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Original Article

Do Wortmannin and Thalidomide induce apoptosis by autophagy inhibition in 4T1 breast cancer cells in vitro and in vivo?

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Abstract: The aim of this study was to show the effects of autophagy inhibitor Wortmannin and antiangiogenic-proapoptotic Thalidomide on autophagy and apoptosis markers in 4T1 breast cancer cells in vitro and in vivo. The half-maximal inhibitory concentration (IC50) values of 4T1 cells for Wortmannin and Thalidomide were evaluated by Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. After cancer formation in 28 BALB/C female mice, drugs were administered for seven days. Cells and tissue sections were evaluated for anti-phosphoinositide 3-kinase (PI3K), anti- the microtubule-associated protein 1 light chain3 (MAPLC3β), anti-caspase 8, anti-caspase 9, and anti-caspase 3 immunoreactivities by immunohistochemical staining and apoptosis by Terminal Transferase dUTP Nick End Labeling (TUNEL) assay. Both PI3K and MAPLC3β immunoreactivities decreased in all treatments when compared to control group except Thalidomide treatment in primary cancer tissue. The caspase 3, 8, and 9 immunoreactivities were increased in all treatment groups and TUNEL positive cells were the highest in the Wortmannin and Thalidomide group. Our findings suggest that autophagy is an important mechanism for 4T1 cells and both Wortmannin and Thalidomide treatments inhibit autophagy and induce apoptosis. In primary cancer tissues, autophagy was not effective as in vitro. The treatment of Wortmannin and Thalidomide increased the apoptotic cells in vivo independent from autophagy inhibition. Different results may be because of microenvironment. Further studies must be done to elucidate the effect of microenvironment.

Keywords: Breast cancer, wortmannin, thalidomide, autophagy, apoptosis

Introduction

Breast cancer is the most common non-skin cancer in women [1]. For the treatment of the breast cancer, investigation of molecular mechanisms which effect the cell proliferation, survival and death are now at the forefront. Autophagy is a process where intracellular molecules or organelles are enveloped in double-membraned vesicles, known as an autophagosomes, which combine with lysosomes where the contents are degraded and reused in the cytosol [2]. The autophagic progress can be divide into four distinct stages: 'initiation, nucleation, autophagic vesicle maturation, fusion with the lysosome and degradation of vesicle content' [3].

Autophagy may play a dual role in cancer whether induction or suppression of tumorigenesis, according to type, time and metabolism of cancer tissue. In the early stages of cancer, autophagy suppresses tumor growth. At the later stages of cancer, autophagy supports the cancer cells in order to survive in hypoxic conditions, nutritional deficiency, and therapeutic stresses [3]. In addition, cancer cells may be dependent on autophagy to use as an energy source [3, 4]. Furthermore, autophagy supports cancer cells in metastasis [5]. These findings suggest that

autophagy inhibition could be used as a new strategy to treat progressive cancer cases.

The autophagy inhibitors can be classified into four groups: 1) Repressors of autophagosome formation (3-methyladenine, Wortmannin, Viridiol), 2) Repressors of lysosomal acidification (Chloroquine, hydroxychloroquine), 3) Inhibitors of autophagosome lysosome fusion (Bafilomycin, Concanamycin) and 4) Silencing expression of autophagy related proteins [5]. Many studies have reported that using 3-methyladenine or chloroquine/hydroxychloroquine with chemotherapeutic agents induced chemosensitivity and cytotoxic effects in gastric cancer, glioblastoma, and breast cancer cell lines [6-8].

Wortmannin is a steroid metabolite of the Penicillium funiculosum. It is an irreversible phosphoinositide 3-kinase (PI3K) inhibitor that suppresses the PI3K-AKT signal pathway which affects cell cycle progression and apoptosis [9, 10]. In addition to this, Wortmannin is widely used as an autophagy inhibitor [4] and can inhibit autophagosome formation by blocking the class III PI3K pathway in the early nucleation phase [4]. In one study using size- adjustable micelles coloading with wortmannin and doxorubicin suppressed melanoma and breast cancer in mice [4]. In another study usage of wortmannin and cisplatin synergistically enhanced chemoradiotherapy in ovarian cancer models [11]. Although wortmannin is mentioned as an autophagy inhibitor, there are limited studies showing its use in cancer treatment.

Glutamic acid derivative, Thalidomide has an antitumor property [12, 13]. The real mechanism has not yet been clarified, but it is known that the effects are related to anti-inflammatory, anti-angiogenic, anti-proliferative, and pro-apoptotic activities [12, 14-16]. The antiangiogenic effect mechanism of Thalidomide appears to involve blocking of growth factors (VEGF, FGF) [12, 14-17]. The effects of thalidomide have been investigated in a number of cancer types, including advanced breast cancer, gliomas, lung cancer, lymphoma, multiple myeloma, and prostate cancer [18].

In this study, we evaluated autophagy inhibitor Wortmannin and antiangiogenic-proapoptotic Thalidomide's effects on 4T1 cells in vitro and in vivo.

Material and methods

The study was carried out in Manisa Celal Bayar University, Faculty of Medicine, Department of Histology and Embryology. The study was conducted with the approval of the Experimental Animal Research Ethics Committee of Manisa Celal Bayar University Faculty of Medicine (20.478.486).

Cell culture

4T1 murine breast cancer cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle Medium and Ham's F-12 medium (1:1) (Biological Industries, Kibbutz Haemek, Israel) containing 10% fetal bovine serum (FBS, Biological Industries, Kibbutz Haemek, Israel), 1% penicillin-streptomycin (Capricorn Scientific, Ebsdorfergrund, Germany), 0.5% Non-essential aminoacids (NEAA, Lonza, Verviers, Belgium), 1% L-glutamine (Capricorn Scientific, Ebsdorfergrund, Germany) at 37°C in 5% CO_2.

Determination of IC50 of wortmannin and thalidomide by MTT (Methylthiazolyldiphenyl-tetrazolium bromide) assay

Cytotoxicity of Wortmannin (Sigma-Aldrich, St. Louis, Missouri, USA) and Thalidomide (Cayman Chemical Company, Ann Arbor, Michigan, USA) on murine 4T1 breast cancer cells was measured by the 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells (0.8×10^5 cells/well) were seeded in a 96-well plate overnight. Then serial dilution (10 µM, 1 µM, 0.1 µM, 0.01 µM, 0.001 µM) of Wortmannin and Thalidomide was applied to the plate and incubated for 24 h. Afterwards, the MTT solution was added to all the wells and the plates were incubated for 4 hours to allow the formation of formazan crystals. Then the formazan crystals were solubilized by dimethyl sulfoxide (DMSO) (Fisher Scientific, New Hampshire, USA) and the absorbance [optical density (OD)] was measured using a spectrophotometer (Bio-Tek Instruments, Vermont, USA) at 570 nm. The assay was done in triplicate, and the cytotoxic effect of Wortmannin and Thalidomide was analyzed using the following formula: Cell viability (%) = (OD sample/OD control) × 100%.

Wortmannin and thalidomide treatments

Groups were designated as Group 1 (Control group, no drug treated), Group 2 (0.01 μM Wortmannin treated), Group 3 (0.1 μM Thalidomide treated), Group 4 (0.01 μM Wortmannin and 0.1 μM Thalidomide treated). After the 4T1 breast cancer cells (8×10^4 cells/mL) were cultured in the 24-well plate, the IC50-determined Wortmannin (0.01 μM) and Thalidomide (0.1 μM) dose was applied to cells and incubated for 24 hours.

Indirect immunocytochemical staining of cells

After drug treatment, 4% paraformaldehyde was used for fixation of cells. Then cells were washed with phosphate buffered saline solution (PBS) and 0.1% Triton X-100 (A4975, AppliChem, Darmstadt, Germany) was used to permeabilize the cells. Endogenous peroxidase activity was inhibited by 3% hydrogen peroxide (H_2O_2, K31355100, Merck, Darmstadt, Germany) for 5 min at room temperature then blocking serum was applied to cells for 1 hour. After the removal of blocking serum, anti-MAP LC3β (sc-271625, Santa Cruz, Dallas, Texas, USA), anti-PI3K (sc-1637, Santa Cruz, Dallas, Texas, USA), anti-caspase 8 (sc-70501, Santa Cruz, Dallas, Texas, USA), anti-caspase 9 (sc-56076, Santa Cruz, Dallas, Texas, USA) and anti-caspase 3 (sc-56053, Santa Cruz, Dallas, Texas, USA) primary antibodies were incubated at 4°C for overnight. After washing 3 times with PBS, the secondary antibodies, biotinylated IgG and peroxidase-conjugated streptavidin (85-9043, Invitrogen, Camarillo, CA, USA) were applied for 30 min for each step. Cells were then incubated with diaminobenzidine (00-2020, Invitrogen) and counterstained with Mayer's hematoxylin (02274390059, J.T. Barker, Deventer, The Netherlands). Then cells were mounted on to slides with mounting medium (AML060, Scytek, UT, USA), and examined under a light microscope (Olympus BX43, Olympus Corp., Tokyo, Japan).

All experimental procedures were repeated three times and intensities of 100 cells were evaluated in three different areas. The staining intensities were evaluated by two observers who are blinded to experiments. The mean values of the staining intensities were calculated using the H-Score (H-Score = $\sum$ Pi (i+1), i; the intensity of staining with a value of (±), (1), (2) or (3) (mild, moderate or strong respectively) and Pi; the percentage of cells stained with each intensity, varying between 0-100%).

TUNEL assay

Terminal Transferase dUTP Nick End Labeling (TUNEL) staining method was used to determine apoptotic cell death. Following the deparaffinization, TUNEL staining was performed according to the manufacturer's instructions of ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Millipore, Massachusetts, USA). TUNEL positive cells were detected after staining and the results were evaluated statistically.

Animal experiment

The study was carried out in Manisa Celal Bayar University Experimental Animal Research and Application Center using 28 BALB/C strain 8-10 weeks old female mice weighing 20 ± 8 grams. Mice were observed for two weeks for any signs of health problems for adaptation. During the study the animals were maintained under stable conditions such as 22°C temperature, 30-70% humidity, 12/12 hr light/dark cycle. They were fed with standard mouse feed and tap water. In order to establish the breast cancer model in mice, metastatic 4T1 mouse breast cancer cells were applied to female mice at 1×10^6/100 μl cells per animal by subcutaneous injection at the right first breast level. After 15 days of cancer formation mice were separated into four groups, Group 1 (Cancer group, no drug treated), Group 2 (0.7 mg/kg ip Wortmannin treated), Group 3 (200 mg/kg sc Thalidomide treated), Group 4 (0.7 mg/kg ip Wortmannin and 200 mg/kg sc Thalidomide treated).

Histopathologic evaluation

After the seven days of drug treatment mice were anesthetized with 80-100 mg/kg ketamine (Ketalar, 002038, Eczacıbası Health Products Industry & TradeCo., Luleburgaz, Turkey) and 5-10 mg/kg xylazine (Alfazyne, 080412511, Alfasan, Woerden, Holland) intraperitoneally. Then mice were sacrificed and tissue samples were taken and fixed in 10% buffered formalin solution for 48 hours. After formalin removal in running water, samples were embedded in paraffin blocks following routine tissue follow-up procedure. Sections of approxi-

Table 1. The IC50 values of wortmannin and thalidomide on 4T1 breast cancer cells

Drug	IC50 Value ± SD (μM)
	4T1 Cells
Thalidomide	0.117 ± 0.02
Wortmannin	0.0132 ± 0.007

mately 5 μm thickness were taken from the paraffin blocks with rotator microtome.

Immunohistochemistry

For immunohistochemical staining, sections were incubated at 60°C overnight and then held in xylene and rehydrated through a series of ethanol solutions. Sections were washed with distilled water and phosphate-buffered saline (PBS, P4417, Sigma-Aldrich, St. Louis, Mo, USA) for 10 min and then treated with 0.1% trypsin (T7409, Sigma-Aldrich) at 37°C for 10 min and washed with PBS. Sections were incubated in a solution of 3% H_2O_2 for 5 min to inhibit endogenous peroxidase activity. After washing in PBS, sections were incubated with non-immune serum for 1 h and then sections were incubated with primary antibodies: anti-MAP LC3β (sc-271625, Santa Cruz, Dallas, Texas, USA), anti-PI3K (sc-1637, Santa Cruz, Dallas, Texas, USA), anti-caspase 8 (sc-70501, Santa Cruz, Dallas, Texas, USA), anti-caspase 9 (sc-56076, Santa Cruz, Dallas, Texas, USA) and anti-caspase 3 (sc-56053, Santa Cruz, Dallas, Texas, USA) overnight at 4°C in a humidity chamber. Sections were washed 3 times for 5 min each with PBS, followed by incubation with biotinylated secondary antibody and then with streptavidin conjugated to horseradish peroxidase in PBS for 30 min. After washing 3 times with PBS, sections were incubated with DAB for 5 min to determine the visibility of the immunohistochemical reaction. After washing with distilled water, sections were counterstained with Mayer's hematoxylin and washed with distilled water. Then mounted with entellan then viewed under a light microscope (Olympus BX43, Olympus Corp., Tokyo, Japan).

Statistical analysis

All data are expressed as the mean ± SD, and statistical analysis was done with GraphPad Prism 7.0. Multiple comparisons were performed using one-way ANOVA test followed by Tukey correction. The value of $P<0.05$ was considered significant.

Results

The cytotoxic effect of wortmannin and thalidomide on 4T1 breast cancer cell line

Cytotoxicity towards 4T1 breast cancer cell line was evaluated for Wortmannin and Thalidomide. IC50 values were obtained from the MTT assay. In this experiment, thalidomide and wortmannin exhibited IC50 values of 0.117 ± 0.02 μM and 0.0132 ± 0.007 μM, respectively (**Table 1**).

In vitro effects of wortmannin and thalidomide on 4T1 cells

We used Wortmannin to inhibit autophagy by the PI3K pathway. We observed moderate cytoplasmic PI3K immunoreactivity in half of the cells in Control group. PI3K immunoreactivity was mild in Wortmannin and Wortmannin-Thalidomide treatment groups. Also the positive stained cells were much more in Wortmannin-Thalidomide treatment when compared to Wortmannin group. In the Thalidomide group PI3K immunoreactivity was mild to moderate (**Figures 1A-D**, **3A**).

Moderate to strong cytoplasmic MAPLC3β immunoreactivity was observed in the control group as autophagy marker in most of the cells. In wortmannin and wortmannin-thalidomide treatment groups it was seen mild MAPLC3β immunoreactivity in a few cells. In thalidomide group MAPLC3β immunoreactivity was moderate in half of the cells (**Figures 1E-H**, **3B**).

Caspase 8 immunoreactivity was mild in a few cells in the control and the thalidomide groups. In the wortmannin treatment group Caspase 8 immunoreactivity was increased and almost all cells were stained. While in wortmannin -Thalidomide treatment group Caspase 8 immunoreactivity was strong in a few cells (**Figures 1I-L**, **3C**).

Caspase 9 immunoreactivity was mild in a few cells in the control group. In the wortmannin treatment group Caspase 9 immunoreactivity was strong, and all the cells were stained. In the thalidomide group Caspase 9 immunoreactivity was mild to moderate while in the wort-

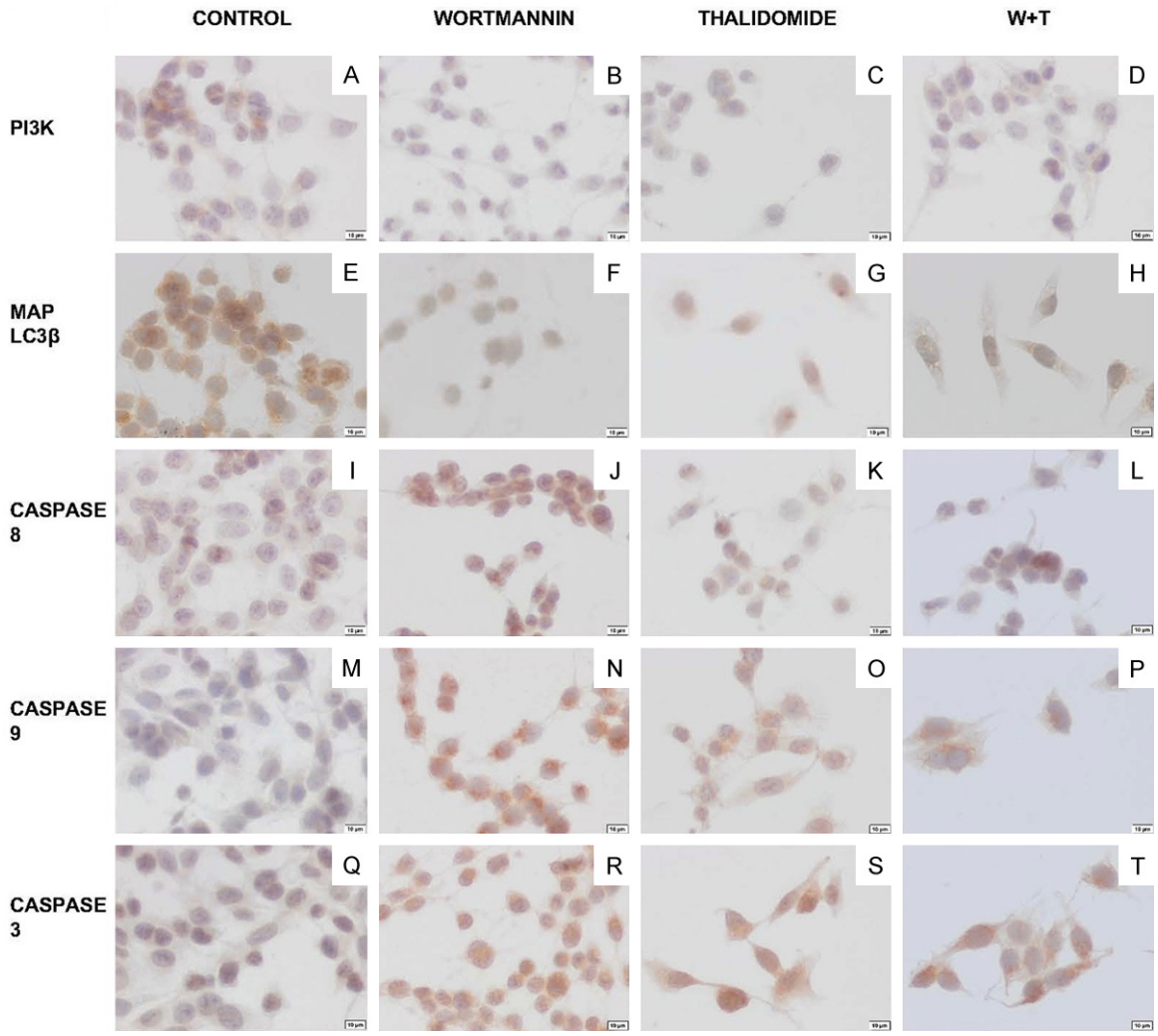

Figure 1. Distributions of PI3K (A-D) MAPLC3β (E-H), Caspase 8 (I-L), Caspase 9 (M-P), and Caspase 3 (Q-T) immunoreactivities of Control, Wortmannin, Thalidomide and Wortmannin-Thalidomide treatment groups.

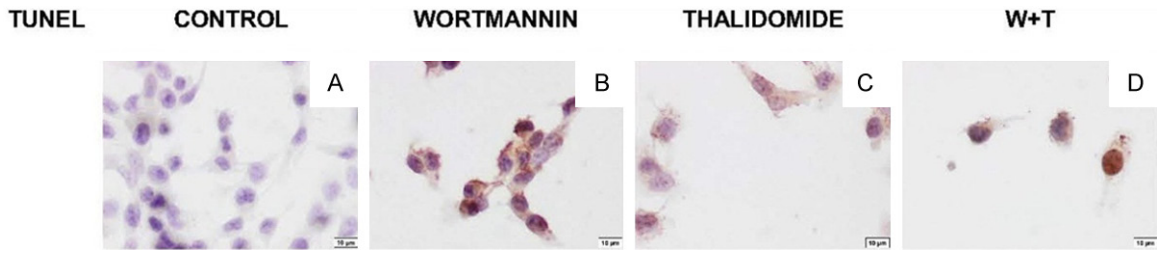

Figure 2. TUNEL staining of Control (A), Wortmannin (B), Thalidomide (C), and Wortmannin-Thalidomide (D) treatment groups.

mannin-thalidomide treatment group it was strong almost in all cells (**Figures 1M-P**, **3D**).

Caspase 3 immunoreactivity was mild in the control in half of the cells. In the other groups, Caspase 3 immunoreactivity and the stained cell number was observed to be increased (**Figures 1Q-T**, **3E**).

In all groups there were TUNEL positive cells, but an increased number of apoptotic cells were observed especially in wortmannin

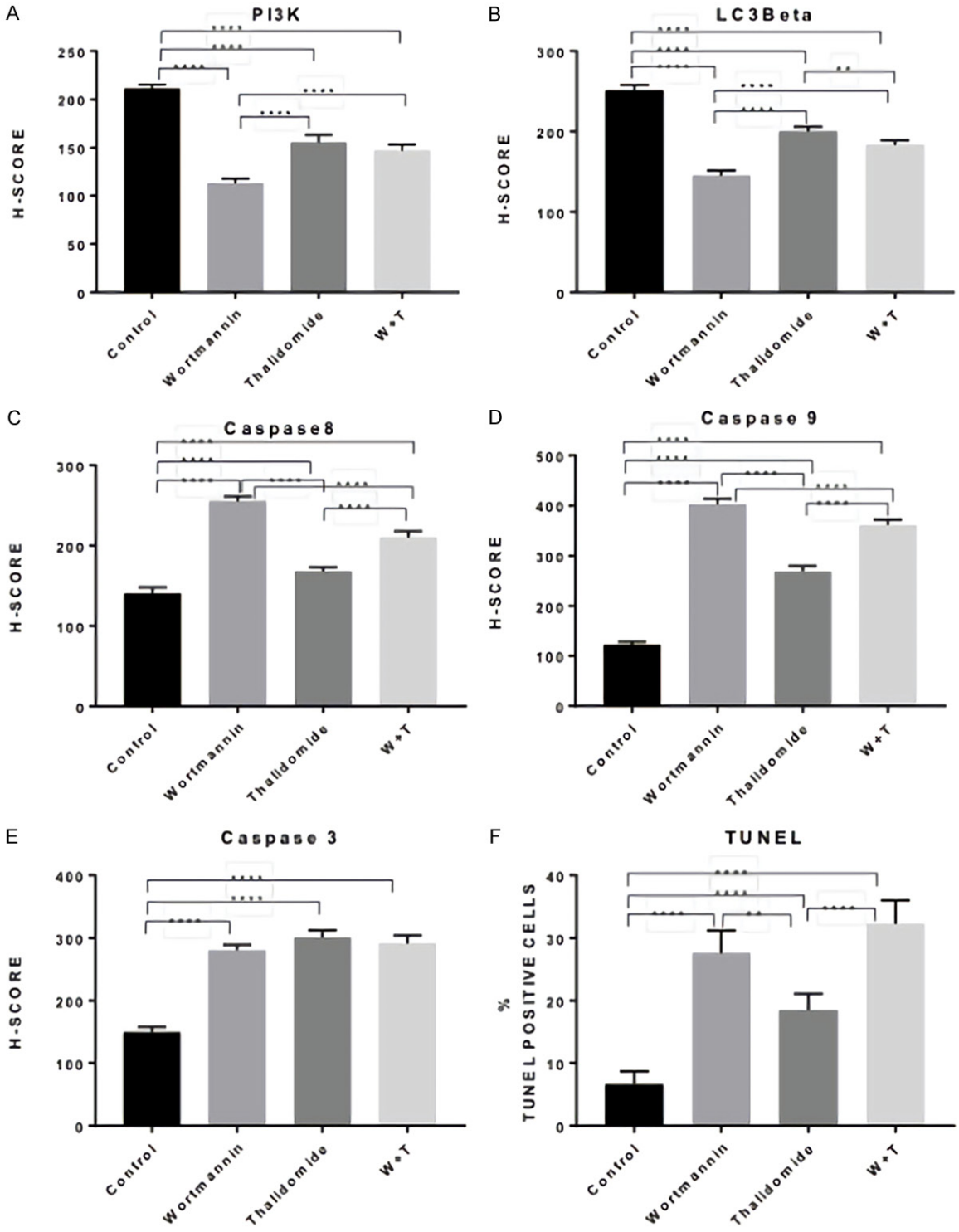

Figure 3. Immunohistochemical staining intensity (H-Score) of PI3K (A), MAPLC3β (B), Caspase 8 (C), Caspase 9 (D), Caspase 3 (E) primary antibodies, and % of TUNEL positive cells (F) of control, Wortmannin, Thalidomide and Wortmannin-Thalidomide treatment groups. Experiments were performed in triplicate and repeated three times with similar results. Bars display mean ± S.D. One-way ANOVA ($P<0.05$) and Tukey correction were performed using GraphPad Prism 7® to test the differences of anti-PI3K (A), anti-MAPLC3β (B), anti-Caspase 8 (C), anti-Caspase 9 (D), anti-Caspase 3 (E) immunoreactivity, and % of TUNEL positive cells between Control, Wortmannin, Thalidomide, and Wortmannin-Thalidomide treated clams (****:<0,0001, MAPLC3β**: 0,0035, TUNEL**: 0,0014).

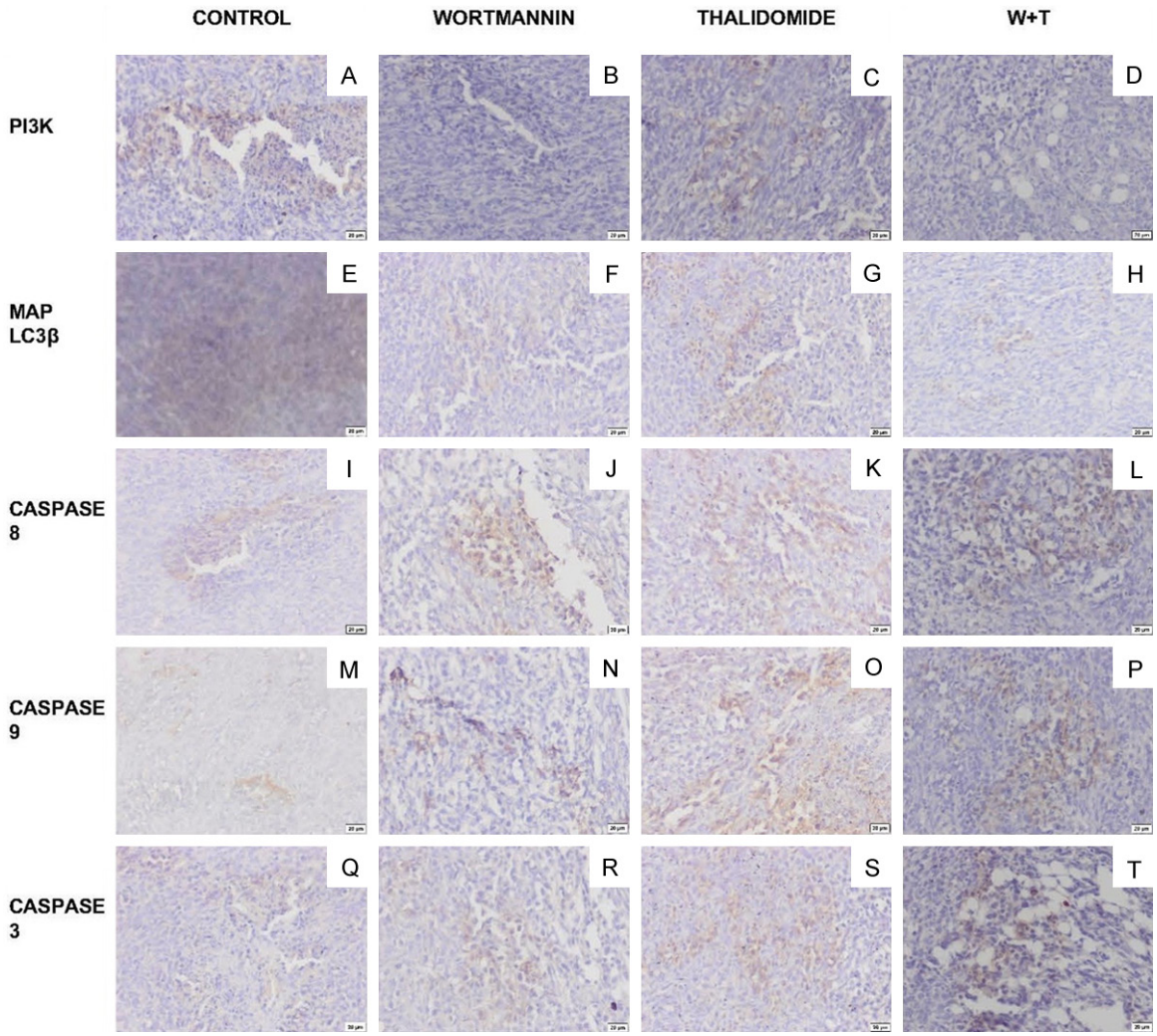

Figure 4. Distributions of PI3K (A-D) MAPLC3β (E-H), Caspase 8 (I-L), Caspase 9 (M-P), and Caspase 3 (Q-T) immunoreactivities of Control, Wortmannin, Thalidomide, and Wortmannin-Thalidomide treatment groups in primary tumor tissues

and wortmannin-thalidomide treated groups (**Figures 2A-D**, **3F**).

In vivo effects of wortmannin and thalidomide on primary tumor tissues

In primary tumor tissues we observed moderate PI3K immunoreactivity in the control and the thalidomide groups while it was negative in both the wortmannin and the wortmannin-thalidomide treatment groups (**Figures 4A-D**, **6A**). MAPLC3β immunoreactivity was mild to moderate in the control, mild in wortmannin and wortmannin-thalidomide treatment, moderate in thalidomide groups (**Figures 4E-H**, **6B**).

In primary tumor tissues, Caspase 8 immunoreactivity was mild to moderate in the control, thalidomide and wortmannin-thalidomide treatment groups while it was moderate in wortmannin group (**Figures 4I-L**, **6C**).

Caspase 9 immunoreactivity was mild to moderate in control, moderate in wortmannin and wortmannin-thalidomide treatment groups, moderate to strong in thalidomide group (**Figures 4M-P**, **6D**).

In primary tumor tissues, Caspase 3 immunoreactivity was mild in control, mild to moderate in wortmannin, moderate in both thalidomide and wortmannin-thalidomide treatment groups (**Figures 4Q-T**, **6E**).

In all groups of primary tumor tissues there were TUNEL positive cells, but an increased

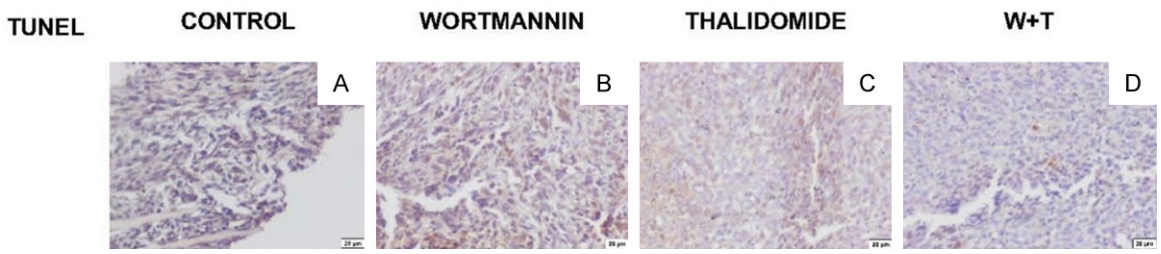

Figure 5. TUNEL staining of Control (A), Wortmannin (B), Thalidomide, (C) and Wortmannin-Thalidomide (D) treatment groups in primary tumor tissues.

number of apoptotic cells were observed especially in wortmannin treated group (**Figure 5A-D**).

Discussion

Autophagy supports cells to preserve homeostasis and adapt to stressful conditions, such as hypoxia, nutrient deprivation, and anticancer therapy. Previous studies have shown that autophagy which protects cancer cells and decreases chemosensitivity can be induced by chemotherapeutic drugs in several tumors such as oral squamous carcinoma, head and neck cancers, and gastric cancer [6, 19-22]. The combination of chemotherapeutic drugs with autophagy inhibitor Chloroquine (CQ) was seen to induce cytotoxic effects and chemosensitivity in glioblastoma cells [6, 7]. Lei et al. demonstrated that autophagy inhibition with 3-Methyladenine (3-MA) or CQ enhanced chemosensitivity to cisplatin in gastric cancer both in vivo and in vitro [6]. Cotzomi-Ortega et al. showed that inhibition of autophagy with CQ decreased cell viability and increased cell death more in metastatic cell lines (66cl4 and 4T1) than in the non-metastatic cell line (67NR) [8]. In this study, we observed wortmannin and thalidomide treatments inhibit autophagy and induce apoptosis on 4T1 cells in vitro. In primary tumor tissues only an effect of wortmanin and combination treatments were seen.

The class III PI3K family member, VPS34 (PIK3C3) controls autophagy at the initiation and maturation stages by making autophagosomes with regulator proteins of autophagy. Autophagy can be blocked with the PI3K inhibitors (3-MA, Wortmannin, LY294002, SAR405, and Viridiol) by inactivating the production of PI3P, which is essential for the recruitment of other ATG proteins at the isolation membrane or phagophore [5, 23, 24]. In this study, we used wortmannin which is an irreversible PI3K inhibitor to suppress autophagy.

Thalidomide is considered a potent teratogen, but is now used to treat various types of cancer (prostate cancer, glioblastoma, glioma, renal cell carcinoma, colon cancer, and advanced breast cancer) with antiangiogenic and immunomodulatory properties [25-28].

In the breast cancer model, thalidomide has been shown to increase the antitumor effect of carboplatin, even though the mechanism has still not been adequately described [12, 28].

Thalidomide can restrict angiogenesis by inhibiting PI3K/AKT pathway [27]. It is suggested that thalidomide has inhibitory effects towards PI3K by stabilizing PTEN (phosphatase and tensing homolog gene) and thus induces limb anomalies [29]. Another study demonstated PI3K inhibition with thalidomide [30]. This study investigated whether thalidomide exhibited antitumor feature by suppressing autophagy over PI3K.

To confirm inhibition of autophagy we examined the PI3K and MAPLC3β (The microtubule-associated protein 1 light chain3) immunoreactivities on 4T1 cells and primary tumor tissues. PI3K immunoreactivity, in the control group was moderate, while in the other groups the intensity and the positive stained cells were observed to be decreased and showed a statistically significant ($P<0.001$) in vitro. While wortmannin was more effective to inhibit PI3K when compared to thalidomide or combination groups ($P<0.001$) in cells. In primary tumor tissues thalidomide was not effective to inhibit PI3K, but wortmannin and combination groups were more effective ($P<0.001$).

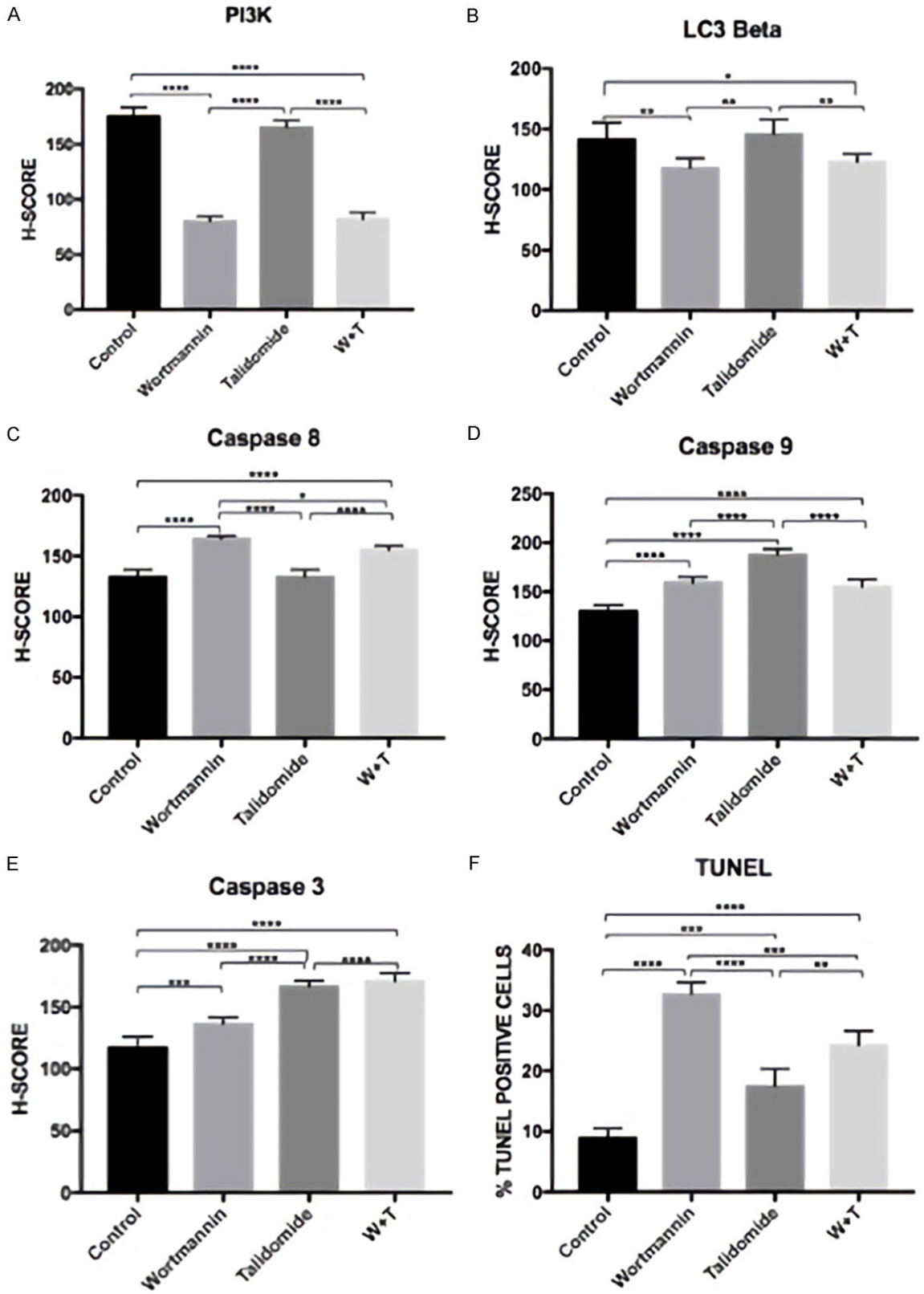

Figure 6. Immunohistochemical staining intensity (H-Score) of PI3K (A), MAPLC3β (B), Caspase 8 (C), Caspase 9 (D), Caspase 3 (E) primary antibodies, and % of TUNEL positive cells (F) of Control, Wortmannin, Thalidomide and Wortmannin-Thalidomide treatment groups in primary tumor tissues. Experiments were performed in triplicate and

repeated three times with similar results. Bars display mean ± S.D. One-way ANOVA (P<0.05) and Tukey correction were performed using GraphPad Prism 7® to test the differences of anti-PI3K (A), anti-MAPLC3β (B), anti-Caspase 8 (C), anti-Caspase 9 (D), anti-Caspase 3 (E) immunoreactivity, and % of TUNEL positive cells between Control, Wortmannin, Thalidomide, and Wortmannin-Thalidomide treated clams (****: P<0,0001, MAPLC3β Control vs Wortmannin**: 0,0095, MAPLC3 β Wortmannin vs Thalidomide**: 0,0090, MAPLC3β Thalidomide vs W+T: 0,0065, MAPLC3β Control vs W+T: 0,0102, Caspase8*: 0,0235, Caspase3***: 0,0006, TUNEL Control vs Thalidomide***: 0,0004, TUNEL Wortmannin vs W+T***: 0,0002, TUNEL Thalidomide vs W+T**: 0,0025).

MAPLC3β is a ATG8 family member which contributed in autophagosome formation. It is defined as an autophagy marker [4]. Rao et al demonstrated that the ratio of LC3-II/LC3-I decreased in the treatment of chemotherapeutic drug doxorubicin and the autophagy inhibitor wortmannin in 4T1 cells [4]. In this study, we observed decreased MAPLC3β immunoreactivity in all treatment groups when compared to the control group (P<0.001), except thalidomide treated primary tumor tissue. The treatment group with the lowest MAPLC3β immunoreactivity was wortmannin treated groups both in vitro and in vivo. These findings suggested that autophagy was an active cell survival mechanism in 4T1 murine breast cancer cells but not in primary breast cancer tissues. Therefore, inhibition of autophagy with wortmannin is more successful in cell culture than in primary breast cancer tissues.

Autophagy inhibits apoptosis by degradation of proapoptotic proteins and mitochondria which is undergoing mitochondrial outer membrane permeabilization (MOMP) [24]. In previous studies, it was shown that inhibition of autophagy induces apoptosis in several tumors [8, 23, 24, 31-35]. Under nutrient deficient conditions, autophagy inhibition induced cell death in HeLa cells [24, 34]. Thorburn et al demonstrated that when autophagy was inhibited, PUMA (modulator of apoptosis) protein levels were selectively increased [36]. CQ treatment suppresses autophagy induced by PI3K inhibition and potentiates apoptosis [37]. Apoptosis in lung and colon cancer with 5-FU and cisplatin increased with autophagy inhibition through 3-MA [5, 32, 33]. Similarly, wortmannin was used as an adjuvant for cisplatin during chemotherapy which increase apoptosis and reduce the resistance to ovarian cancer cells [24, 35].

In this study, all treatments were effective to induce apoptosis when we compared to the control group (P<0.001). Both intrinsic (caspase 9) and extrinsic (caspase 8) pathways were activated in all treatments. Wortmannin treatment used both intrinsic (caspase 9) and extrinsic (caspase 8) pathway. Thalidomide and combination treatments used intrinsic pathway, rather than an extrinsic pathway of apoptosis both in vitro and in vivo.

In summary, our study suggests that cancer cells use autophagy as a survival mechanism and inhibition of autophagy enhances apoptosis in vitro. Autophagy inhibition was not significant as cell culture in primary cancer tissues, but still significant enough to induce apoptosis. This diversity may be related to the microenvironment of the cancer. The limitation of the study was not studying any chemotherapeutic drug with wortmannin and thalidomide treatment. Further studies will help to understand microenvironment effects on treatment of the cancer with these drugs.

Disclosure of conflict of interest

None.

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