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A preliminary study

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BACKGROUND: *Breast cancer (BC) is a major public health problem diagnosed in more than 2 million women worldwide in 2018, causing more than 600,000 deaths. 90% of deaths due to breast cancer are caused by metastasis. Metastasis is a complex process that is divided into several steps, including separation of tumor cells from the primary tumor, invasion, cell migration, intravasation, vasculature survival, extravasation, and colonization of the secondary site. Astaxanthin (AXT) is a marine-based ketocarotenoid that has many different potential functions such as anti-oxidant, anti-inflammatory and oxidative stress-reducing properties to potentially reduce the incidence of cancer or inhibit the expansion of tumor cells. This study aims to investigate the effects of astaxanthin as a new metastasis inhibitor on T47D human invasive ductal carcinoma breast cancer cell.*
MATERIAL AND METHODS: *To investigate the effects of the astaxanthin as a new metastasis inhibitor on T47D cell, expression levels of anti-maspin, anti-Kai1, anti-BRMS1, and anti-MKK4 were examined by western blot. Also, we evaluated differences of these suppressors expression levels in tissue sections of 10 patients diagnosed with in situ and invasive ductal carcinoma by immunohistochemistry method.*
RESULT: *250 μM astaxanthin increased the activation of all metastasis suppressing proteins. Also, these metastasis suppressors showed higher expression in invasive ductal carcinoma tissues than in situ ductal carcinoma patients.*
CONCLUSION: *We think that astaxanthin is a promising therapeutic agent for invasive ductal carcinoma patients. The effects of astaxanthin on metastasis in breast cancer should be investigated further based on these results.*

KEY WORDS: Breast, cancer, metastasis

Introduction

The population growth, aging of the increasing population, and changes in lifestyles all over the world increase cancer cases and mortality rates every year. Breast cancer in women ranks first among the causes of female deaths in developing countries, surpassing lung cancer. According to the statistics of Globocan, approximately 520,000 people died of cancer worldwide in 2012, and breast cancer accounts for 25% of these deaths [1].
Metastasis is a complex process that is divided into several steps, including separation of tumor cells from the primary tumor, invasion, cell migration, intravasation, vasculature survival, extravasation, and colonization of the secondary site. Metastasis accounts for most cancer-related morbidity and mortality [2]. The metastatic cascade is a gradual process that consists of the separation of cells from the primary tumor, their spread through the lymphatic or circulatory system, followed by the formation of micrometastatic foci. They form secondary tumors in distant organs after colonization and growth.

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These distant organs include structures such as the brain, lungs, and bones [3,4]. Therefore, effective prevention and treatment of metastasis is the main focus of research in breast cancer. Metastasis suppressors, BRMS1, NM23, Maspin, KAI1, KISS1, and MKK4 are proteins that block metastasis without inhibiting primary tumor formation [5].

Breast cancer metastasis suppressor 1 (BRMS1) was identified as a suppressor of breast cancer metastasis in the late 1990s. In vitro and in vivo studies have shown that the strong metastasis-suppressing effect of BRMS1 is not limited to breast cancer. BRMS1 mRNA expression is also observed in the human bladder cancer cell line T24 [5-7]. BRMS1 DNA from many species, including yeast, mouse, rat, rabbit, cow, and human, shows that BRMS1 is evolutionarily conserved [6].

CD82, one of the members of the tetraspanin family, shows reduced expression in malignant tumors and is closely related to the malignancy and prognosis of the tumor. Metastasis suppressor protein CD82 / KAl1 is a biological marker to determine the metastatic potential of solid tumors [8]. They show high expression in healthy cells, but they are decreased or they show no expression in tumor tissues [9]. Clinically, protein expression of CD82 is negatively associated with malignancy and metastatic capacity of tumors. Detection of CD82 protein expression can be used as an important indicator to evaluate tumor metastasis and prognosis [10]. There is a trend towards a decrease in CD82 protein and mRNA expression levels in tissues during the transformation of healthy breast tissue into invasive ductal breast cancer [1].

Maspin (mammary serine protease inhibitor) is a member of the superfamily of serine protease inhibitors / no inhibitors. While maspin expression is down-regulated in breast, prostate, stomach, and melanoma cancers; it is overexpressed in pancreatic, gallbladder, colorectal, and thyroid cancers. This suggests that maspin may exhibit different activities in different cell types. Since tumor metastasis requires detachment of tumor cells from the basement membrane and invasion through the stroma, selectively increasing adhesion by maspin expression may contribute to the inhibition of tumor metastasis [11]. Maspin, which is generally silenced in cancer cells, shows suppressive activity against tumor growth and metastasis. Maspin is involved in processes that are important for both tumor growth and metastasis, such as cell invasion, angiogenesis, and more recently apoptosis.

Mitogen-activated protein (MAP) kinase kinase 4 (MKK4) is a component of the stress-activated MAP kinase signaling modules. Mitogen-activated protein (MAP) kinase signaling pathways are important mediators of cellular responses such as growth factors, hormones, cytokines, and environmental stresses versus extracellular signals [12]. Some MKKs can phosphorylate and activate JNK and p38. MKK4 can activate both JNK and p38 [13]. While some studies focus on the tumor and metastasis suppressor role of MKK4, some studies indicate that MKK4 has a pro-oncogenic role. While MKK4's loss of function may play a role in the formation of some primary tumors, it may also be linked to more advanced stages of cancer progression [14-16].

Astaxanthin is a reddish pigment that belongs to the carotenoid group. Astaxanthin occurs naturally in some algae and causes a pink or red color in seafood such as salmon, trout, lobster, shrimp. Astaxanthin, first discovered in lobsters in 1983, is used for pigmentation in aquaculture. Astaxanthin was approved for use as a food supplement in 1991 due to its antioxidant properties, biological and physiological activities[17]. Astaxanthin alleviates endothelial dysfunction, as well as a variety of interesting biological activities, including proapoptotic, anticancer, antioxidant, and anti-inflammatory effects. However, its role in malignant cells is still under investigation [18]. The anti-proliferative activity of astaxanthin has been demonstrated in several cancer lines, including human hepatocarcinoma (CBRH-7919), rat breast cancer cells (SHZ-88), and Lewis mouse lung carcinoma cells [12,20]. Astaxanthin, which has antioxidant properties, has affected tumor growth in many different types of cancer. Astaxanthin has made pancreatic cells sensitive to the chemotherapy drug Gemcitabine with its antioxidant properties [21,22]. The same antioxidant property has shown anti-inflammatory properties in reducing gastric inflammation as well as prostate tumor growth [23]. The combination of astaxanthin and vitamin C has been effective in preventing and reducing stomach inflammation [24]. Astaxanthin negatively affects breast cancer cell viability [25]. These inhibitory properties are provided by the apoptosis and autophagy caused by the antioxidant property of astaxanthin. The antioxidant property of astaxanthin allows it to cause apoptosis and autophagy by targeting cancer cells without significantly affecting healthy cells [26,27].

In the light of all this information, we aim to investigate the effect of astaxanthin on maspin, CD82 (Kai1), BRMS1, and MKK4 metastatic suppressor proteins in T47D ductal carcinoma cell line in culture to examine the anti-metastatic effect of astaxanthin at a molecular level besides comparing the expressions of metastasis suppressing proteins in ductal carcinoma samples with and without metastasis from the pathology archive.

Materials and Methods

In vivo Ductal Carcinoma Study Protocol

The expression levels of anti-maspin, anti-Kai1, anti-BRMS1, and anti-MKK4 will be examined by immunohistochemistry on in situ and invasive ductal carcinoma tissue samples in paraffin block from 10 patients previously diagnosed in the pathology archive as a result of the power analysis performed with the reference study

titled "Expression and Regulation of Tumor Suppressor Gene Maspin in Breast Cancer" in the Department of Biostatistics of ESOGÜ Faculty of Medicine, and the ethics committee approval given by Eskişehir Osmangazi University Non-Invasive Clinical Research Ethics Committee (date 09.10.2018 and Decision No: 13).

Histochemistry and Immunohistochemistry

All cases underwent standard histological examination, including microscopic analysis with standard H&E staining. Immunohistochemical staining for Maspin, MKK4, BRMS1, and CD82 was performed on sections of formalin-fixed paraffin-embedded tissue from the invasive and in situ ductal carcinoma breast cancer tumors.
The 5 μ sections are taken from the paraffin block are treated with 3% H_2O_2 after deparaffinization and dehydration steps. Blocking is done for 10 minutes at room temperature after washing. After the antigen retrieval stage, polyclonal rabbit antibody to Maspin, MKK4, BRMS1, and CD82 were applied overnight at 4°C using the following dilutions: anti-maspin, anti-MKK4, anti-BRMS1 (1/200) and anti-CD-81 (1/2000) (Proteintech Lab, USA). Upon three rinses in Tris-buffered saline (TBS) and incubation with the secondary antibody, positive red staining was detected using standard avidin and biotinylated horseradish peroxidase (ABC) technique with 3-Amino-9-ethylcarbazole (AEC) as the chromogen. Slides were then counterstained in Mayer's hematoxylin for 10 seconds, dehydrated in graded alcohol, mounted.

In vitro Ductal Carcinoma Study Protocol

T47D human ductal carcinoma cells, procured from ATCC, were used in our experiment. These cells are obtained from a 60-year-old Caucasian female diagnosed with invasive ductal carcinoma of the breast and are widely used in in vitro studies. They are of epithelial origin. The cells were grown in a 37°C, 5% CO_2 incubator with DMEM (Dulbecco's Modified Eagle's Medium), 10% fetal bovine serum, and 1% penicillin-streptomycin (100 U/mL–100 μg/mL) as a monolayer. When the cells filled 75–85% of the 75 cm^2 flasks, the cells were re-passaged to prevent inhibition and plated into new flasks containing 10 mL of medium. In the passaging process, 0.05% trypsin/0.53 mM EDTA solution was used to remove the cells.

Determination of Stock Solution and Astaxanthin Dose

Astaxanthin (Sigma, SML 0982, Germany) was dissolved in DMSO to prepare a 50 mM stock solution. Increased doses of 1.9 μM to 2000 μM were given to T47D cells.

MTT Viability Method

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method is a colorimetric method that is frequently used for the determination of cell viability and measured by a spectrophotometer. MTT is a substance that is actively absorbed by the cells and reduced to a colored, water-insoluble formazan by a reaction of mitochondria. The MTT reduction property of cells is taken as a measure of cell viability, and the dye density obtained as a result of MTT analysis is proportional to the number of live cells. The cytotoxicity of astaxanthin on cells or the enhancing viability effect on cells was determined by the MTT method. T47D cells were seeded at 5000 cells per well, and after 24 h, the doses of astaxanthin were given to the cells. After 24, 48, and 72 h, the MTT was prepared by dissolving in phosphate-balanced salt solution and filtered. Cells were incubated at 37 °C for 4 h by adding 1/10 MTT to each well of 96 wells. After the incubation period, the medium in each well was pipetted and 100 μL of DMSO was added, and the wells were shaken for 5 to 8 min on the heated shaker. The absorbance of the formazan dye was measured at 570 nm with the ELISA device. The optical density read from the drug-treated wells was converted to the percentage of living cells versus control. The obtained data were expressed as mean % fraction ± standard error deviation of the control. One-way analysis of variance (ANOVA) and then Tukey test or Games–Howell multiantifaceted comparison test was used for statistical evaluation.

Protein Isolation from Cell Culture

T47D cells were counted in the Thoma lam 24 h before and grown in plates at 500,000 cells per well. The cells were treated with astaxanthin at doses of 0, 125, 250, and 500 μM. After 24 h, the cells were discarded and washed with cold PBS, then scraped from the flask surfaces with a sterile cell scraper and collected in an Eppendorf tube. Thereafter, the cells were then treated for 20 min with lysis buffer, including a protein inhibitor cocktail, vortexed every 5 min at 4 °C and centrifuged at 14,000 g for 5 min. The supernatant was removed from the pellet and stored until the day of use in a –80 °C refrigerator.

Western Blot

Cells were washed with ice-cold PBS and then boiled in sodium dodecyl sulfate (SDS) buffer (90 mM Tris–HCl, pH 6.8; 2.5% sodium dodecyl sulfate, 15% glycerol) at 100 °C for 5 min and the protein concentrations were determined using Qubit 2.0 Fluorometer (Invitrogen, Thermo Fisher Scientific Inc., USA). The proteins (50

μg/well) were separated according to their molecular weight in 7.5% or 10% SDS-PAGE gels and transferred to the nitrocellulose membrane at 25 V for 25 min after electrolysis. The membranes were blocked in TBS containing 5% BSA and 0.05% Tween 20 for 1 h at room temperature. Then, we added Maspin (11722-1-AP), MKK4 (51142-1-AP), CD82 (10248-1-AP), and BRMS1 (16096-1-AP), respectively, and incubated them overnight at 4 °C. After washing with TBST 3 times for 5 min at room temperature, they were incubated with Goat anti-Rabbit Horseradish Peroxidase secondary antibody (sc-2004, Santa Cruz, USA) for 1 h. The bands were visualized and analyzed using the Bio-Rad ECL and Li-Core imaging system (Li-Cor, USA).

Statistical analysis

Statistical analyzes were performed using IBM SPSS Statistics 21 package program. Significance level was accepted as $p<0.05$. MTT results were expressed as mean % fraction ± standard error deviation of the control. One-way analysis of variance (ANOVA) and then Tukey test or Games–Howell multifaceted comparison test was used for statistical evaluation.

Results

In vivo Histopathological Results

We observed in figure 1 a high grade in situ ductal carcinoma with central necrosis is seen. Neoplastic epithelial cells with high grade atypia and pleomorphism was shown (Fig. 1). This neoplasm is confined to the duct covered by the basement membrane. In figure 2, we observed invasive ductal carcinoma section. Small groups and tumor cells with infiltrative cords and prominent collagen bands are observed between them in invasive ductal carcinoma (Fig. 2).

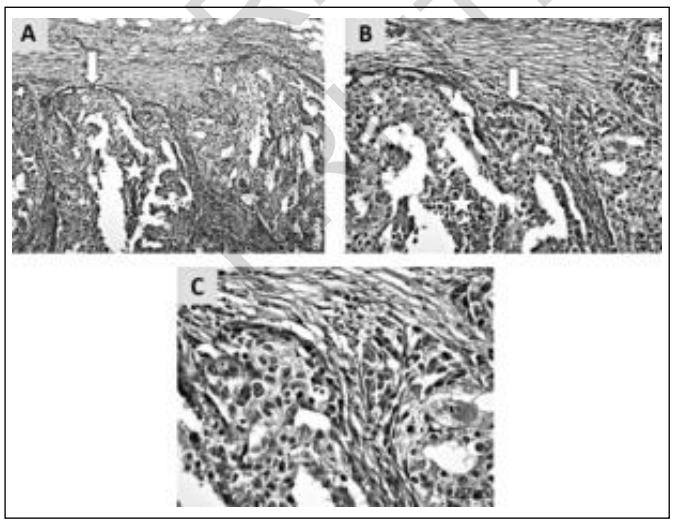

Fig. 1: High grade in situ ductal carcinoma. Arrows show border of basal membrane. Star shows central necrosis (HE, 10x, 20x, 40x).

In vivo Immunohistochemistry Results

Although BRMS1 expression was observed only partially in the nucleus in in situ ductal carcinoma, intense BRMS1 staining was observed in both nucleus and cytoplasm in grade 2 invasive ductal carcinoma (Fig. 3). CD82 expression, similar to BRMS1, although partial expression was observed in the nucleus in in situ ductal carcinoma, but intense cytoplasmic staining was observed in grade 2 invasive ductal carcinoma (Fig. 4). Although maspin expression was partially observed in the

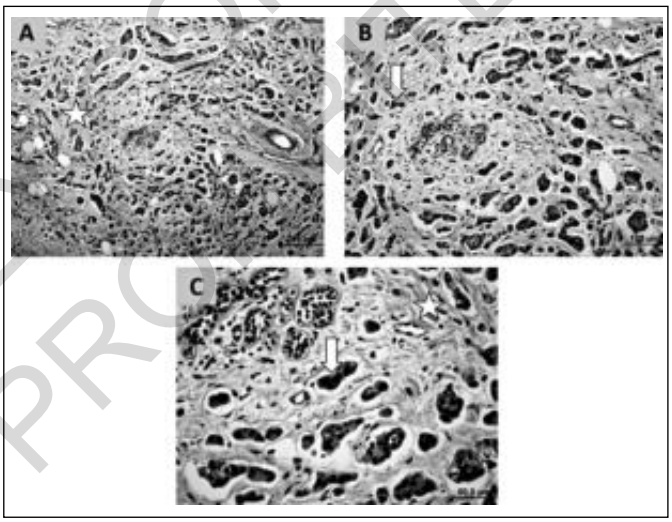

Fig. 2: Invasive ductal carcinoma. Arrows show infiltrative tumor cell cords. Stars show collagen bundles (HE, 10x, 20x, 40x).

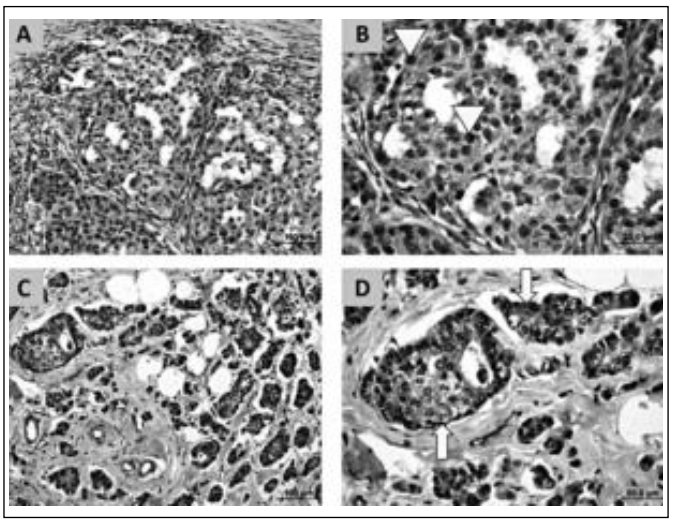

Fig. 3: BRMS1 staining in in situ ductal carcinoma (A, B) and invasive ductal carcinoma (C, D). Arrow heads show nucleus staining, arrows show both nucleus and cytoplasmic staining (20x, 40x).

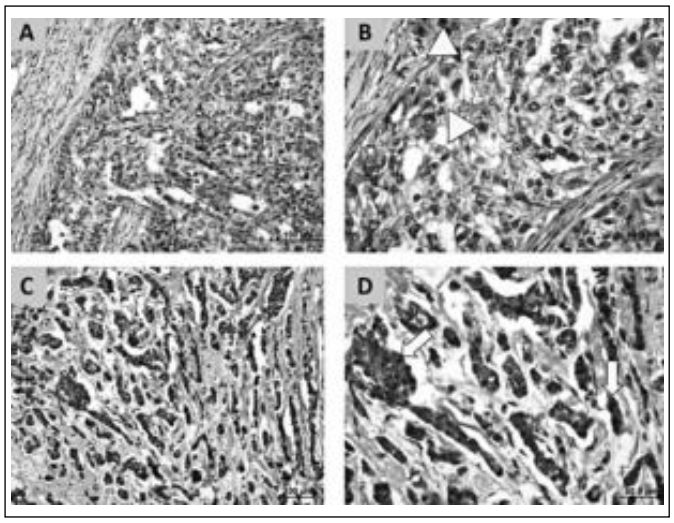

Fig. 4: CD82 staining in in situ ductal carcinoma (A, B) and invasive ductal carcinoma (C, D). Arrow heads show nucleus staining, arrows show both nucleus and cytoplasmic staining (20x, 40x).

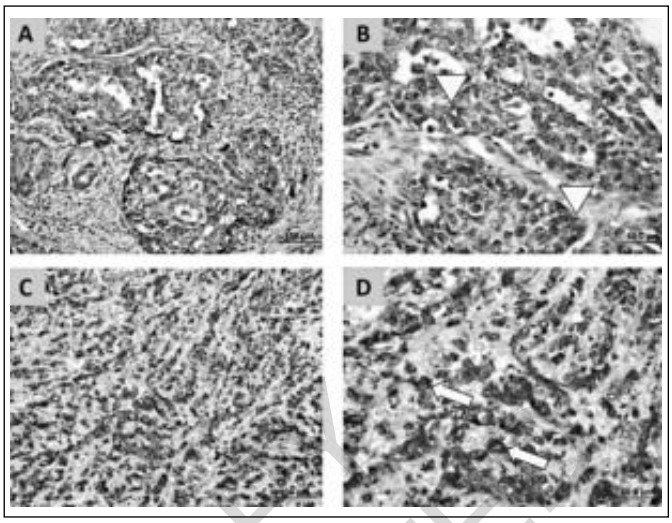

Fig. 6: MKK4 staining in in situ ductal carcinoma (A, B) and invasive ductal carcinoma (C, D). Arrow heads show pale cytoplasmic staining, arrows show intense cytoplasmic staining (20x, 40x).

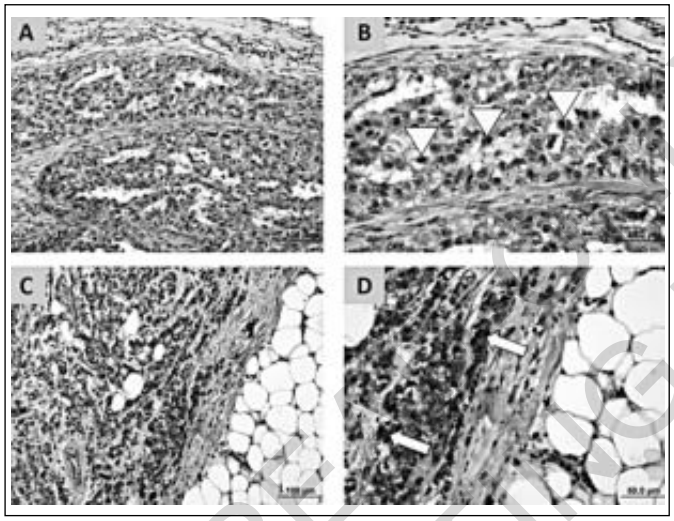

Fig. 5: Maspin staining in in situ ductal carcinoma (A, B) and invasive ductal carcinoma (C, D). Arrow heads show nucleus staining, arrows show intense cytoplasmic staining (20x, 40x).

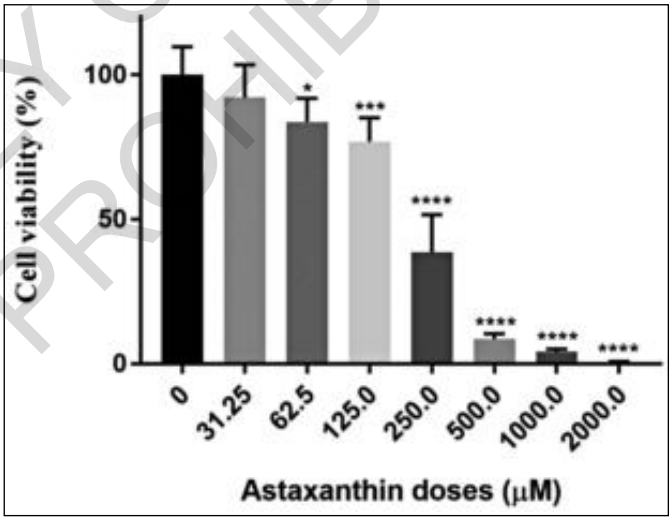

Fig. 7: 0-2000 μM dose dependent of astaxanthin effect on T47D cell viability.

nucleus and cytoplasm of in situ ductal carcinoma, intense cytoplasmic staining was observed in grade 2 invasive ductal carcinoma (Fig. 5). Although MKK4 expression was partially observed in the cytoplasm of in situ ductal carcinoma, intense cytoplasmic staining was observed in grade 2 invasive ductal carcinoma (Fig. 6).

In vitro Invasive Ductal Carcinoma Cells MTT Results

T47D cells were treated with astaxanthin doses that range between 0-2000 μM. Although the viability of T47D cells treated with 92.3 μM astaxanthin for 24 hours decreased to 92.3%, there was no statistically significant difference when compared to control cells ($p>0.05$). In the 62.5 μM astaxanthin, the cell viability ratio decreased to 82.8%, and a significant difference of $p<0.05$ was detected when compared to control cells. 125.0 μM astaxanthin further decreased the viability to 76.9% ($p<0.001$ vs control cells). In the 250.0 μM astaxanthin, the viability lowered to 38.6% by decreasing more than 50% ($p<0.0001$ vs control cells). In the 500.0 μM and 1000.0 μM astaxanthin, the viability even decreased under 10% (8.7% and 4.1, respectively), and a significant difference of $p<0.0001$ between control cells were detected at both doses. Finally, at the highest dose of astaxanthin applied to the cells, almost no cells were observed (0.7%, $p<0.0001$ vs control) (Fig. 7) (Table I).

In vitro Western Blot Results

The changes in anti-maspin, BRMS1, CD-82, and MKK-4 expressions of 125 and 250 μM astaxanthin applied to invasive ductal carcinoma T47D cells were examined in our study. Administration of Astaxanthin in increasing doses increased the expression of BRMS1 protein. In T47D invasive ductal carcinoma without astaxanthin, BRMS1 expression increased 3.4-fold in the 125 μM astaxanthin group and 18.8- fold in the 250 μM astaxanthin group compared to the non-treated group. It is known that increased BRMS1 expression reduces proliferation in metastatic areas. Based on this, it is suggested that astaxanthin provides its anti-proliferative effect with BRMS1 activation. Maspin protein is known to inhibit angiogenesis by associating with the p53 tumor suppressor pathway. Although 125 μM astaxanthin significantly suppressed maspin expression, 250 μM astaxanthin increased maspin expression 4.7-fold. Considering that the IC50 dose of the astaxanthin we used in our study was 212 μM, we think that 250 μM astaxanthin showed its anti-angiogenic effect through maspin protein. Cancer cells show high metastatic activity in the absence of MKK-4 protein. High expression of MKK-4 suppresses metastasis in this case. While the level of MKK-4, another metastasis suppressing protein, decreased with 125 μM astaxanthin application; 250 μM astaxanthin increased MKK-4 expression 3-fold. While expression of CD-82, another metastasis suppressor protein, decreased in 125 μM astaxanthin application; 250 μM astaxanthin increased CD-82 expression 5.1-fold. This led us to the idea that astaxanthin also prevents metastasis (Fig. 8).

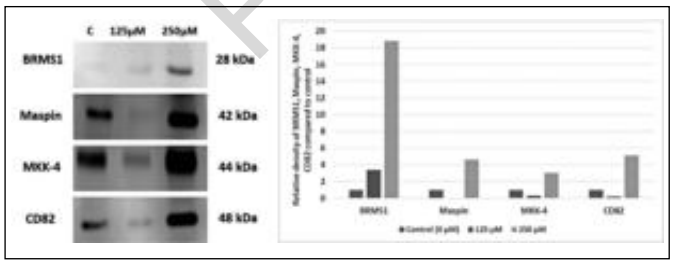

Fig. 8: Effects of 0, 125 and 250 μμ astaxanthin treatments on the BRMS1, Maspin, MKK-4 and CD82 proteins in T47D human invasive ductal carcinoma cells compared to untreated cells.

Discussion

Breast cancer, which has a high incidence worldwide and has a gradual increase in its incidence in recent years, constitutes 30% of all cancers seen in women in Europe. Distant metastasis, one of the main causes of death in breast cancer, is an important biological feature of malignant tumors. The metastatic process is a complex, biological and clinical situation consisting of specific and interrelated steps such as separation of tumor cells from the basal lamina, intravasation of migrating cells into lymphatic and vascular systems, extravasation, and growth in the new host organ/tissue where they migrate [28]. Many genes, including genes related to metastasis, play a role in the regulation of the metastatic process. Although they do not affect the growth of the primary tumor, metastasis suppressor genes are considered to suppress metastasis [29].

Table I

Astaxanthin doses(μM)	MTT Cell viability (%) (mean±SD)	p values
Control (Untreated)	100.0 ± 9.7	–
31.3	92.3 ± 11.3	ns
62.5	83.8 ± 8.2	p<0.05
125.0	76.9 ± 8.2	p<0.001
250.0	38.6 ± 13.1	p<0.0001
500.0	8.7 ± 1.8	p<0.0001
1000.0	4.1 ± 1.0	p<0.0001
2000.0	0.7 ± 0.2	p<0.0001

Astaxanthin (3,3'-dihydroxy-β-carotene-4,4'-dione, AXT), which is commonly found in plants and seafood, belongs to the xanthophyll carotenoids family [30]. In general, carotenoids consist of hydrocarbon (β-carotene, α-carotene, γ-carotene, and lycopene) and oxygenated carotenoids (neoxanthin, fucoxanthin, lutein, zeaxanthin, astaxanthin, and canthaxanthin) are important bioactive compounds. The United States Food and Drug Administration and the European Commission approved AXT for use as a food coloring. Among the carotenoids, AXT has a variety of biological activity against cancer, inflammation, and age [31]. AXT has been shown to have anticancer activity in various types of cancer in recent studies [26,32].

The antiproliferative activity of astaxanthin has been demonstrated in many cancer lines, including human hepatocarcinoma (CBRH-7919), rat breast cancer cells (SHZ-88), and Lewis mouse lung carcinoma cells, and has higher sensitivity for the human hepatocarcinoma (CBRH-7919) cell line, IC50 values ™are about 39 μM [20]. Astaxanthin significantly inhibits cell growth in the HepG2 cell line at a concentration of 42 μM [33], the IC50 value in LM3 and SMMC-7721 is 100 μM [34]. Although astaxanthin did not significantly suppress the viability of breast cancer (MCF-7 and MDA-MB-468) cells in one study [35], in another study, H.pluvialis extract showed a synergistic effect with astaxanthin, showing cytotoxic activity on breast adenocarcinoma [36]. In another study, the 33 mM dose of astaxanthin triggered cell death in MCF-7, while the application of astaxanthin in the form of nanoparticles against MDA-MB-231 showed anticancer properties and the IC50 dose was determined to be 84 μM [37]. On the other hand, we found the IC50 dose of astaxanthin applied to T47D invasive ductal car-

cinoma cells as 212 μM in our study. The reason for such different doses is thought to be due to the synthetic origin of astaxanthin. Régnier et al. Revealed that synthetic astaxanthin is 90 times less effective on human umbilical vein endothelial cells than natural astaxanthin [38]. Although astaxanthin does not show toxic effects on healthy breast epithelial cells, it has been accepted as a chemopreventive agent in breast cancer [19,26,39].

In our study, we aimed to observe the expression difference of the metastasis suppressing proteins BRMS1, MKK-4, Maspin, and CD82 in invasive and in situ breast cancer cells as well as to examine the effect of astaxanthin on metastasis suppressing proteins. As a result, the expression of metastasis suppressing proteins in tissue sections of invasive ductal carcinoma patients were found to be much higher than in in situ ductal carcinoma patients samples. Also, in the in vitro part of our study, we found that 125 μM astaxanthin suppressed MKK-4, maspin, and CD82 expression on T47D invasive ductal carcinoma cell line and we observed that it activates at a dose of 250 μM. Based on this, we can say that astaxanthin has a dose-dependent effect. Also, we observed its anti-proliferative effect with increasing BRMS1 activation depending on the increasing dose of astaxanthin.

Breast cancer metastasis suppressor-1 (BRMS1) is one of the metastasis suppressor genes. It has been shown to potentially inhibit tumor progression without inhibiting the growth of orthotopic tumors in different tumor types, including non-small cell lung cancer, ovarian, melanoma, and breast cancers. The mRNA expression level of BRMS1 was found to be much lower in breast tumor metastasizing to the brain compared to primary breast tumors. BRMS1 mRNA expression was found to be low in the primary tumor when healthy breast tissue and primary breast tumor were compared [40,41]. A decrease in BRMS1 mRNA expression is observed in breast cancer cell lines such as MDA-MB-435, MDA-MB-231, and MCF10AT compared to healthy breast tissue [6]. Zhang et al. stated in their study that BRMS1 expression level is inversely proportional to the long-term survival of breast cancer patients. As a result, the literature states that BRMS1 plays an important suppressive role in human breast cancer [29]. In our study, although BRMS1 expression was observed only partially in the nucleus in in situ ductal carcinoma, intense BRMS1 staining was observed in both nucleus and cytoplasm in grade 2 invasive ductal carcinoma. While BRMS1 expression was low in T47D cancer cells in our cell culture study, BRMS1 expression increased due to the increased dose of astaxanthin. This highlights the use of astaxanthin while it increases the expression of BRMS1, which plays an important role in suppressing human breast cancer.

CD82, also known as KAI1, is a member of the TM4SF protein family and is another metastatic-suppressor protein [42]. In general, proteins belonging to the TM4SF family play a role in cell morphology, cell proliferation, fusion, motility, cell signaling, and regulation of the immune system [43,44]. KAI1 / CD82 has been reported to suppress metastasis in the rat prostatic adenocarcinoma model [45]. Song et al. Stated that overexpression of CD82 in breast cancer cells causes in vitro invasion and suppression of in vivo metastasis (20). CD82 interacts with other tetraspanin proteins, integrins, and chemokines to regulate migration, adhesion, and signaling of cells [46]. Jee et al. Stated that the increased regulation of the CD82 protein is responsible for the marked reduction of invasion and metastatic potential of non-small cell lung cancer cells. Also, the high expression of CD82 is associated with decreased integrin expression. Odintsova et al. Reported that CD82 contributes to epithelial growth factor (EGF) -induced signaling and identified its association with EGF receptor (EGFR) and tetraspanin, which are critical for EGFR desensitization [47]. Similarly, in another study, CD82 expression has been shown to significantly suppress in vitro invasion of breast cells [48]. In our study, CD82 expression, similar to BRMS1, although partial expression was observed in the nucleus in in situ ductal carcinoma, but intense cytoplasmic staining was observed in grade 2 invasive ductal carcinoma.

Maspin is a serine protease inhibitor-associated with inhibiting angiogenesis and inducing apoptosis [49]. Maspin expression is characterized by more aggressive breast cancer phenotype [50]. Cytoplasmic Maspin expression is associated with poor prognosis, while nucleus maspin expression is associated with a good prognosis [51-53]. However, recent evidence suggests that the nuclear localization of maspin in cancer cells is required for its tumor suppressor activity and that chromatin-bound maspin in the nucleus is required to effectively prevent cells from metastasizing [54]. Maspin gene expression is down-regulated in breast cancer. Maspin gene expression decreases as breast cancer moves from in situ to invasive. Researchers observe that maspin expression decreases in advanced metastatic carcinoma cases [55,56]. Dabiri et al. showed in their study aiming to shed light on the role of Maspin gene expression in breast cancer that maspin gene expression decreased in grade II and III breast cancer samples [57]. Strien et al. Reported that maspin expression was significantly decreased in metastases compared to primary tumors (PTs) [51]. In contrast to these studies, Helal and El-Guindy indicated that expression of maspin was upregulated in invasive ductal carcinoma of the breast. Subcellular localization of maspin can strongly affect cancer prognosis. Cytoplasmic maspin was significantly related to high tumor grade and positive nodal metastasis whereas, nuclear maspin was related to less aggressive tumors [58]. These results are consistent with our study. Although maspin expression was partially observed in the nucleus and cytoplasm of in situ ductal carcinoma, intense cytoplasmic staining was observed in grade 2 invasive ductal carcinoma. Also, 250 μM astaxanthin significantly increased the maspin reac-

tion. This suggests that astaxanthin has an anti-angiogenic effect, indicating this by increasing maspin expression.

Members of the MAPK family, MKK4, and its downstream regulator JNK chemically play vital roles in cell cycle arrest and apoptotic processes [59]. JNK phosphorylation activates substrates of MKK4-dependent transcription factors or proapoptotic proteins. Functional suppression of JNK activation and inactivation of MKK4 has recently been recognized as an important cellular survival mechanism, causing cancer cells to avoid apoptosis [60,61]. Also, MKK4 dysregulation occurs during the development of clinical cancer metastases. While it is stated that impaired expression of MKK4 in prostate and ovarian tumors support metastasis [14], decreased mRNA level of MKK4 has been shown to cause metastasis from breast cancer to the brain [15,16]**.** All this information led to the idea that activation of the MKK4-JNK signaling pathway would be a promising therapeutic approach as it induces proliferation inhibition of tumor cells and prevention of metastasis [62]. Based on this, the fact that 250 μM astaxanthin increases MKK4 expression in our study strengthens our therapeutic use of astaxanthin in terms of preventing cell proliferation and metastasis. Also, more intense MKK4 staining was observed in our invasive ductal carcinoma samples compared to our in situ sections. In conclusion, we show in our study that Maspin, CD82, MKK4, and BRMS1 are an important driving force of metastasis in breast cancer, astaxanthin targets these metastasis suppressors and is a promising treatment method for invasive ductal carcinoma patients.

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Riassunto

Il cancro al seno (BC) è un grave problema di salute pubblica diagnosticato in oltre 2 milioni di donne in tutto il mondo nel 2018, e che causa oltre 600.000 decessi. Il 90% dei decessi seno sono causati da metastasi, processo complesso suddiviso in diversi passaggi, tra cui la separazione delle cellule tumorali dal tumore primario, l'invasione, la migrazione cellulare, la invasione e la sopravvivenza intravascolare, l'emigrazione extravascolare e la colonizzazione secondaria. L'astaxantina (AXT) è un chetocarotenoide di origine marina che ha molte diverse potenziali funzioni in termini di proprietà antiossidanti, antinfiammatorie e di riduzione dello stress ossidativo in grado di ridurre potenzialmente l'incidenza del cancro o inibire l'espansione delle cellule tumorali. Questo studio mira a indagare gli effetti dell'astaxantina come nuovo inibitore della metastasi sulle cellule di carcinoma mammario invasivo umano di carcinoma duttale T47D.

Per studiare gli effetti dell'astaxantina come nuovo inibitore della metastasi sulla cellula T47D, sono stati esaminati mediante western blot i livelli di espressione di anti-maspin, anti-Kai1, anti-BRMS1 e anti-MKK4. Inoltre, abbiamo valutato le differenze dei livelli di espressione di questi soppressori in sezioni di tessuto di 10 pazienti con diagnosi di carcinoma duttale in situ e invasivo con immunoistochimica.

Risultato: 250 μM di astaxantina hanno aumentato l'attivazione di tutte le proteine™ che sopprimono le metastasi. Inoltre, questi soppressori di metastasi hanno mostrato una maggiore espressione nei tessuti di carcinoma duttale invasivo rispetto ai pazienti con carcinoma duttale in situ.

In conclusione riteniamo che l'astaxantina sia un promettente agente terapeutico per le pazienti con carcinoma duttale invasivo. Gli effetti dell'astaxantina sulle metastasi nel cancro al seno dovrebbero essere ulteriormente studiati sulla base di questi risultati.

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