Huaier extract enhances the treatment efficacy of paclitaxel in breast cancer cells via the NF-κB/IκBα pathway

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Abstract. Breast cancer is considered as the most common malignant disease in women. Huaier extract, a type of traditional Chinese medicine, has been found to have antitumor activity. In the present study, we aimed to investigate whether the combined treatments of paclitaxel and Huaier extract may improve treatment efficacy in breast cancer cells. Human breast cancer cell lines MCF-7 and MDA-MB-231 were used to evaluate the antitumor efficacy of Huaier extract and paclitaxel both *in vitro* and *in vivo*. Using proliferation assays and flow cytometry, we found that both Huaier extract and paclitaxel decreased cell viability and induced cell apoptosis in a time- and dose-dependent manner. The combined treatments were more effective than monotherapy. Huaier extract induced cycle arrest in the G0/G1 phase, and paclitaxel arrested the cell cycle in the G2/M phase. Furthermore, the results of real-time PCR and western blotting revealed that Huaier extract decreased p65 and c-Met expression and increased IκBα expression, while paclitaxel increased p65 expression and reduced IκBα and c-Met expression. Consistent with the *in vitro* results, both Huaier extract and paclitaxel exerted a significant inhibitory effect on xenografted tumor growth, and the combined therapies revealed the most marked inhibitory effect. Collectively, our results indicated that Huaier extract increased the antitumor effect of paclitaxel therapy in breast cancer cells. The molecular mechanisms may be involved in the inhibition of the NF-κB pathway and c-Met expression.

Introduction

Breast cancer is the most frequently diagnosed cancer in women. Globally, ~1.3 million new cases are diagnosed annually (1). China accounted for 12.2% of all newly diagnosed global cases and 9.6% of all breast cancer-related deaths worlwide in 2008 (2), and since 1990s the incidence in China has increased more than twice as fast as that of global rates, particularly in urban areas (3). Although treatments of breast cancer have significantly been improved in surgery, radiotherapy and molecular targeted therapy, chemotherapy is still the major method. Chemotherapy reduced the risk of disease recurrence and metastasis (4). However, the chemotherapy resistance is still a major limitation in clinical treatment. Hence, new drugs or new therapeutic combinations are required to enhance chemosensitization and therapeutic efficacy (5).

Paclitaxel, one of the most important anticancer drugs, has been widely used for chemotherapy in ovarian, non-small cell lung cancer, and head and neck cancer, for almost 40 years, and it is also the first-line chemotherapy drug used in breast cancer (6,7). Paclitaxel prompts tubulin polymerization by binding β-tubulin. By stabilizing the microtubule polymer and preventing microtubules from disassembly, paclitaxel arrests the cell cycle in the G2/M phases and induces cell death (8-10). However, chemotherapy resistance limits the effect of paclitaxel and influences the prognosis of patients. Thus, it is particularly important to enhance the sensitivity of cancer cells to paclitaxel.

Trametes robiniophila Murr. (Huaier), a type of traditional Chinese medicine, has been widely used in China for ~1,600 years (11). Huaier extract is the fungus extracted twice with hot water and the effective ingredient is proteoglycan, containing 41.53% polysaccharides, 12.93% amino acids and 8.72% water (12-14). However, the anticancer effect of proteoglycan is less effective than that of the Huaier extract (15). It has been demonstrated that the main anticancer mechanisms of Huaier are achieved by inhibiting the growth and proliferation of cancer cells, inducing apoptosis, decreasing drug resistance in cancer cells, and improving immunity as well (16,17). *In vitro* experiments revealed that Huaier extract inhibited the growth of hepatocarcinoma and human lung adenocarcinoma cells (18,19). Huaier extract could inhibit the growth of ERα-positive breast cancer cells through the ERα/NF-κB pathway (20). A recent study reported that Huaier extract synergized with tamoxifen to increase autophagy and cell apoptosis in MCF-7 breast cancer cells, which was more effective than monotherapy (21).

In 1986, NF-κB was discovered as a nuclear factor that binds to the enhancer element of the immunoglobulin κ light-chain of activated B cells (22). Aberrant activation of NF-κB is frequently found in various solid tumors and hematological

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malignancies. NF-κB family members and their regulated genes are associated with tumor development, tumor cell proliferation, survival, angiogenesis, invasion, metastasis and drug resistance (23). IκB is the specific intracellular inhibitor of NF-κB. In most cell types, NF-κB is present in an inactive form, where it is complexed with IκB in the cytoplasm (24). A previous study observed persistent activation of NF-κB in hepatocellular carcinoma cell lines, and suggested that inhibition of NF-κB signaling markedly sensitized HCC cells to doxorubicin-induced apoptosis (25). In the chemotherapy, drug resistance is an important problem, as well as the expression levels of multidrug resistant (MDR) proteins, apoptosis and anti-apoptosis genes are the main factors of tumor chemosensitivity (26). Moreover, NF-κB is closely associated with apoptosis and mediates cell proliferation and survival. NF-κB can activate a variety of anti-apoptosis genes, such as Bcl-xL, Bfl/A1 and Bcl-2. Furthermore, the downregulation of NF-κB and different Bcl-2 family members leads to enhanced chemosensitization or radio-sensitization of multiple human cancers (27).

In the present study, we demonstrated for the first time that Huaier extract combined with paclitaxel induced cell apoptosis and enhanced paclitaxel drug sensitivity in breast cancer cells via the NF-κB pathway.

Materials and methods

Cell lines and reagents. ER-positive breast cancer cell line MCF-7 and triple-negative breast cancer cell line MDA-MB-231 were obtained from The Cell Bank of the Chinese Academy Sciences (Shanghai, China). Huaier extract was kindly provided by Gaitianli Medicine Co. Ltd. (Jiangsu, Chain). One gram Huaier extract was dissolved in 10 ml of complete RPMI-1640 medium and sterilized with a 0.22-μm filter to obtain 100 mg/ml stock solution at 4°C. Paclitaxel was purchased from Yangzijiang Medicine Co. Ltd. (Jiangsu, China). p65, IκBα and c-Met monoclonal antibodies were purchased from Abcam (Cambridge, UK). Rabbit anti-human polyclonal β-actin antibody was purchased from Bioworld Technology Inc. (St. Louis, MO, USA). The secondary anti-rabbit antibody was purchased from Qiaoyi Biotechnics Co. Lit (Shanghai, China).

Cell culture. Both MCF-7 and MDA-MB-231 cells were cultured in RPMI-1640 medium (Gibco-BRL, Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (FBS; Biological Industries Israel Beit-Haemek Ltd., Kibbutz Beit-Haemek, Israel), 100 U/ml penicillin and 100 μg/ml streptomycin. MCF-7 and MDA-MB-231 cells were routinely cultured at 37°C with 5% CO_2 in a humidified incubator.

Cell viability assay (CCK-8 assay). Cell viability was determined by Cell Counting Kit-8 (CCK-8) assay. Both MCF-7 and MDA-MB-231 cells were seeded into 96-well plates at a density of $5x10^4$ cells/well in 100 μl RPMI-1640 medium. After being cultured in 5% CO_2, in a 37°C incubator overnight, the RPMI-1640 medium in each well was treated with different concentrations of solutions (0, 2, 4, 8, 10 and 16 mg/ml of Huaier extract; 0, 0.001, 0.01, 0.1, 1 and 10 μg/ml of paclitaxel, or combined therapies) and incubated for 0, 24 or

48 h. Afterwards, 10 μl of CCK-8 solution was added to each well for another 2 h at 37°C. The absorbance at 450 nm was read using a microplate reader (BioTek, Winooski, VT, USA). The inhibitory rate of cell growth was calculated based on the following equation: Cell growth inhibition rate = (1 - experimental OD450/control OD450) x 100%. Based on the statistical analysis of results, 4 and 8 mg/ml of Huaier extract and 0.01 and 0.1 μg/ml of paclitaxel were used to explore cell functional studies and mechanisms. For combined therapies *in vitro*, the cells were treated with 4 mg/ml Huaier extract + 0.01 μg/ml paclitaxel (H4 + P0.01) and 8 mg/ml Huaier extract + 0.1 μg/ml paclitaxel (H8 + P0.1) for 48 h. Experiments were repeated three times, independently.

Flow cytometric analysis of the cell cycle and apoptosis. Cell apoptosis was performed using an FITC Annexin V apoptosis detection kit (BD Biosciences, San Jose, CA, USA). All groups of cells were strictly treated under manual processing and analyzed using a Beckman Coulter Cytomics FC500 flow cytometer (Beckman Coulter, Inc., Brea, CA, USA). The data were analyzed by EXPO32 ADC analysis software.

Cell cycle analysis was performed using the standard method with some modifications. Cells were fixed overnight with 75% ethanol at 4°C. The following day, the fixed cells were washed with phosphate-buffered saline (PBS). Then, the cells were suspended with 200 μl RNase A at 37°C for 10 min, followed by the addition of 250 μl propidium iodide (PI) (100 μg/ml) to stain the DNA of cells in the dark for 15 min. Finally, the cell cycle was analyzed with a Beckman Coulter Cytomics FC 500 flow cytometer, and the data was analyzed using MultiCycle AV for Windows (version 295) software.

Real-time PCR analysis. Total RNA from treated cells was extracted using TRIzol and was used to synthesize cDNA using PrimeScript RT reagent kit (both from Takara, Dalian, China) according to the manufacturer's instructions. Then, real-time PCR was carried out using PowerUp SYBR-Green Master Mix (Life Technologies, Thermo Fisher Scientific). The reaction was conducted using the following parameters: 95°C for 30 sec, 40 cycles at 95°C for 5 sec and 60°C for 30 sec. Internal control and primers for real-time PCR were obtained from the reference. Real-time PCR primers were synthesized by SBS Genentech Co. Ltd. (Shanghai, China). The specific primers for P65, IκB, c-Met and the reference gene (β-actin) are listed as follows: p65 forward, 5'-ACAACCCCTTCCAAGTT CCT-3' and reverse, 5'-TGGTCCCGTGAAATACACCT-3'; IκB forward, 5'-TGAGGACCAGCAGTGTCTTG-3' and reverse, 5'-CATCGTTGATCACAAGTCGG-3'; c-Met forward, 5'-CATCTCAGAACGGTTCATGCC-3' and reverse, 5'-TGCACAATCAGGCTACTGGG-3'; β-actin forward, 5'-GCTACAGCTTCACCACCACAG-3' and reverse, 5'-GGT CTTTACGGATGTCAACGTC-3'. The experiments were repeated at least three times and the data were analyzed using $2^{-\Delta\Delta Ct}$.

Western blot analysis. MCF-7 and MDA-MB-231 cells were treated with different concentrations of Huaier extract (4 and 8 mg/ml) and/or paclitaxel (0.01 and 0.1 μg/ml) for 48 h. The cells were lysed and total protein was separated using 10% sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE) and transferred (300 mA, 2 h) onto polyvinylidene fluoride (PVDF) membranes. After blotting with 5% non-fat milk, the membranes were incubated with primary antibodies (anti p65 1:5,000, anti p-p65 1:1,000, anti IκBα 1:1,000, anti c-Met 1:1,000, β-actin 1:5,000) at 4˚C overnight. Then, the membranes were washed using Tris-buffered saline with Tween-20 (TBS-T) buffer and incubated with a secondary horseradish peroxidase (HRP)-labeled anti-rabbit antibody at room temperature for 1 h. Subsequently, the membranes were washed again with TBS-T buffer three times (10 min each time). The target proteins were visualized with a chemiluminescence system (Gene Company Ltd., Shanghai, China) and normalized to β-actin from the same membrane.

Xenograft tumorigenicity assay. MCF-7 and MDA-MB-231 cells ($5x10^6$ in 0.1 ml PBS) were subcutaneously injected into four week old female nude mice. Treatments were started at the second week after injection. The mice were randomly assigned to control (double-distilled water), Huaier extract, paclitaxel or combined treatment groups. The dose of Huaier extract was 250 mg/ml and paclitaxel was 10 mg/kg. The Huaier group was administered 50 mg of Huaier extract by gavage once every two days, while paclitaxel was administered by intraperitoneal injection (IP) once a week or both were administered in the combined treatment group. Tumor growth was assessed every week and the tumor volume was calculated using the formula: 0.5 x (length x width²). After six weeks, the mice were sacrificed and the xenografted tumors were removed for flow cytometric and western blot analyses.

Statistical analysis. Statistical analyses were performed with SPSS for Windows 21.0 (SPSS, Inc., Chicago, IL, USA). Student's t-tests and one-way ANOVA were performed to determine statistical significance. All data are presented as mean ± standard error of the mean (SEM) of three experiments, and P<0.05 was considered statistically significant.

Results

Combination of Huaier extract and paclitaxel exhibits a more effective inhibitory effect on cell proliferation compared with monotherapies. As shown in Fig. 1A and B, Huaier extract reduced the viability of MCF-7 and MDA-MB-231 cells in a time- and concentration-dependent manner. Compared with the control group, 10 and 16 mg/ml of Huaier extract significantly decreased cell viability in both cell lines at 24 h (P<0.01). At 48 h, the dosing >4 mg/ml of Huaier extract significantly reduced the cell viability in both cell lines (P<0.01). We observed that the proliferation of MCF-7 and MDA-MB-231 cells was significantly inhibited by paclitaxel treatment in a time- and dose-dependent manner (Fig. 1C and D). At 48 h, cell viability was significantly decreased by paclitaxel treatment as low as 0.01 µg/ml (P<0.05). The combined therapies sharply decreased the viability of both MCF-7 and MDA-MB-231 cells, and the extent of reduced cell viability by combined therapies was significantly greater than the monotherapies of Huaier extract or paclitaxel treatment (Fig. 1E and F). Compared with the 0.1 µg/ml of paclitaxel treatment alone, the inhibition rate of cell viability in the combined treatments

(H8P0.1) was 1.382±0.161 times in the MCF-7 cells, and 2.83±0.751 times in the MDA-MB-231 cells (Fig. 1G and H). In order to establish a synergistic mode of action between Huaier and paclitaxel, we performed an isobolometric analysis. The results revealed that the point was on the left. This suggested that the combined action was supra-additive in both cell lines (Fig. 1I and J).

Huaier extract combined with paclitaxel to induce apoptosis in MCF-7 and MDA-MB-231 cells. The four quadrants of the apoptosis results: upper left represents cell debris, upper right represents late-stage apoptotic, lower left represents living and lower right represents early-stage apoptotic cells. Compared with the control group, Huaier extract (H4P0 and H8P0) significantly increased cell apoptosis in MCF-7 and MDA-MB-231 cells, and 8 mg/ml of Huaier extract revealed a more significant effect on cell apoptosis than 4 mg/ml Huaier extract (Fig. 2A and B; P<0.001). Similar to Huaier extract treatment, paclitaxel treatment (H0P0.01 and H0P0.1) significantly induced cell apoptosis in MCF-7 and MDA-MB-231 cells, and 0.1 µg/ml of paclitaxel exhibited a more significant effect, particularly in the increase of early-stage apoptosis (Fig. 2A and B; P<0.001). The combined treatments of Huaier extract and paclitaxel revealed a more significant effect on cell apoptosis than the monotherapies (Fig. 2A and B). Compared with paclitaxel treatment (H0P0.01 and H0P0.1) alone, the apoptosis rate in the combined therapy group (H4P0.01 and H8P0.1) increased by 2.139 and 1.431 times, respectively, in MCF-7 cells (P<0.001). Similarly, in MDA-MB-231 cells, the apoptosis rate of the combined therapy group (H4P0.01 and H8P0.1) increased by 1.131 (P<0.05) and 2.245 times (P<0.001), respectively, compared with paclitaxel treatment alone (H0P0.01 and H0P0.1).

Huaier extract synergizes with paclitaxel to induce cell cycle arrest in MCF-7 and MDA-MB-231 cells. After being exposed to Huaier extract for 48 h, the percentage of cells in the G0/G1 phase significantly increased in both MCF-7 cells (from 41.268±0.11 to 55.704±0.45%; P<0.001) and MDA-MB-231 cells (from 35.026±0.23 to 55.659±1.54%; P=0.001), while the percentage of cells in the S phase was decreased (Fig. 3A and B). Moreover, a higher concentration of Huaier extract (H8P0) exhibited a stronger effect on cell cycle arrest in the G0/G1 phase in both cell lines compared with the lower dose (H4P0). Notably, paclitaxel treatment (H0P0.01 and H0P0.1) also significantly induced cell cycle arrest in both cell lines, but cell cycle arrest was observed in the G2/M phase (Fig. 3A and B; P<0.001). Similar to Huaier extract treatment, paclitaxel treatment reduced the percentage of MCF-7 and MDA-MB-231 cells in the S phase (P<0.001). Furthermore, we analyzed the cell cycle in MCF-7 and MDA-MB-231 cells after combined therapies and found significant arrest in both G0/G1 and G2/M phases while the proportion of S phase was sharply decreased (P<0.001).

Huaier extract and paclitaxel target p65, IκBα and c-Met in MCF-7 and MDA-MB-231 cells. c-Met plays an important role in cell proliferation, invasion and chemoresistance. The phosphorylation of c-Met activates multiple downstream signaling
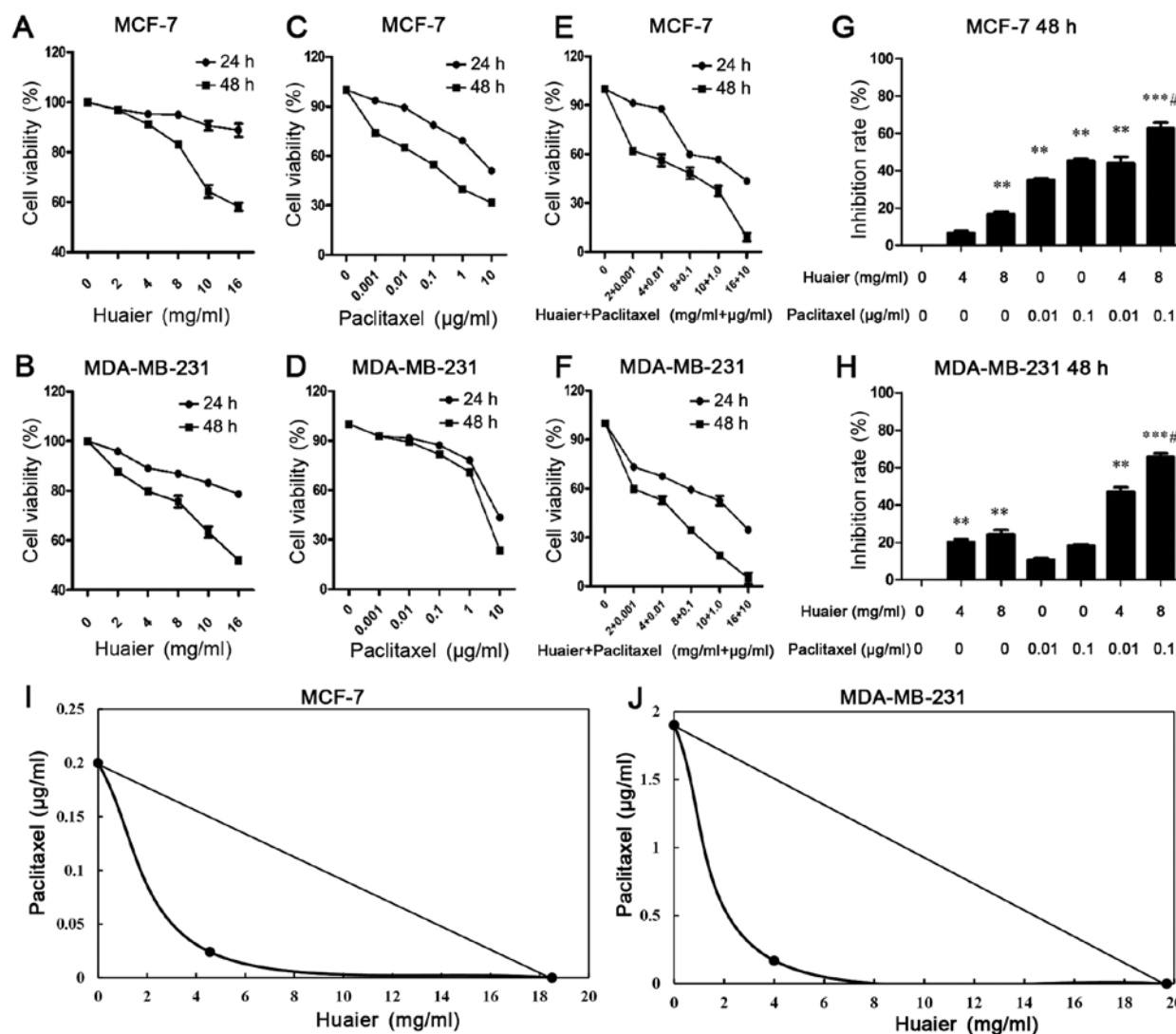
Figure 1. Combined treatments inhibit cell proliferation more significantly than monotherapies. Both (A and B) Huaier and (C and D) paclitaxel inhibited cell viability in a dose- and time-dependent manner. (E and F) Combined treatments significantly inhibited cell viability more than monotherapies in both cell lines. The inhibition rates of (G) MCF-7 and (H) MDA-MB-231 cells in the H8P0.1 group were higher than that in the H0P0.1. (I and J) The results of isobolometric analysis. The point was on the left, indicating that the combined action was supra-additive in both cell lines. The data were analyzed by one-way ANOVA (n=3), and P<0.05 was statistically significant (**P<0.01, ***P<0.001 vs. control group; #P<0.05, vs. paclitaxel alone).

pathways, including Ras/MAPK, PI3K/Akt and NF-κB. Thus, we wanted to detect whether Huaier and combined treatment mediated c-Met expression. As shown in Fig. 4A and B and G, after being treated with Huaier extract (H4P0 and H8P0) for 48 h, the expression of p65 and c-Met at the mRNA and protein levels was significantly decreased, while the expression of IκBα was significantly increased after treatment with H8P0 for 48 h (P<0.01). Therefore, Huaier extract suppressed the NF-κB/IκBα pathway. In contrast to the Huaier extract treatment, paclitaxel treatment (H0P0.01 and H0P0.1) for 48 h significantly increased the expression of p65 at the mRNA and protein levels in MCF-7 and MDA-MB-231 cells (P<0.05), while the expression of IκBα and c-Met at the mRNA and protein levels was significantly reduced (Fig. 4C, D and G). These results implied that paclitaxel activates the NF-κB pathway. We also examined the expression of p65, IκBα and c-Met in MCF-7 and MDA-MB-231 cells after combined treatments with Huaier extract and paclitaxel (Fig. 4E, F and G). The combined treatments (H4 + P0.01 and H8 + P0.1) significantly

decreased the expression of p65 and c-Met at the mRNA and protein levels, while the expression of IκBα mRNA and protein was significantly increased in both cell lines after combined treatments (P<0.05). These results were similar but more significant than those of the Huaier extract treatment alone.

Combined Huaier extract and paclitaxel treatments inhibit tumor growth in a xenograft model. To ascertain the inhibitory effect of Huaier extract and paclitaxel on breast cancer cells, we examined the antitumor effect of Huaier extract, paclitaxel and combined treatment in nude mice. Compared with the control group (825.8±33.7 mm³), the tumor volume in the Huaier (367.3±22.1 mm³; P<0.05), paclitaxel (223.9±28.2 mm³; P<0.01) and combined treatment group (158.1±25.2 mm³; P<0.01) was significantly smaller in MCF-7 xenografted tumors (Fig. 5A). However, there was no significant difference in the volume between the combined group and paclitaxel group (P=0.144). Similar results were observed in the MDA-MB-231 xenografted tumors (Fig. 5B), but the
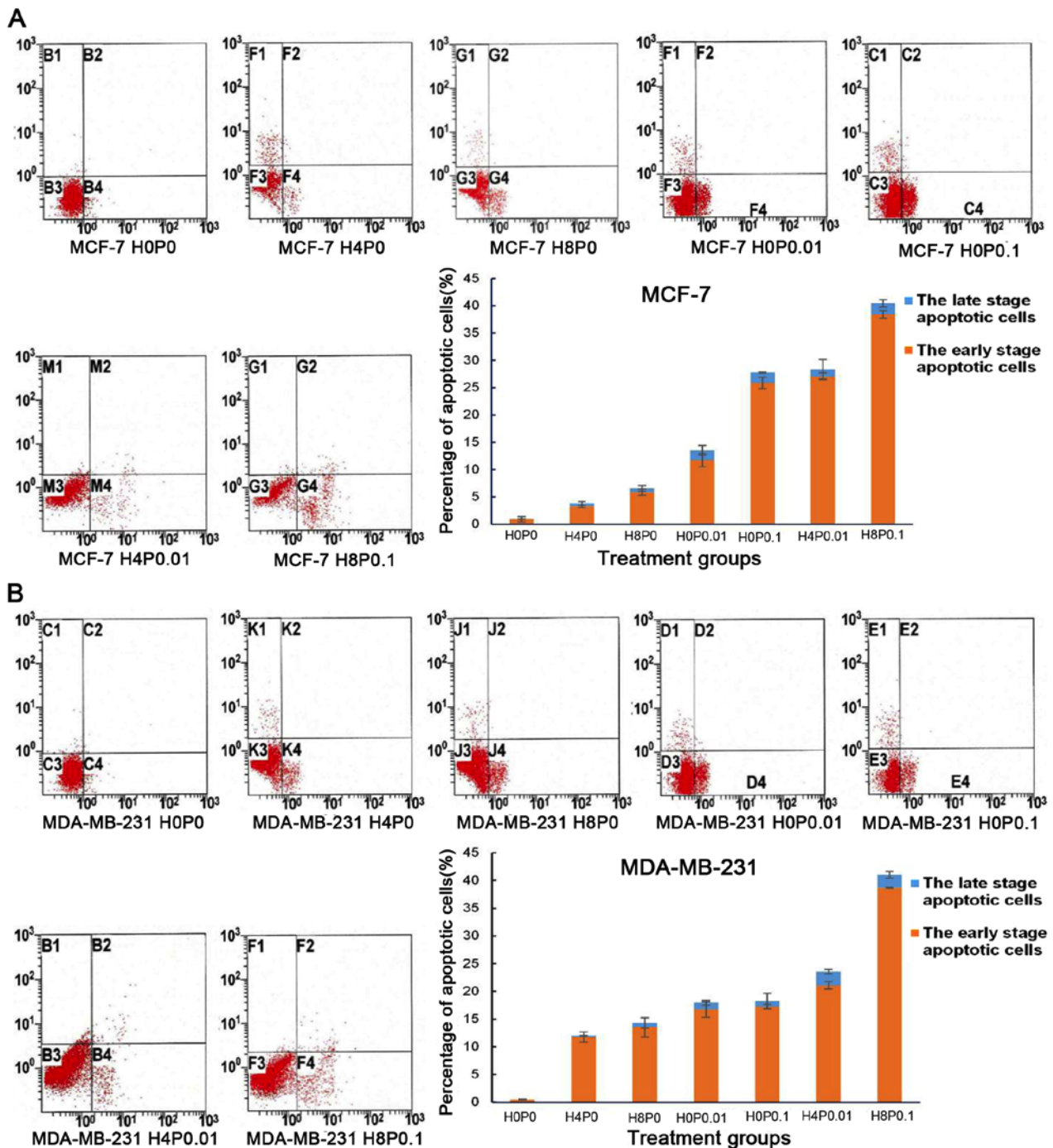
Figure 2. Huaier extract combined with paclitaxel induces apoptosis in MCF-7 and MDA-MB-231 cells. The four quadrants of the results: upper left represents cell debris, upper right represents late-stage apoptotic, lower left represents living and lower right represents early-stage apoptotic cells. (A) The apoptosis rates of MCF-7 cells treated with 4 and 8 mg/ml Huaier extract were 4.30±0.030% (**P<0.01) and 7.57±0.012% (***P<0.001) compared with the untreated group 0.94±0.057%. The apoptosis rates of MCF-7 cells treated with 0.01 and 0.1 μg/ml paclitaxel were 13.54±0.89% (***P<0.001) and 28.67±0.12% (***P<0.001) compared with the untreated group. The apoptosis rates of MCF-7 cells treated with H4P0.01 and H8P0.1 were 28.97±1.78% (***P<0.001) and 41.03±0.69% (***P<0.001) respectively, compared with the untreated MCF-7 group. (B) The apoptosis in MDA-MB-231 cells was induced by the same treatment as in MCF-7 cells. The apoptosis rates of MDA-MB-231 cells treated with 4 and 8 mg/ml Huaier extract were 12.28±0.100% (***P<0.001) and 14.29±0.045% (***P<0.001) respectively, compared with the untreated group. The apoptosis rates of MDA-MB-231 cells treated with 0.01 and 0.1 μg/ml paclitaxel were 14.97±0.54% (***P<0.001) and 18.27±1.39% (***P<0.001) compared with the untreated group. The apoptosis rates of MDA-MB-231 cells treated with H4P0.01 and H8P0.1 were 23.56±0.40% (***P<0.001) and 41.02±0.62% (***P<0.001) respectively, compared with the untreated group. The combined therapies of Huaier extract and paclitaxel (H4P0.01 and H8P0.1) induced the most apoptotic effect in (A) MCF-7 and (B) MDA-MB-231 cells. The data were analyzed by one-way ANOVA (n=3).

tumor volume in the combined group was smaller than that in the paclitaxel group (P<0.05). After the mice were sacrificed, we assessed the tumor weight (Fig. 5C and D). The tumor weight in each group was consistent with that of tumor volume in both cell lines (P<0.05). Moreover, compared with the paclitaxel group, the tumor weight in the combined group was lighter in both xenografted tumors (P<0.05). To examine the antitumor mechanisms of the Huaier extract, paclitaxel and
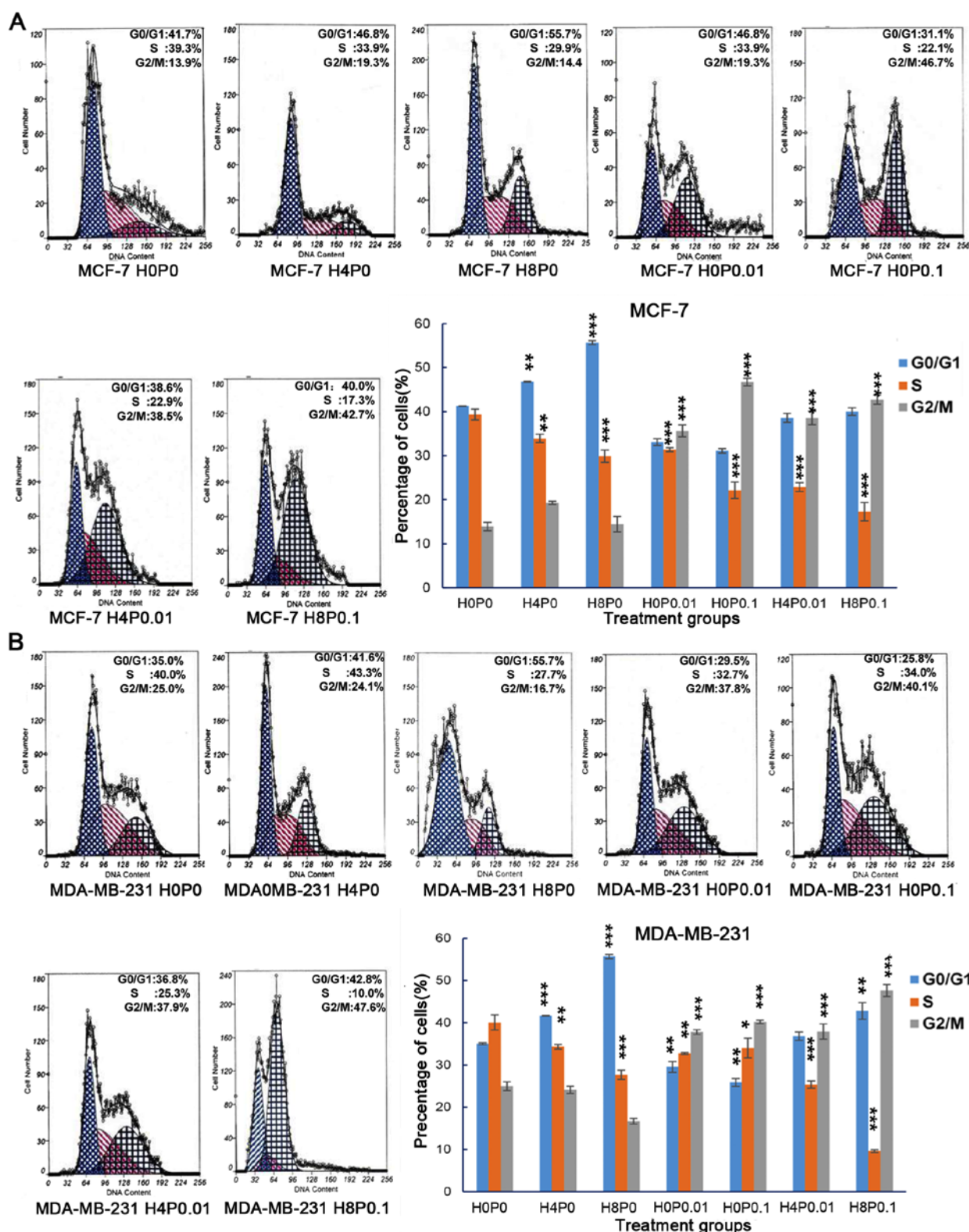
Figure 3. Huaier extract synergizes with paclitaxel to induce cell cycle arrest in MCF-7 and MDA-MB-231 cells. Huaier extract (4 or 8 mg/ml) induced G0/G1 arrest and decreased the S phase in (A) MCF-7 and (B) MDA-MB-231 cells. Paclitaxel treatment (0.01 or 0.1 μg/ml) induced G2/M arrest and decreased the S phase in (A) MCF-7 and (B) MDA-MB-231 cells. Combined treatment induced G0/G1 and G2/M arrest in MCF-7 and MDA-MB-231 cells, however the proportion of S phase cells was decreased. The data are presented as the mean ± SD of three separate experiments (*P<0.05, **P<0.01, ***P<0.001 vs. the control group).

the combined treatment *in vivo*, we analyzed the apoptosis and the expression of p65 and IκBα in xenografted tumors. As shown in Fig. 5E and F, the combined treatments caused the most significant apoptosis compared to the control and monotherapy groups. Furthermore, combined treatment significantly decreased the expression of p65 and c-Met, but increased IκBα expression in xenografted tumors compared with those from monotherapies (Fig. 5G and H).
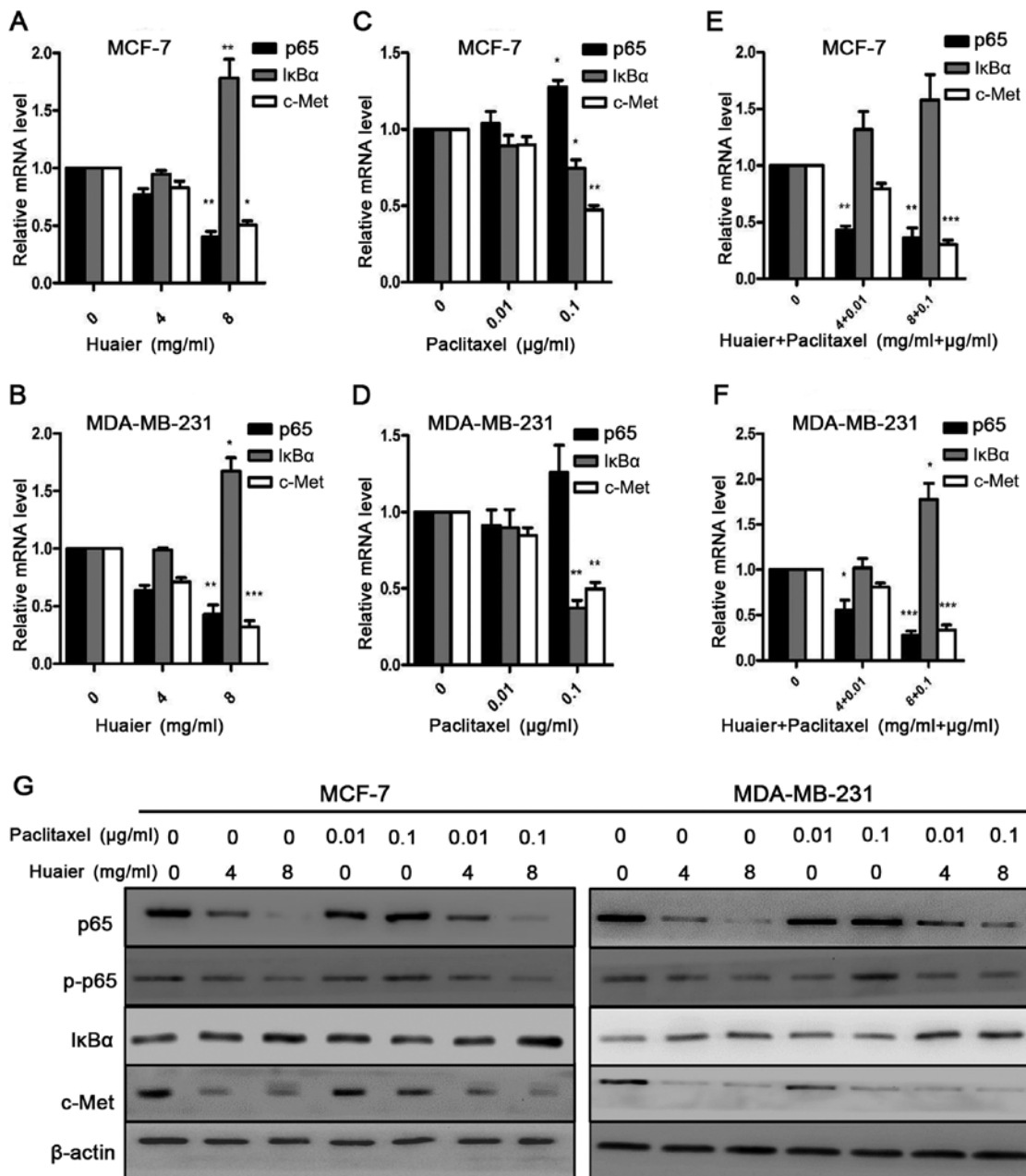
Figure 4. Huaier extract and paclitaxel target p65, IκBα and c-Met in MCF-7 and MDA-MB-231 cells. Huaier extract mediated the expression of p65, IκBα and c-Met at the mRNA levels in (A) MCF-7 and (B) MDA-MB-231 cells. Paclitaxel treatment mediated the expression of p65, IκBα and c-Met at the mRNA levels in (C) MCF-7 and (D) MDA-MB-231 cells. The effect of the combined therapies on the expression of p65, IκBα and c-Met at the mRNA levels in (E) MCF-7 and (F) MDA-MB-231 cells. (G) The protein expression levels of p65, IκBα and c-Met were altered in line with the mRNA expression level. The data are presented as the mean ± SD of three separate experiments (*P<0.05, **P<0.01, ***P<0.001 vs. the control group).

Discussion

Breast cancer, as a highly heterogeneous tumor, is significantly different in pathological type, molecular classification and prognosis. Estrogen receptor-positive breast cancer accounts for ~70% of breast cancer and triple-negative breast cancer (TNBC) accounts for ~10-20% (28). Although ER-positive breast cancer patients can benefit from endocrine therapy, chemotherapy is still an important systemic therapy after surgery for preventing recurrent and metastasis. Chemotherapy is the only treatment for TNBC presently, since TNBC is invasive, recurs and metastasizes easily and thus TNBC cannot benefit from endocrine and target therapy.

Recently, traditional Chinese medicines (TCMs) have been considered as anticancer drugs for their ability to kill cancer cells, and reduce the side-effects of chemotherapy. Huaier extract has been found to not only have an antitumor effect, but also enhance the sensitivity of cancer cells to chemotherapy drugs and improve immunity and long-term prognosis in cancer patients (29,30). Bao et al (18) found that the Huaier extract inhibited the proliferation of human hepatocellular carcinoma Hep-G2 cells and induced cell apoptosis in a concentration-dependent manner. In the present study, after treating MCF-7 and MDA-MB-231 cells with Huaier extract for 24 and 48 h, we found that Huaier extract inhibited cell proliferation, reduced cell viability
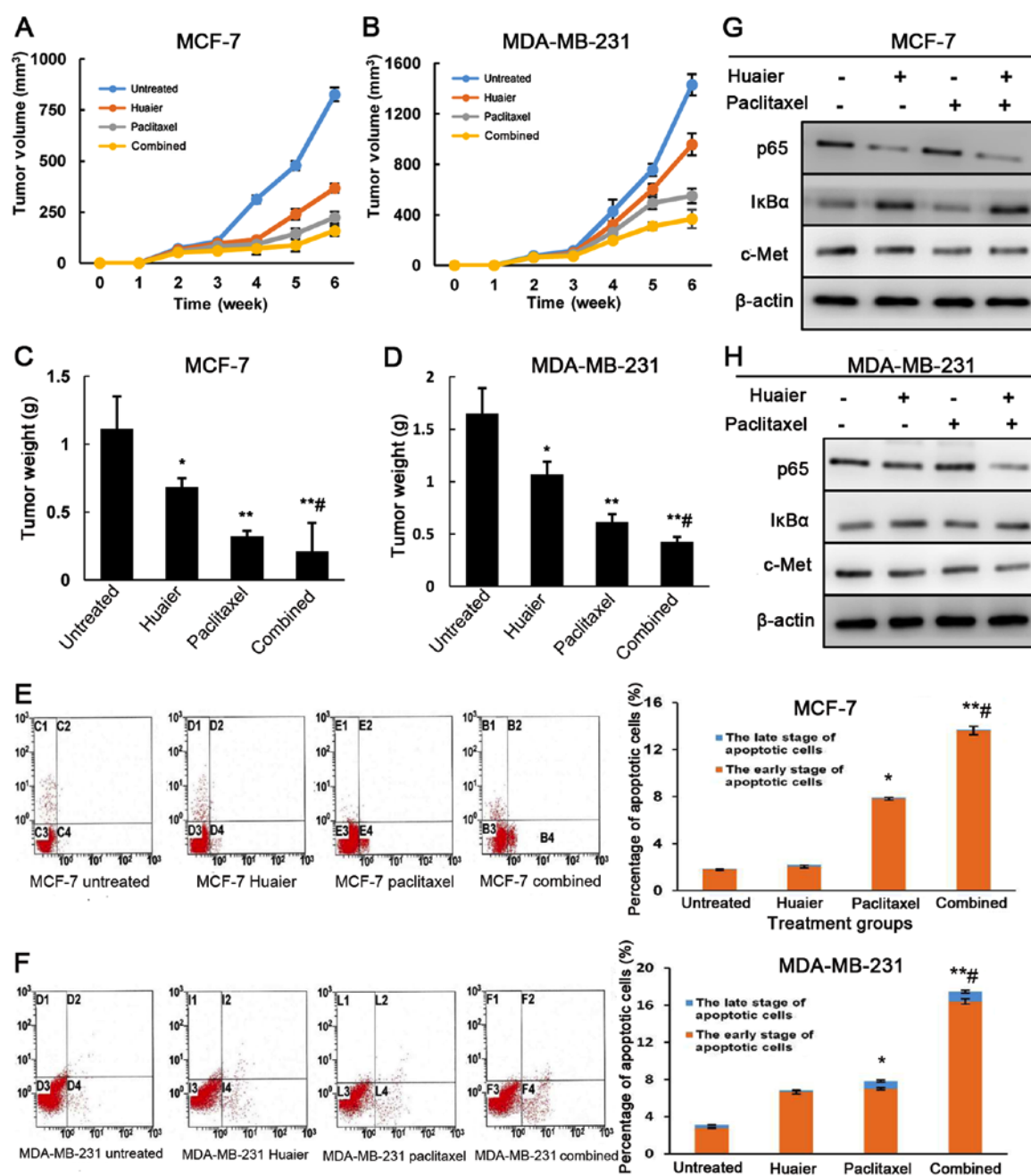
Figure 5. Huaier extract enhances the paclitaxel treatment efficacy in a xenograft model. Mice were sacrificed six weeks after being injected with either Huaier extract or paclitaxel or a combination of both. (A and B) The growth curves of xenograft tumors from MCF-7 and MDA-MB-231 cells at different time-points. In both cell lines, compared with the control group, the tumor volume in the Huaier (P<0.05), paclitaxel (P<0.01) and combined treatment groups (P<0.01) was significantly smaller. (C and D) The tumor weight from the MCF-7 and MDA-MB-231 cells in different groups. The tumor weight of the combined treatment group was significantly lower than that of the control group (P<0.01). (E and F) Flow cytometric analysis of apoptosis in MCF-7 and MDA-MB-231 xenograft tumors. The apoptosis rates of MCF-7 xenograft tumors treated with Huaier, paclitaxel and a combination of both were (2.05±0.18%), (7.82±0.05%)* and (13.62±0.09%)**# compared with the untreated group (1.94±0.03%). The apoptosis rates of MDA-MB-231 xenograft tumors treated with Huaier, paclitaxel and a combination of both were (6.63±0.21%), (6.92±0.83%)* and (16.40±1.06%)**# compared with the untreated group (2.78±0.33%). (G and H) Combination treatment decreased the level of p65 and increased IκBα. The data are presented as the mean ± SD of three separate experiments (*P<0.05, **P<0.01, ***P<0.001 vs. the control group; #P<0.05 vs. the paclitaxel group).

and promoted cell apoptosis in a time- and dose-dependent manner, results which were consistent with a study from Zhang *et al* (14). We also found that Huaier extract could induce cell cycle arrest in the G0/G1 phase, which was in line with a study by Qi *et al* (21). A previous study demonstrated that paclitaxel treatment caused cell cycle arrest in the G2/M phase, inhibited mitotic progression and induced apoptotic cell death (31). In the present study,

we found that combined therapies of Huaier extract and paclitaxel treatments in breast cancer cells induced cell cycle arrest in the G0/G1 and G2/M phases, but reduced the percentage of cells in the S phase. These results revealed that both Huaier extract and paclitaxel treatments inhibited cell proliferation activity and induced cell apoptosis in breast cancer cells, and the combined therapies achieved the most effective efficacy.

Our research also studied the relationship between the anticancer effect of paclitaxel and the NF-κB pathway. Current studies have suggested that paclitaxel functions by binding to tubulin, promoting the formation of stable microtubules and inhibiting microtubule depolymerization. Thus, paclitaxel interferes with the normal function of mitotic spindle formation, arrests the cell cycle in the G2/M phase and induces cell death in cancer (32). Our results revealed that paclitaxel reduced IκBα expression, but increased p65 expression. As the inhibitory protein of NF-κB, the reduction of IκBα expression was associated with NF-κB activation. Similar results were also observed in a study in which paclitaxel induced the degradation of the IκBα protein, which in turn activated NF-κB and induced cell apoptosis (33). Bava et al (34) reported that paclitaxel treatment after 30 min may lead to the translocation of NF-κB into the nucleus of HeLa cells. NF-κB activation plays an important role in the resistance to cytotoxic agents (such as doxorubicin), microtubule disrupting agents (such as paclitaxel) and 5-fluorouracil (27,31). Our data indicated that paclitaxel treatment for 48 h may induce drug resistance via NF-κB activation.

In a previous study, the combination of Huaier extract with chemotherapy drugs promoted tumor cell apoptosis and improved sensitivity of tumor cells to chemotherapy drugs (15), but the mechanism is still not clear. Zhang et al (35) reported that Huaier increased the sensitivity of MCF-7/A breast cancer cells to adriamycin (ADM) which was related to the decrease in the expression of drug resistant gene MDR-1. Another study revealed that Huaier extract synergized with tamoxifen (TAM) to increase apoptosis and G0/G1 arrest in ER-positive breast cancer cells (21). However, in the actively proliferative cancer cells, the proportion of G0/G1 and S phase cells was much higher than the proportion of G2/M phase cells. Paclitaxel mainly induced cell cycle arrest in the G2/M phase, while Huaier extract caused cell cycle arrest in the G0/G1 phase. Therefore, paclitaxel and Huaier complemented each other and the anticancer effect produced from their combination treatment was significantly improved. Moreover, the inhibition of NF-κB (p65) was more effective after combined therapies. A previous study suggested that the inhibition of NF-κB can improve tumor cell sensitivity to chemotherapy drugs (36). Our results revealed that compared with monotherapies, the combined therapies increased cell cycle arrest in the G0/G1 and G2/M phases, more significantly promoted cell apoptosis, inhibited NF-κB and enhanced antitumor efficacy.

The encoding product of proto-oncogene c-met is the hepatocyte growth factor receptor (also known as c-Met), which is a transmembrane tyrosine kinase. c-Met plays an important role in cell proliferation, invasion and angiogenesis (37). In breast cancer tissue particularly TNBC, c-Met was abnormally expressed (38). The phosphorylation of c-Met activated multiple downstream signaling pathways, including Ras/MAPK, PI3K/Akt and NF-κB (39). Activation of the PI3K/Akt pathway increased cell survival and inhibited apoptosis. Akt can regulate the NF-κB pathway. In a future study we would like to further investigate the relationship between drug resistance reversion by Huaier and c-Met/Akt/NF-κB. In the present study, both the Huaier extract and paclitaxel inhibited the expression of c-Met, and their combination in a high-dose group revealed the most inhibitory effect on c-Met expression.

These results revealed that combined treatments may enhance the antitumor effect of paclitaxel therapy in breast cancer cells.

Our results demonstrated that Huaier-induced inhibition of the NF-κB pathway resulted in the suppression of cell proliferation and the promotion of apoptosis. Paclitaxel-induced NF-κB activation was associated with chemoresistance and a decreased antitumor effect. Huaier reversed the effect of paclitaxel on the activation of NF-κB, and thus, suppressed cell proliferation and increased the antitumor effect. The NF-κB pathway may play an important role in the combined treatment. In future, further research into the molecular mechanisms and the proteins involved in the cell cycle and proliferation as well as resistance-associated genes regulated by NF-κB is warranted in order to obtain more valuable insights.

In summary, Huaier extract synergized with paclitaxel to suppress proliferation and increase apoptosis in ER-positive breast cancer cells and TNBC. The mechanisms were involved in inducing cell cycle arrest and cell apoptosis and inhibition of the NF-κB pathway. Moreover, the combined treatments of Hauier extract and paclitaxel were more effective on antitumor activity than the monotherapies, which may increase the anticancer effect of breast cancer cells to paclitaxel therapy.

Acknowledgements

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