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Regular paper

Formononetin enhances the chemosensitivity of triple negative breast cancer *via* BTB domain and CNC homolog 1-mediated mitophagy pathways

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This study aimed to investigate the effects of formononetin on triple negative breast cancer (TNBC). Clinical samples were collected from patients with TNBC. Overall survival rates were evaluated using the Kaplan-Meier method. Gene expression was determined using immunohistochemistry, immunofluorescence and western blot. Cellular functions were determined using CCK-8, colony formation and propidium iodide (PI) staining. Xenograft assay was performed to further verify the effects of formononetin (FM) on TNBC. We found that FM combined therapy suppressed the metastasis of TNBC and increased the overall survival rates of TNBC patients. Moreover, FM suppressed the proliferation and induced mitochondrial damage and apoptosis of TNBC cells. FM increased the expression of the BTB domain and CNC homolog 1 (BACH1) in TNBC tissues as well as cells. However, BACH1 knockdown antagonized the effects of FM and promoted the survival of TNBC cells. FM suppressed the tumor growth of TNBC. Taken together, FM suppressed the aggressiveness of TNBC *via* BACH1/p53 signaling. Therefore, FM may be an alternative strategy for TNBC.

Keywords: triple negative breast cancer, formononetin, chemosensitivity, BACH1, mitophagy

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Abbreviations: BACH1, BTB domain and CNC homolog 1; FM, formononetin; PI, propidium iodide; TNBC, triple negative breast cancer

INTRODUCTION

Breast cancer is the most common tumor among women worldwide (Braden *et al.,* 2014; Burstein *et al.,* 2019). Recent decades witness an increase in the incidence and mortality of breast cancer in China (Ding *et al.,* 2020; Fan *et al.,* 2014). Triple negative breast cancer (TNBC) is characterized by a high proliferative index, histological grade, and metastatic states (Garrido-Castro *et al.,* 2019). Although great advances have been made in chemotherapy, radiotherapy, and surgery for TNBC (Bianchini *et al.,* 2016). However, the overall survival rates of TNBC are still unsatisfactory (Newman and Kaljee 2017). The high recurrence and metastatic properties of TNBC neutralize the clinical outcomes (Xu *et al.,* 2020). Therefore, a new strategy for TNBC is urgently needed.

Traditional Chinese medicine (TCM), with high efficiency and few side effects, is widely applied in the treatment of breast cancer (Chan *et al.,* 2021; Yang *et al.,* 2021). Formononetin (FM), extracted from *astragalus membranaceus* and s*patholobus suberectus* and with anti-inflammatory and anti-carcinogenic properties, is used as adjuvant therapy for breast cancer (Ma *et al.,* 2020; Xin *et al.,* 2019; Yu *et al.,* 2020). Previous studies reveal that formononetin exerts its anti-cancer function *via* modulating several signaling. For instance, formononetin suppresses the chemoresistance of TNBC *via* inactivating autophagy (Li *et al.,* 2021). Formononetin induces prostate cancer cell mitochondrial and apoptosis *via* regulating IGF-1/IGF-1R pathways (Huang *et al.,* 2013). Additionally, formononetin inhibits the immune suppressiveness of cervical cancer through inactivating MYC/STAT3/PD-L1 signaling (Wang *et al.,* 2022). This study explored the effects of NP (vinorelbine and cisplatin) combined with formononetin on TNBC.

MATERIALS AND METHODS

Patients

Clinical samples were collected from patients with refractory TNBC undergoing chemotherapy (vinorelbine and cisplatin, NP) with or without FM. at People's Hospital of Dongtai City from April 1, 2019 to March 31, 2021. The samples were immediately stored in liquid nitrogen at –80°C. This study was approved by the Ethical Committee of People's Hospital of Dongtai City. All patients signed confirmed consent. The inclusion criteria are: (1) the patients diagnosed with mTNBC; (2) women under the age of 70; (3) no serious complications occurred after the operation; (4) general condition score: ECoG 0-2; (5) patients in the combined group insisted on taking formononetin for at least 8 months. The exclusion criteria are: the mTNBC patients with an estimated ≤3 months of survival time; (2) patients accompanied by severe impairment or insufficiency of heart, liver and kidney functions; (3) patients with poor compliance and unable to adhere to treatment.

Immunohistochemistry

Sections were deparaffinized. Then the slides were blocked with 0.1% Triton X-100. After washing with 10 PBS, the section was incubated in 3% H_2O_2. The sections were incubated with primary antibody against BTB domain and CNC homolog 1 (BACH1) (ab128486, 1: 150, Abcam, USA) at 4°C overnight in shade. The next day, the section was incubated with a secondary antibody at 37°C for 1.5 h in the shade. The slices were counterstained using hematoxylin for 10 min. Finally, the sections were visualized using a microscope (Nikon, Japan).

Cell culture and transfection

Breast cancer cell line MCF7 was purchased from ATCC. Cells were incubated with DMEM containing 10% FBS at 37°C in 5% CO_2.

Cells were treated with 1.5 μM of vinorelbine, 2 μM of cisplatin, 80 μmol/L of FM or 10 mM of N-acetylcysteine (NAC).

Cells were transfected with shBACH1 (sh1, F: 5'-CCGGCCAGCAAGAATGCCCAAGAAACTC-3' and R: 5'-AATTCAAAAACCAGCAAGAATGCCCAAGA-3'; sh2, F: 5'-CCGGGCCCATATGCTTGTGTCATTACTCGA-3'and R: 5'-AATTCAAAAAGCCCATATGCTTGTGTCATT-3', sh-p53 (5'-GACUCCAGUGGUAAUCUAC-3') and the negative control (NC, F: 5'-CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTTG-3' and R: 5'-AATTCAAAAACAACAAGATGAAGAGCACCAA CTCGAGTTGGTGCTCTTCATCTTGTTG-3') by using Lipofectamine® 3000 for 48 h.

Western blot

Protein was collected from TNBC tissues and cells. Protein concentrated with BCA kit (Beyontine, Shanghai). The protein was isolated using 12% SDS-PAGE. The separated protein was moved onto the PVDF membrane, which was then blocked using 5% skimmed milk. Afterwards, the membranes were incubated with primary antibodies, such as anti-cyto C (ab133504, 1:2000, Abcam, USA), anti-caspase3 (ab32351, 1:5000, Abcam, USA), anti-Bcl-2 (ab32124, 1:1000, Abcam, USA), anti-Bax (ab32503, 1:1000, Abcam, USA), anti-BACH1 (ab300130, 1:1000, Abcam, USA), anti-p53 (ab32389, 1:10000, Abcam, USA), anti-PARK2 (ab73015, 1:1000, Abcam, USA), anti-PINK1 (ab300623, 1:1000, Abcam, USA) and anti-GAPDH (ab9485, 1: 2000, Abcam, USA), and then with secondary antibodies (ab6721, 1:5000, Abcam, USA). Finally, the bands were captured using an ECL kit and analyzed using ImageJ software.

MDA and SOD determination

The release of MDA and SOD was determined using specific commercial kits (Beyotime, Shanghai).

Immunofluorescence

Cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Afterwards, cells were sealed with 5% bovine serum. Then cells were incubated with primary antibodies against Hsp60 (ab190828, 1:200, Abcam, USA), BACH1 (ab300130, 1:100, Abcam, USA), cyto-C (ab133504, 1:100, Abcam, USA) and then with secondary antibody. Then cells were counterstained with DAPI. The results were visualized using an immunofluorescence microscope (Zeiss, Germany).

CCK-8 assay

After 48-hour transfection, cells were collected. Then cells were plated into 24-well plates and cultured for 0, 12, 24, and 48 h. After being supplemented with CCK-8 regents and cultured for another 2 h, cells were detected by a microplate reader at the wavelength of 450 nm.

Colony formation assay

After transfection, cells were plated into a 24-well plate. After 2 weeks of culture, cells were fixed and stained with 0.1% crystal violet. Subsequently, the colonies were visualized using a microscope.

Table 1. Baseline demographics and disease

Characteristics	**N=88**
Female, n (%)	88 (100)
Age, years, median(range)	52 (23-84)
Postmenopausal, n (%)	53 (60.23)
ECOG performance status, n (%)	
0	56 (63.64)
1	32 (36.36)
LDH concentration, n (%)	
<1 xULN	45 (51.14)
≥1 x ULN to <2.5 x ULN	38 (43.18)
≥2.5xULN	2 (2.27)
Unknown	3 (3.41)
Target lesion size, mm, median (range)	48.5 (9-194)
No. of metastatic organ sites, n (%)	
1	26 (29.55)
2	48 (54.55)
≥3	14 (15.90)
Visceral± nonvisceral disease, n (%)	55 (62.50)
Prior NP therapy, n (%)	56 (63.64)
Disease-free interval	
<12 months	39 (44.32)
≥12 months	49 (55.68)
Previous FM therapy, n (%)	32 (36.36)
No. of previous lines of therapy for recurrent/metastatic disease, n%)	
0	85 (96.59)
1	3 (3.41)

Propidium iodide (PI) staining

After transfection, cells were plated into a 24-well plate. Then cells were treated with PI solution (2 μg/mL). Finally, PI positive cells were captured by a fluorescence microscope (Leica, Germany).

Xenograft assay.

18 BALB/c nude mice (6–8 weeks, 18–22 g) were purchased from the Animal Center of Nanjing Medical University. Mice were randomly divided into three groups: control group, NP+FM group, and NP+FM+CDDO-ME (CDDO-ME) group. Each mouse was inoculated subcutaneously with 3×10^4 cells. The tumor was measured every three days. Tumor size was calculated as followed: $V=lw^2/2$. At 21 days, mice were euthanized, and tumor were collected. This study was authorized by the Animal Care Broad of People's Hospital of Dongtai City.

Statistical analysis

All data were analyzed using SPSS 20.0. The difference was analyzed using the Student t-test and ANOVA assay. The survival rates of patients were analyzed using Kaplan Meier and log-rank test. $P<0.05$ was deemed as a significant difference.

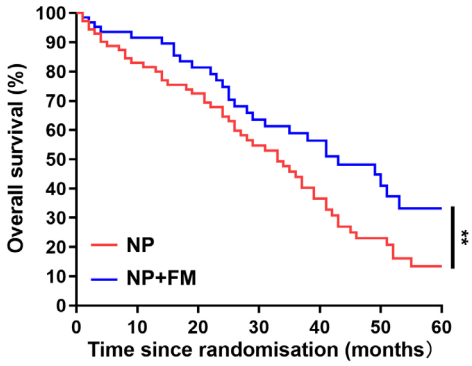

Figure 1. The survival rates of TNBC patients.
The overall survival rates of TNBC patients. **P<0.01.

RESULTS

The characteristics of TNBC patients

As shown in Table 1, the participants were all female. 68% of the TNBC patients had increased LDH levels. 45% showed visceral metastasis and 33% with recurrence. Additionally, combined therapy significantly improved the overall survival rate of TNBC patients (Fig. 1).

FM promotes oxidative stress and mitochondrial damage in TNBC

Previous studies reveal that FM suppresses the progression of multiple myeloma *via* inducing oxidative stress. Then we determined the release of oxidative stress in TNBC. FM enhanced the effects of NP on the release of MDA and GSH (Figs 2A and B). FM enhanced mitochondrial aggregation induced by NP (Fig. 2C). Moreover, FM+NP markedly increased cyto C protein expression compared with the NP group (Fig. 2D).

FM suppresses the aggressiveness of TNBC cells.

To further verify the effects of FM on TNBC, we determined the MCF7 cellular functions. Compared to the control group, FM treatment significantly suppressed the cell viability of MCF7 cells (Fig. 3A). This was consistent with the results from the colony formation assay. FM markedly inhibited the proliferation of MCF7 cells (Fig. 3B). Additionally, FM significantly enhanced the apoptosis of MCF7 cells (Fig. 3C). FM remarkably increased the protein expression of BAX and Caspase3 and decreased Bcl2 (Fig. 3D).

FM increases BACH1 expression

BACH1 is evidenced to play a vital role in mitochondrial function. We then determined the potentials of BACH1 in TNBC. The online database showed that BACH1 expression was decreased in invasive breast cancer tumors (Fig. 4A). To further verify this, we determined BACH1 expression in TNBC patients. As shown in Fig. 4B, BACH1 expression in patients administrated with NP+FM. Moreover, the protein expression of BACH1 was markedly increased in cells treated with NP+FM (Figs. 4C and D).

BACH1 transmits ROS signaling to mitochondria

BACH1 suppresses the aggressiveness of cancer cells *via* increasing the release of mitochondrial ROS (Hao *et al.,* 2021). We, therefore investigated the potentials of BACH1 mitochondrial ROS. Figure 5A showed the transcription efficiency of sh-BACH1. BACH1 knockdown antagonized the effects of FM and increased the cell ability of MCF7 cells (Fig. 5B). Moreover, BACH1 knockdown suppressed the protein expression of cyto-C, and cleaved caspase3 and -9 (Fig. 5C). Additionally, BACH1 knockdown suppressed mitochondrial aggrega-

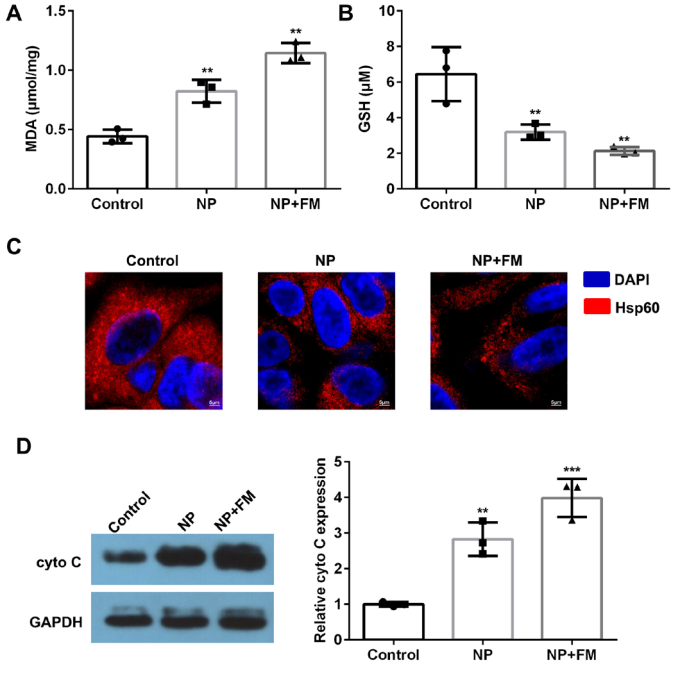

Figure 2. FM increases oxidative stress and mitochondrial damage in TNBC.
The release of MDA (**A**) and GSH (**B**) in MCF7 cells. (**C**) Mitochondrial aggregation determined by immunofluorescence. (**D**) The protein expression of cyto C in MCF7 cells determined using western blot. **P<0.01, ***P<0.001.

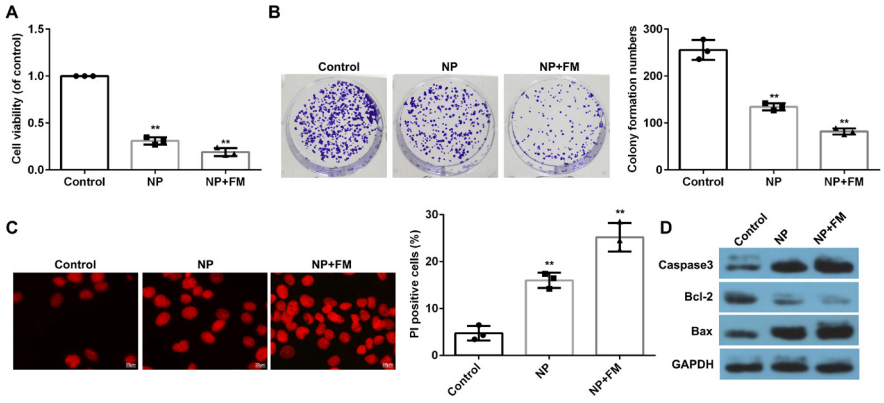

Figure 3. FM suppresses the aggressiveness of MCF7 cells.
(**A**) MCF7 cell viability determined using CCK-8 assay. (**B**) The proliferation of MCF7 cells detected using a colony formation assay. (**C**) The apoptosis of MCF7 cells detected using PI staining. (**D**) The protein expression determined using western blot. ***P*<0.01.

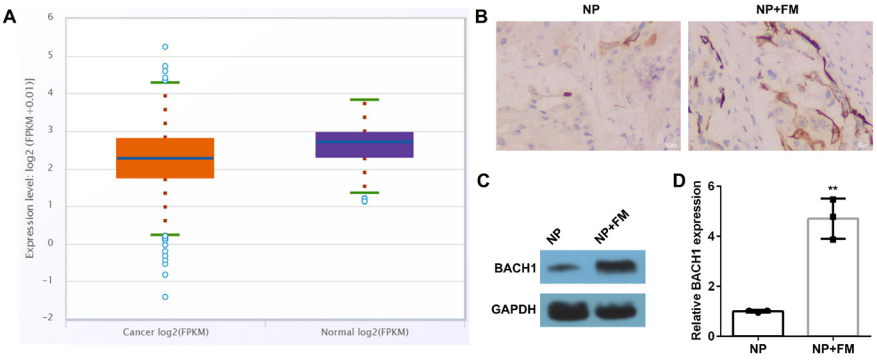

Figure 4. FM increases BACH1 expression.
(**A**) The expression of BACH1. (**B**) The expression of BACH1 in TNBC patients determined using immunohistochemistry. (**C and D**) BACH1 protein expression detected using western blot. ***P*<0.01.

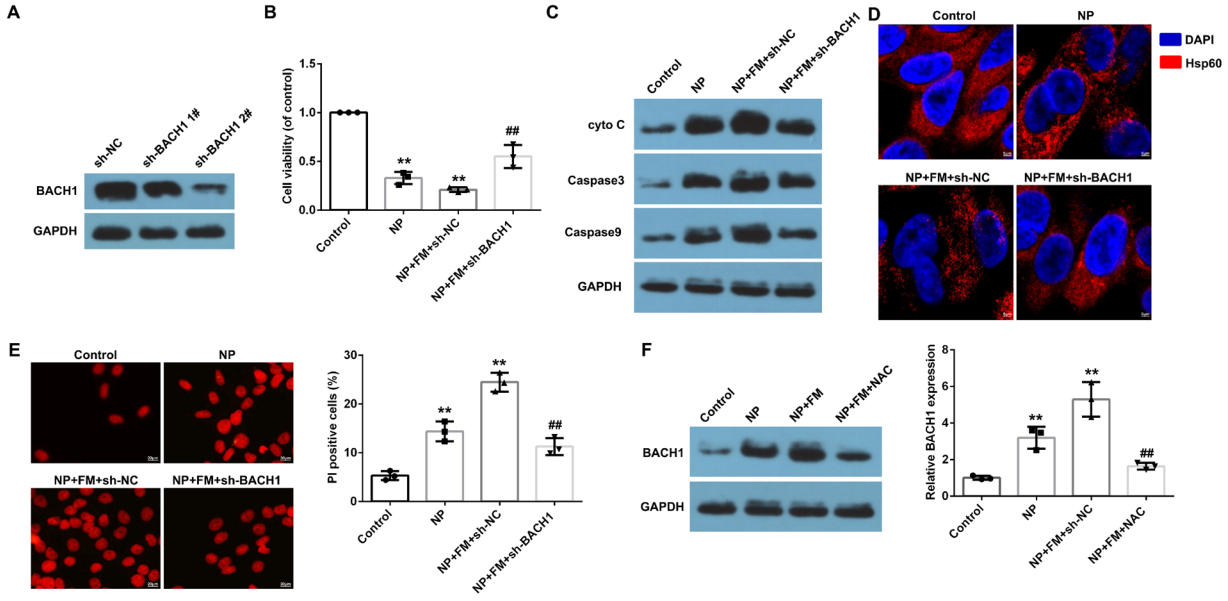

Figure 5. BACH1 transmits ROS signaling to mitochondria.
(**A**) BACH1 protein expression detected using western blot. (**B**) MCF7 cell viability determined using CCK-8 assay. (**C**) Caspase3 and -9 protein expression detected using western blot. (**D**) Mitochondrial aggregation determined by immunofluorescence. (**E**) The apoptosis of MCF7 cells detected using PI staining. (**F**) BACH1 protein expression detected using western blot. **P<0.01, ##*P*<0.01.

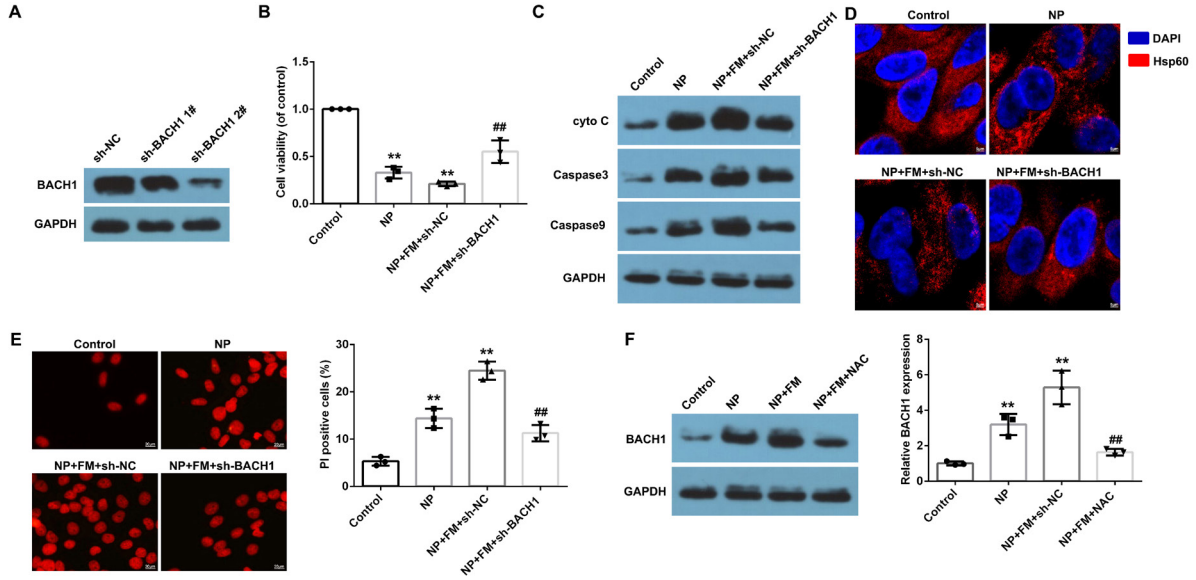

Figure 6. FM suppresses mitophagy via inducing BACH1-mediated activation of p53.
(**A**) The interaction between BACH1 and p53 predicted using STING. (**B**) p53 expression determined using immunofluorescence. (**C**) p53 protein expression determined using western blot. (**D**) Mitochondrial aggregation determined by immunofluorescence. (**E**) cyto-C and caspase-9 protein expression determined using western blot. (**F**) PINK1 and PARK2 protein expression determined using western blot. (G) Cell viability determined using CCK-8 assay. (**H**) The apoptosis of MCF7 cells detected using PI staining. $^{**}P<0.01$, $^{\#\#}P<0.01$.

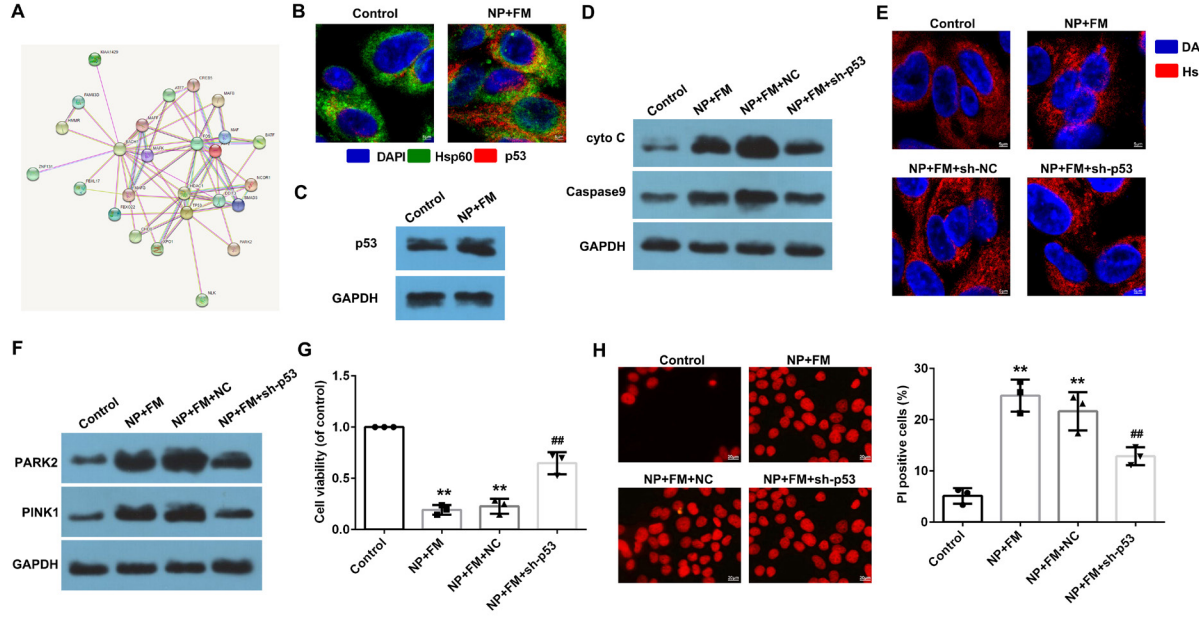

Figure 7. FM suppresses the tumor growth of TNBC *via* regulating BACH1.
The tumor size (**A**), volume (**B**), and weight (**C**) of TNBC in vivo. $^{**}P<0.01$, $^{***}P<0.001$, $^{\#\#}P<0.01$.

tion (Fig. 5D). The apoptosis rates were significantly decreased in the sh-BACH1 group (Fig. 5E). To further confirm the roles of BACH1 in mitochondrial ROS. Cells were exposed to NAC (an ROS inhibitor). As shown in Fig. 5F, NAC antagonized the effects of FM and decreased the protein expression of BACH1.

FM regulates mitophagy *via* inducing BACH1-mediated activation of p53

We further investigated the potential underlying mechanisms. The online database STING predicted the potential genes interacting with BACH1 (Fig. 6A). Then we found that BACH1 may regulate the expression of p53. To further verify this, cells were treated with FM and/or sh-p53. As shown in Figs 6B and C, FM promoted p53 translocation from cytoplasm to mitochondria. p53 deficiency suppressed protein expression of cyto C and Caspase9 mitochondrial aggregation (Fig. 6D) as well as mitochondrial aggregation (Fig. 6E). Moreover, p53 knockdown decreased the protein expression of PINK1, PARK2 (Fig. 6F). p53 knockdown promoted the cell

viability and inhibited the apoptosis of MCF7 cells (Figs 6G and H).

FM suppresses the tumor growth of TNBC *via* regulating BACH1

To further verify the effects of FM on TNBC, in vivo assay was performed. As shown in Fig. 7A–C, FM suppressed the tumor size, volume and weight, which was abated by BACH1 deficiency.

DISCUSSION

In this study, FM suppressed improved the clinical outcome of TNBC patients. FM induced oxidative stress and apoptosis of MCF7 cells suppressed tumor growth. Additionally, FM suppressed mitophagy *via* inactivating BACH1/p53 signaling pathways.

Formononetin (FM) possesses anti-tumor properties in various cancers (Tay *et al.,* 2019). For instance, FM suppressed the proliferation and metastasis of ovarian cancer cells (Zhang *et al.,* 2018). FM suppresses the growth and migration of gastric cancer (Yao *et al.,* 2019). In breast cancer, FM induced breast cancer cell cycle rest (Chen *et al.,* 2011). FM inhibits Taxol-chemoresistance of breast cancer cells *via* suppressing autophagy signaling (Wu *et al.,* 2021). However, seldom study focuses on the mitochondrial functions in breast cancer cells. In this study, FM transmitted ROS to mitochondria to release cyto C and induced the cascades of caspase-3 and caspase-9, the activation of which induced the apoptosis of MCF7 cells. These findings suggest that FM may exert its anti-tumor functions *via* impeding the mitochondrial function in breast cancer cells.

BACH1, a member of the cap'n'collar (CNC) b-Zip family, plays a key role in regulating oxidative stress (Wiel *et al.,* 2019). BACH1 is a key regulator of mitochondrial metabolisms in cancer (Lignitto *et al.,* 2019). For instance, BACH1 knockdown induces mitochondrial respiration and increases the chemosensitivity of papillary thyroid cancer cells to metformin (Yu *et al.,* 2022). Moreover, BACH1 is overexpressed in TNBC patients and High levels of BACH1 predict poor overall survival and disease-free survival rates (Ou *et al.,* 2019). Overexpressed BACH1 inhibits glycolysis as well as suppresses lactate catabolism in the tricarboxylic acid (TCA) cycle, and promotes breast cancer bone metastasis (Lee *et al.,* 2019; Padilla *et al.,* 2022). These studies dictate that BACH1 may function as an oncogene in breast cancer. In this study, BACH1 was overexpressed in TNBC patients and MCF7 cells. Moreover, overexpressed BACH1 suppressed the release of oxidative stress and cancer cell apoptosis. Moreover, BACH1 alleviated FM-induced mitochondrial damage.

Mitochondrial metabolism plays a vital role in mitochondrial function, requiring intensive integration of mitochondrial morphology and dynamics (Chan, 2020). Mitophagy, erasing damaged mitochondria *via* autophagy, is a key process to maintaining mitochondrial quality (Srinivasan *et al.,* 2017). However, dysfunction of mitophagy may induce the pathogenesis of cancer, such as hepatocellular carcinoma, colon as well as breast cancer (Chen *et al.,* 2019; Deng *et al.,* 2021; Yin *et al.,* 2021). Therefore, to unraveling the underlying mechanisms may provide a novel target for cancer therapy. In this study, FM treatment induced the overexpression of p53. As a tumor suppressor, p53 suppresses tumorgenesis *via* inducing apoptosis, pyroptosis, ferroptosis as well as autophagy. In this study, FM stimulated p53/PINK1/PARK2 signaling pathways *via* inactivating BACH1, which promoted the release of cyto C and caspases as well as mitophagy-mediated mitochondrial dysfunction.

However, there are several limitations to this study. A large number of patients could make the results more convincing. Therefore, future studies will recruit more metastatic TNBC patients. Additionally, mitochondrial damage and mitophagy may induce other forms of death, such as ferroptosis and pyroptosis. Whether FM induced ferroptosis or pyroptosis. This needs further study.

In conclusion, FM improved the clinical outcomes of TNBC patients. Additionally, FM suppressed the aggressiveness of TNBC *via* regulating BACH1 signaling. Therefore, FM may be an alternative strategy for TNBC.

Declarations

Conflict of interest. None.
Funding. None.

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