignant disease that mainly involves the posterior triangle including the buccal mucosa, lips, tongue, upper and lower gums, hard palate, and molars (Estilo et al., 2009). Multiple oral carcinoma types have been characterized to date (for instance, basal cell carcinoma, true carcinoma, nasopharyngeal carcinoma, malignant melanoma, ameloblastoma and mucoepidermoid carcinoma), among which upper squamous cell carcinoma is the most common oral malignancy. OSCC accounts for 90% oral cancer cases, with ~300000 new cases reported annually worldwide (Wei et al., 2016). This multifactorial disease is affected by several genetic alterations and environmental factors. During disease progression, expression changes in multiple genes are induced that affect normal physiological processes of cells and lead to disruption of macromolecular metabolism, signal transduction and cell structure, triggering abnormal proliferation of cells and eventual carcinoma formation (Kademani et al., 2008; Murugan et al., 2009). At present, surgical resection of carcinoma tissue is still the best treatment of choice for patients with OSCC, but this method is not effective for advanced metastases. Although chemotherapy and radiotherapy combined with surgery can be used to treat advanced cancers to a certain extent, these patients have a high rate of tumor recurrence and the low 5-year survival rate remains a significant global problem. Therefore, development of novel, innovative methods for treating OSCC remains an urgent medical requirement.

We also did experiments on apoptosis and autophagy at the same time as we did the pyroptosis (Fig. S3&4), but since the effect of anthocyanin on apoptosis (Ya et al., 2018) and autophagy (Chen et al., 2018) in cancer has been frequently reported. However, pyroptosis is a type of inflammatory caspase-mediated necrotic cell death process which become more and more important (Yuan et al., 2016) and the correlation between anthocyanin and pyroptosis in OSCC has not been clear so far, therefore, we decided to focus on pyroptosis in this study. Typical pyroptosis is mediated by caspase-1 activated by an inflammasome, such as NLRP3. Simultaneously, caspase-1 cleaves the amino- and carboxyl-terminal linkers of gasdermin D (GSDMD) that facilitates binding of the released amino-terminal fragment to the cell membrane to form oligomeric asymptotic pores (Ding et al., 2016; Aglietti et al., 2016; Liu et al., 2016; Sborgi et al., 2016). In addition, caspase-1 processes the precursor of IL-1β to become a mature body (Kayagaki et al., 2011) that either escapes from the pore formed by cleaved gasdermin D or is released through membrane rupture (Ding et al., 2016; Liu et al., 2016), thereby regulating cell death. Data from the current study indicate that after administration of anthocyanin, pyroptosis is activated in OSCC, which triggers death of tumor cells and inhibits their migration and invasion. However, after administration of the caspase-1 inhibitor AC-YVAD-CMK, the effects of anthocyanin on OSCC cells were suppressed. Accordingly, we propose that anthocyanin promotes death of OSCC cells through activating pyoptosis and inhibits carcinoma development.

Anthocyanin belongs to the flavonoid family. A natural pigment, anthocyanin is widely distributed in the flowers, leaves, fruits, and rhizomes of colored plants, generating red, blue, and purple colors.

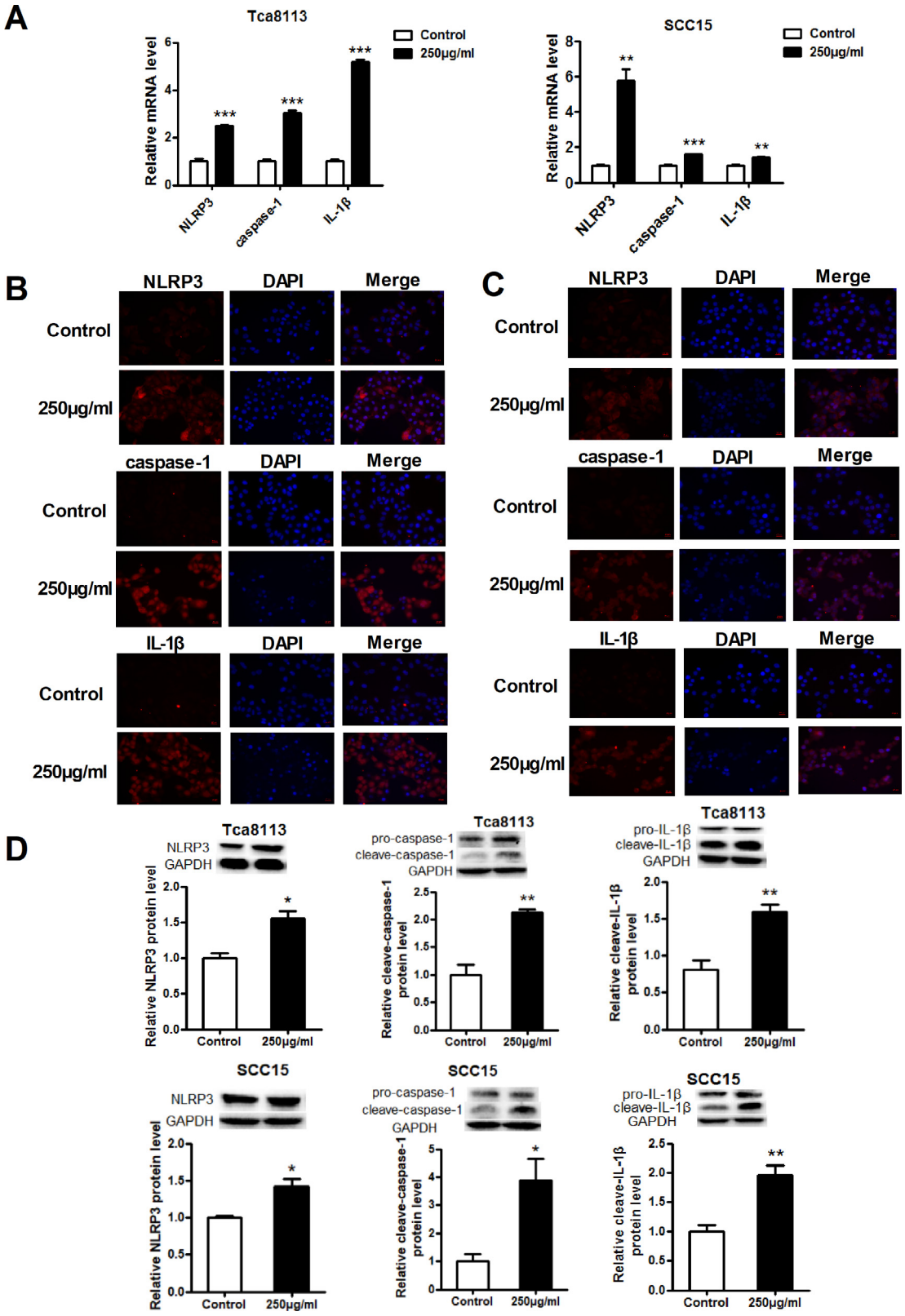

Fig. 2. Anthocyanin is involved in the activation of pyroptosis in OSCC. A. Relative expression of NLRP3, caspase-1 and IL-1β mRNA in Tca8113 and SCC15 cells, detected with qPT-PCR. **B.** The immunofluorescence staining of NLRP3, caspase-1 and IL-1β in Tca8113 cells. **C.** The immunofluorescence staining of NLRP3, caspase-1 and IL-1β in SCC15 cells. **D.** The expression of NLRP3, caspase- 1 and IL-1β protein in Tca8113 and SCC15 cells. GAPDH served as an internal control. $^{*}p < 0.05$ *vs.* Control, $^{**}p < 0.01$ *vs.* Control, $^{***}p < 0.001$ *vs.* Control; $n = 3$.

Anthocyanin has been shown to possess anti-oxidative (Aqil et al., 2012) and anti-inflammatory (Jean-Gilles et al., 2012) properties and reduce obesity (Nascimento et al., 2013). In recent years, anti-tumor activity of anthocyanin has additionally been widely documented (Kim et al., 2012; Wang et al., 2013; Long et al., 2018). A number of mechanisms are proposed to explain the anti-carcinogenic effect of

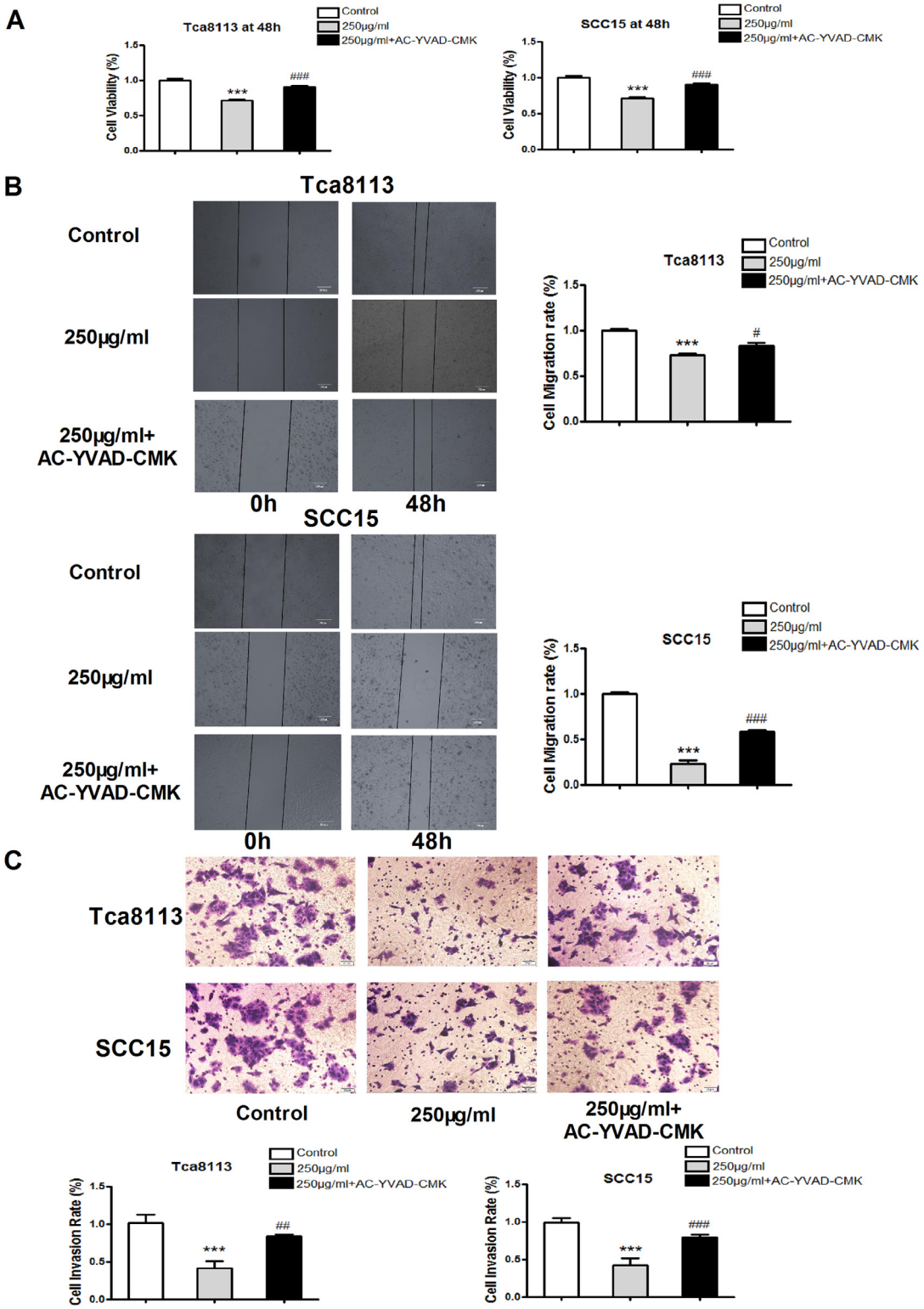

Fig. 3. Caspase-1 inhibitor attenuates the effects of anthocyanin in Tca8113 and SCC15 cells. A. Alterations of viability of Tca8113 and SCC15 cells, as revealed by CCK8 assay. **B.** Migration rate of Tca8113 and SCC15 cells, as measured by scratch-wound assay. **C.** Invasion rate of Tca8113 and SCC15 cells, as revealed by Transwell invasion assay. **D.** Relative expression of NLRP3, caspase-1 and IL-1β mRNA in Tca8113 and SCC15 cells, detected with qRT-PCR. **E.** The immunofluorescence staining of NLRP3, caspase-1 and IL-1β in Tca8113 cells. **F.** The immunofluorescence staining of NLRP3, caspase-1 and IL-1β in SCC15 cells. **G.** The expression of NLRP3, caspase-1 and IL-1β protein in Tca8113 and SCC15 cells. GAPDH served as an internal control. $^{**}p < 0.01$ *vs.* Control, $^{***}p < 0.001$ *vs.* Control, $^{\#}p < 0.05$ *vs.* Anthocyanin 250 μg/ml, $^{\#\#}p < 0.01$ *vs.*Anthocyanin 250 μg/ml, $^{\#\#\#}p < 0.001$ *vs.* Anthocyanin 250 μg/ml; $n = 3$.

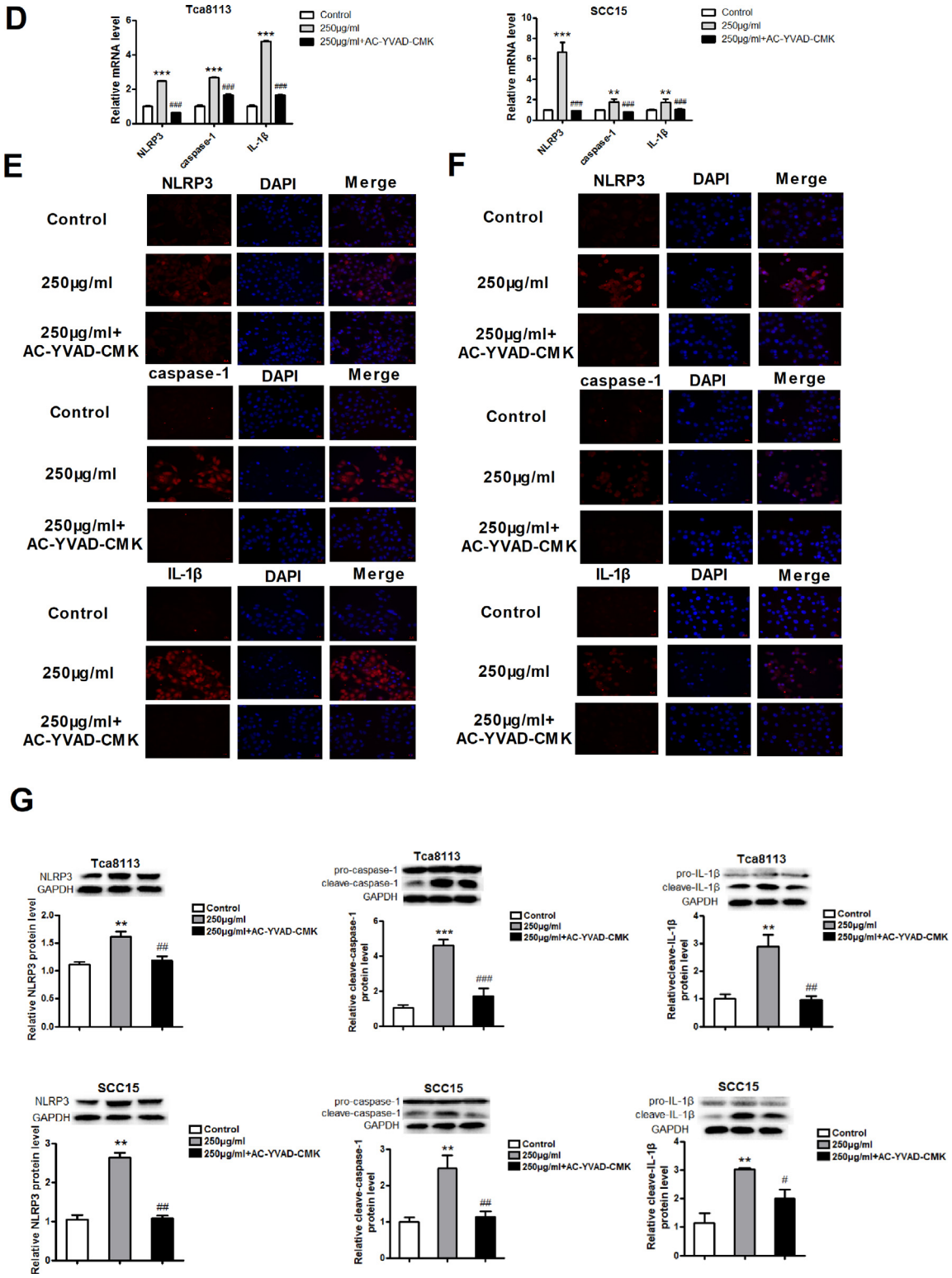

Fig. 3. (*continued*)

anthocyanin (Sun et al., 2017; Li et al., 2018). In this experiment, we chose metformin as the positive control, however, anthocyanin much inhibited the cell viability of OSCC than the effect of metformin (Fig. S5). Therefore, we focused on the potential relationship between anthocyanin and pyroptosis. Treatment of OSCC cells with anthocyanin increased the expression of the pyroptosis-associated factors NLRP3, caspase-1, and IL-1β, which in turn, activated pyroptosis leading to cell death and suppression of OSCC progression. The activating effect of anthocyanin on pyroptosis was inhibited upon treatment with a caspase-1 inhibitor, supporting the theory that anthocyanin could partially suppress the growth of OSCC cells through induction of pyroptosis. However, further studies are required to clarify the specific underlying mechanisms. Interestingly, anthocyanin 250 μg/ml inhibited tumor cells and did not inhibit HaCaT cells at 1000 μg/ml. We speculate the reason for this phenomenon is that there is complex mechanism in malignant cells such as tumor cells and non-malignant cells that we are

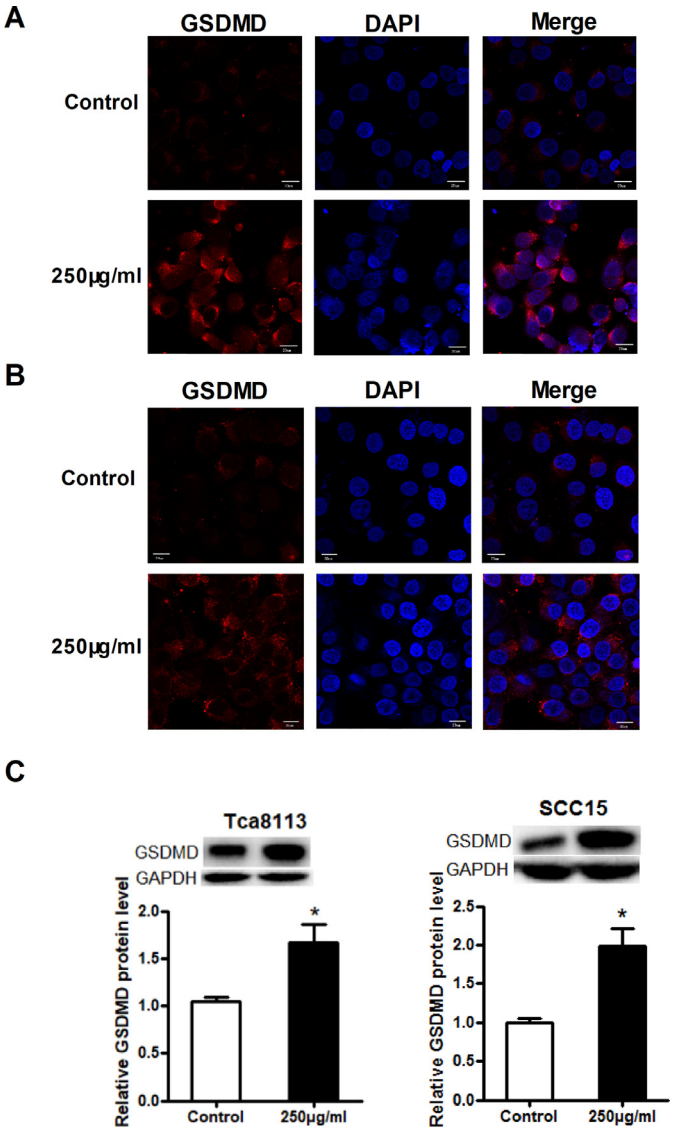

Fig. 4. Validation of the involvement of anthocyanin on pyroptosis in OSCC. A. The immunofluorescence staining of GSDMD in Tca8113 cells, detected with confocal. **B.** The immunofluorescence staining of GSDMD in SCC15 cells, detected with confocal. **C.** The expression of GSDMD protein in Tca8113 and SCC15 cells. GAPDH served as an internal control. $^{*}p < 0.05$ *vs.*Control; $n = 3$.

going to explore in the future. In addition, extremely high concentration of anthocyanin (4000 μg/ml) will also inhibit the viability of HaCaT cell (Fig. S6).

Overall, three significant findings are highlighted in the current study. First, activation of pyroptosis contributes to the pathogenesis of OSCC. Second, activation of caspase-1 triggers the cell death through pyroptosis and may have therapeutic potential for OSCC. Finally, the anti-OSCC activity of anthocyanin is attributed, at least in part, to the activation of caspase-1.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2018.09.223.

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