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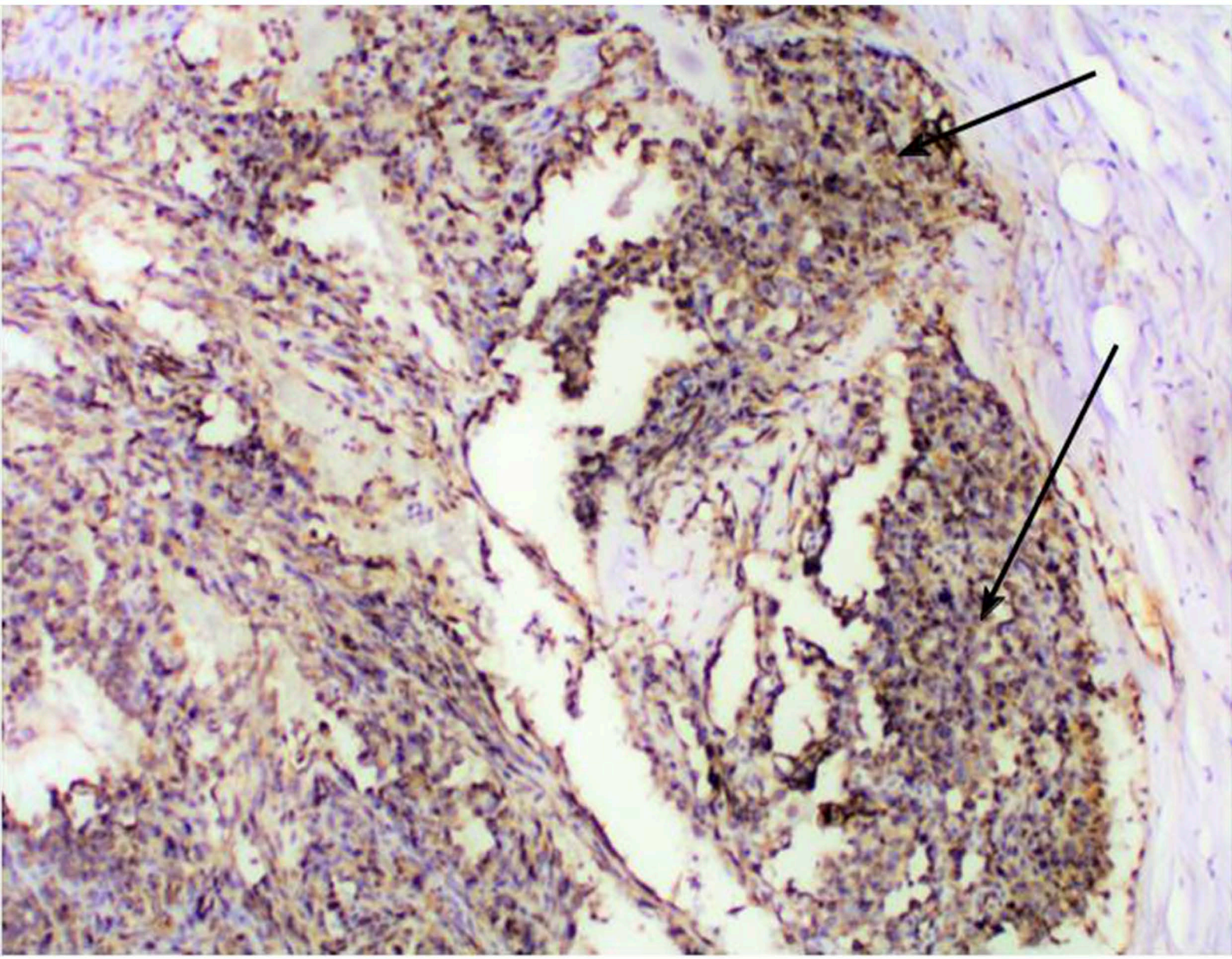

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CASE REPORT

Primary angiosarcoma of the breast: A clinical case and review of the literature

Lena Marinova[1*] Bistra G. Yordanova[2] Doroteya V. Malinova[3]

Abstract: We present here a rare case of primary mammary angiosarcoma in 48-year-old female patient. After 3 years without treatment, the woman presented to the hospital with locally advanced tumor in right mammary gland, involving the overlying skin and bleeding. Radical mastectomy was performed with axillary lymph-node dissection. The CT scan revealed solitary liver metastasis. After an overview of different cases of primary angiosarcoma of the breast published in the literature, we discuss the importance of histological criteria and immunohistochemical methods, as well as the optimal multimodal treatment in these patients. Poorly differentiated primary mammary angiosarcoma (grade 3) is an invasive neoplasm with high risk of local recurrence and distant metastases. The multimodal treatment involves radical mastectomy with or without axillary lymph-node dissection. Adjuvant radiotherapy and adjuvant chemotherapy help the local tumor control, reduce recurrences and increase overall survival.

Keywords: primary mammary angiosarcoma, radical mastectomy, radiotherapy, chemotherapy, multimodal treatment

1 Introduction

Angiosarcoma (AS) is a rare aggressive tumor which arises from endothelial cells lining vascular structures[1,2]. Breast is one of the most common primary sites of angisosarcoma[3]. Mammary AS accounts for 1% of all soft tissue tumors[4,5] and 0.04% of all primary breast tumors[4,6–11]. According to the etiology, angiosarcoma of the breast can be primary and secondary[12]. Secondary tumors are associated with chronic lymphaedema in the upper limb after axillary lymph-node dissection and radiotherapy in patients with mammary carcinoma. The condition is known as Stewart-Treves syndrome[4,8,13,14]. Secondary angiosarcoma is also considered a complication from radiotherapy and breast-conserving surgery (BCS) in patients with breast carcinoma[15]. Primary mammary AS is usually found in young patients (20-50 years) with no history of breast carcinoma[16–19]. Secondary AS is found in older patients (67-71 years), usually 5 to 10 years after radiotherapy for breast carcinoma[4,20–22].

2 Clinical case

We report a case of primary angiosarcoma of the breast in 48-year-old woman. Three years prior to admission to the hospital the woman noticed a lump in the right mammary gland, which slowly had enlarged. In the last three months the lesion had enlarged more rapidly and reached the skin with ulcer formation. The woman was admitted to the University Hospital in Ruse with severe bleeding from the ulcerated area. On examination, the right mammary gland was three times larger than the left one, with bluish colour and palpable firm tumor mass. In the outer upper quadrant of the affected breast the skin was ulcerated and bleeding. On palpation, right axillary lymph nodes were enlarged but painless. Right radical mastectomy was performed with axillary lymph-node dissection. Chest and abdominal CT scan revealed bilateral pneumofibrotic changes in dorsobasal areas of the lungs, no pathological mediastinal and axillary lymphadenopathy, no pleural effusions. In 8th segment of the liver, oval shaped metastasis was found.

Gross examination of the resected breast showed no evidence of mammary gland parenchyma. Subcutaneously, a cavity, 13 cm in diameter was found. It was filled with dark red blood and blood clots. The nipple was not affected. Histology revealed a tumor composed of anastomosing densely packed vascular spaces of variable sizes,

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lined with atypical endothelial cells with severe nuclear polymorphism and hyperhromasia, prominent nucleoli and atypical mitotic figures. Intraluminal papillary projections composed of endothelial cells were found, as well as solid areas of spindle shaped cells between the described vascular spaces (Figure 1). Histological examination of the resected axillary lymph nodes showed sinus histiocytosis. The final diagnosis was angiosarcoma of the right mammary gland, grade 3 (G3). Immunohistochemical study revealed a neoplasm positive for CD34 antibody, confirming the vascular nature of the tumor (Figure 2). The epithelial marker, Cytokeratin 1/3, was negative (Figure 3). Estrogen (ER) was negative (Figure 4) and Progesteron (PR) was negative (Figure 5).

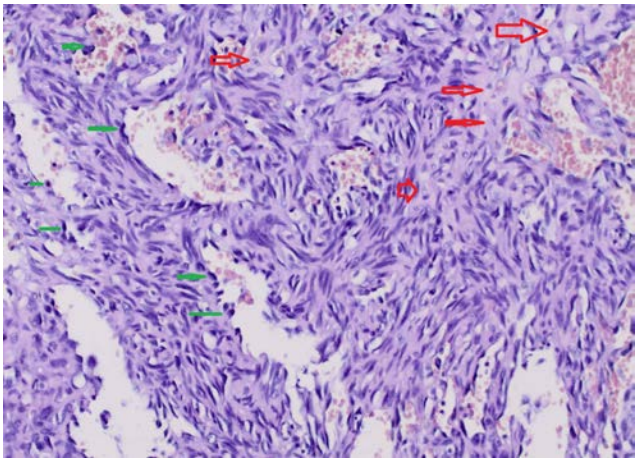

Figure 1. Mammary angiosarcoma (G3) composed of atypical polygonal (red arrow) and spindle shaped endothelial cells with hyperhromatic nuclei (green arrow), H & E, (× 40)

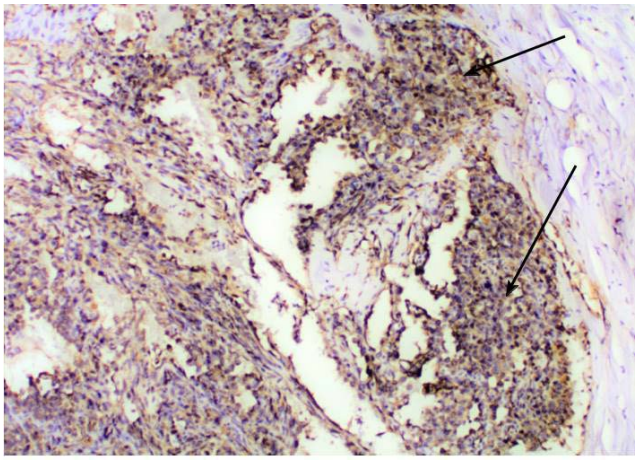

Figure 2. Mammary angiosarcoma (G3). Immunohistochemical study showed positivity for CD34 endothelial marker (× 40)

After the surgery, the woman was assessed for chemotherapy, which was not carried out, due to a deterioration in her condition. Within six months the dis-

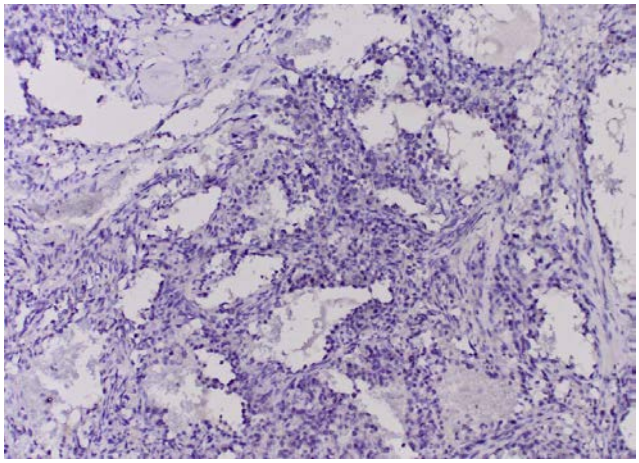

Figure 3. Mammary angiosarcoma (G3). Tumor cells are negative for AE1/AE3 (× 20)

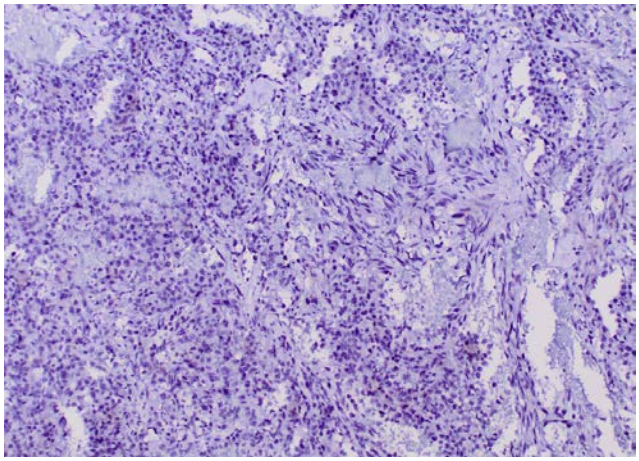

Figure 4. Mammary angiosarcoma (G3). Tumor cells are negative for ER (× 20)

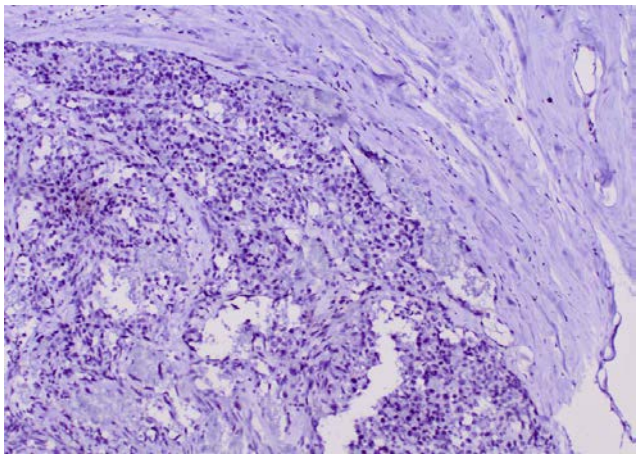

Figure 5. Mammary angiosarcoma (G3). Tumor cells are negative for PR (× 20)

ease quickly progressed. The patient developed a local relapse in the right chest, metastases in the hilar lymph nodes in the right lung with compression of the right pulmonary artery, pulmonary metastases, hepatic metastases, bone and brain metastases. The patient succumbed to her disease eight months after the surgery.

3 Discussion

The first case of primary angiosarcoma of the breast is published in 1907 by Borrman and the first case of seconady mammary angiosarcoma is described by Body *et al*. in 1987[7,20,21]. Primary AS of the breast is aggressive tumor with high risk of local recurrence and distant metastases[8,22,23]. The lack of prospective randomized studies on the role of the systematic therapeutic approach in the literature is due to the rarity of this tumor. The major problems concerning AS are: (1) difficult histological diagnosis and broad differential diagnosis, requiring immunohistochemical analysis; (2) choosing the most reliable multimodal treatment for these patients.

Clinically, patients with primary mammary AS present with rapidly growing painless palpable tumor mass[24,25]. In 2% of the cases patients present with increased breast volume and bluish red coloration of the overlying skin[13,25]. Usually, the axillary lymph nodes are not enlarged. In the majority of cases published in the literature, the size of the tumor is more than 4 cm. in diameter[26].

Preoperative diagnosis with fine needle biopsy (FNB) is difficult. Chen *et al*. reported false negative diagnosis after FNB in 37% of the cases[3]. Differential diagnosis includes hemangioma, benign vascular proliferation[27], hamartoma[28], stromal hyperplasia, phylloides tumor, stromal sarcoma, metaplastic carcinoma, other sarcomas, like fibrosarcoma and liposarcoma[26,29]. Large-core biopsy might facilitate the accurate diagnosis, but such a biopsy is difficult to perform due to the vascular nature of the neoplasm. Final diagnosis usually is made after surgical resection and histological examination of the specimen. Three main histopathological patterns of mammary AS have been described according to the grade of differentiation:

Grade I (G1) is characterized by anastomosing vascular channels, lined by a single layer of endothelial cells; these channels dissect through the stroma causing distortion but little destruction of the preexisting lobules and ducts.

Grade II (G2) is similar to grade II tumors but with increased mitoses, endothelial tufting and foci of papillary formations and/or solid growth pattern.

Grade III (G3) is characterized by marked pleomorphism, mitoses, necrosis and solid growth, may show epithelioid and spindled cytology (Figure 1)[31,32].

Different areas from one and the same tumor can be with different tumor grades. The degree of differentiation can't be defined properly on core-biopsy[1].

Immunohistochemical analysis can prove the endothelial differentiation of the tumor cells. CD31 is one of the most sensitive and specific markers for endothelial cells. Tumor cells are also positive for Factor VIII, Fli1 and CD34[13,31,33–35]. In some cases papillary proliferations are found in the vascular spaces and these areas can resemble ductal carcinoma in situ. Estrogen receptors are reported to be negative in most cases[26,36]. In the case reported here, estrogen and progesterone receptors were both negative (Figure 4 and Figure 5).

Soft tissue sarcomas depending on histology and G are aggressive neoplasms with varying degrees of local invasiven and risk of hematogenous dissemination. In the presented clinical case, there is an aggressive G3 sarcoma with high metastatic potential.

There is no optimal treatment for breast angiosarcoma due to the rarity of this tumor[15]. Similar to other soft tissue sarcomas, prognostic factors include tumor size, degree of differentiation, margin status at the time of surgery[15,37,38]. Disease recurrence rate increases in the case of residual tumor and grade 3 AS[21,37,39,40]. Three studies reported longer disease free survival (DFS) in grade I and grade II tumors, compared to grade III neoplasms[2,37,41]. It has been reported that the five year survival rate in well differentiated AS is 76%, and in poorly differentiated angiosarcomas, it is 15%[13].

3.1 Surgical treatment

Mammary AS is treated surgically, but also with chemotherapy and radiotherapy. The treatment lacks uniformity and criteria for surgery and adjuvant therapy are still discussed in the literature[42]. Breast-conserving therapy is recommended for small, grade I tumors, if there is a chance of achieving negative surgical margins[32]. Some authors recommend aggressive surgical treatment with removal of muscule tissue[21,22]. Radical mastectomy alone or with axillary lymph-node dissection is the preferred surgical treatment[14]. However, nodal metastases are rare in AS and the necessity for nodal dissection is obscure[15]. A study from 2017 reported worse overall survival (OS) in patients with primary AS who received mastectomy, when compared with patients who received breast-conserving surgery[43].

3.2 Adjuvant chemotherapy and radiotherapy

Chemotherapy and radiotherapy have been used in the adjuvant setting of mammary AS, but these therapies need

further examination and clear criteria[12,29,38,44]. Literature lacks prospective randomized trials on the effect of adjuvant chemotherapy and radiotherapy and they are used only for tumors with high risk of recurrence[13]. Radiotherapy is based on tumor characteristics and the type of surgical treatment[2]. Radiotherapy is beneficial for patients with histologically positive surgical margins after mastectomy[45]. In two studies, a benefit to the 5 and 10 year recurrence free survival (RFS), disease free survival (DFS) and overall survival (OS) was observed following radiation treatment[46,47]. Some authors report that Anthracycline-based therapy can improve DFS and OS[48]. A meta-analysis of patients treated with Doxorubicin and a randomized trial of Epirubicin plus Ifosfamide demonstrated longer DFS and OS[49,50]. In two studies, adjuvant chemotherapy had no effect on DFS or OS[2,51]. In the majority of the reported cases patients are treated with Cyclophosphamid, Anthracycline or alkylating agents in combination with pyrimidine analog[24]. Paclitaxel is proven to be active and is commonly used in advanced angiosarcomas from different primary tumor sites[51–53]. T Sher *et al*. report that anthracycline-ifosfamide and gemcitabine-taxane chemotherapy regimens appear to be highly active in 48% of the cases[36].

3.3 Neoadjuvant chemotherapy and radiotherapy

Primary mammary AS can spread through blood to lungs, liver, skin and contralateral mammary gland[13]. Retrospective analysis of 41 patients with metastatic angiosarcomas from different primary tumor sites showed an improved OS from 10.4 to 23.7 months with taxane based regimens compared to non-taxane based adjuvant chemotherapy[54]. Paclitaxel therapy shows promise in the treatment of angiosarcoma[55].

Immunotherapy with IL-2 (interleukin-2) is also part of the treatment[43]. Different drugs that suppress endothelial proliferation can be used[13]. There are few papers that have examined the use of angiogenesis inhibitors like bevacizumab[55,56] and rapamycin[18].

4 Conclusion

The reported primary AS of the breast is a rare aggressive tumor with bad prognosis. The final diagnosis is based on specific histological criteria and immunohistochemical analysis. Prospective randomized trials are requested to reach a consensus on the optimal multimodal treatment. The gold-standard treatment in patients affected by primary angiosarcoma of the breast is surgery. Adjuvant chemotherapy and radiotherapy are recommended in G3 tumors with high risk of recurrence and distant metastases. The presented primary AS of the breast is a rare aggressive G3 sarcoma with high metastatic potential.

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RESEARCH ARTICLE

Primary investigation on effects of *Pinus massoniana* bark extract inducing senescence of hepatoma HepG2 cells

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Abstract: *Pinus massoniana* bark extract (PMBE) is a traditional Chinese medicine used for the treatment of various health disorders. Previous studies have demonstrated that PMBE may induce the apoptosis of hepatoma and colon cancer cells, and one of the potential mechanisms is by activating *p*53 to up-regulate the expression of *p*21. The P53/P21 signaling pathway is also one of the main mechanisms to regulate cell senescence. Therefore, we wonder if PMBE is able to induce hepatoma cells into senescence by inhibiting their growth. In the current study, the effects of PMBE on the viability of human hepatoma HepG2 cells were detected using an MTT assay. The phenotypes of HepG2 cells with PMBE treatment were detected with β-Galactosidase staining assay. The results revealed that the growth of HepG2 cells was inhibited by PMBE at dose-dependent manner when PMBE concentration is above 50 μg/ml, furthermore, PMBE could induce HepG2 cells into senescence at concentration of 40 μg/ml. These findings indicated that PMBE significantly inhibited the growth of HepG2 cells and induced them into senescence, while the potential mechanism and its safety in normal cells require further investigation.

Keywords: *Pinus massoniana* bark extract, cell senescence, HepG2 cells

1 Introduction

Pinus massoniana bark extract (PMBE) is a natural product from *pinus massoniana* lamb, an important tree for afforestation in South China[1]. Its needles and roots are widely-used traditional Chinese medicinal material, and the trunk are a source of resin and tannin[1].

In recent years, PMBE has attracted more and more attention from domestic and foreign scholars. Standard PMBE contains 26.0-28.3% flavonoids (mainly proanthocyanidins, PAs) as important bioactive substances[2]. PAs are primarily known for their powerful anti-oxidative properties[3] and also show other biological activities, such as anti-cancer[4], anti-inflammation[5], anti-microbe[6], cardiovascular protection[7], and nerve protection[8]. According to their connection modes between monomers, PAs can be classified as A-type and B-type. A-type PAs are doubly linked by a 4β-8 (B-type) and 2β-O-7 inter-flavanoid bonds, while B-type PAs are only linked by C4-C8 or C4-C6 bonds[9]. B-type PAs are the main constituents of PMBE and that may determine the chemical activity of the mixture[10]. PMBE has been shown strong effects for antioxidant, anti-cancer, cell cycle arrest, anti-migration, antimicrobial, anti-virus and pro-apoptosis[1,11]. PMBE broadly inhibits the growth of cancer cells from eight major systems of human body[12]. Some scientists found that PMBE can promote hepatoma cell apoptosis through down-regulating the expression of Bcl-2[13,14].

The *p*53/*p*21 signaling pathway is reported one of the main mechanisms regulating cell senescence mainly by implementing widespread changes in gene expression[15]. The *p*53 is master transcriptional regulator and *p*21 is its downstream effector. The *p*53/*p*21 pathway is cyclin-dependent kinase inhibitor (CDKI) and potent negative regulator of cell cycle progression[16]. Chronic activation or over-expression of *p*53 or *p*21 is generally sufficient to induce a growth arrest of senescence[16]. Cellular senescence refers to the essentially irreversible arrest of cell proliferation (growth) that occurs when cells experience potentially oncogenic stress[17]. The permanence of the senescent growth arrest enforces the idea that the senescence response evolved at least in part to suppress the development of cancer[18,19].

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There are many researches on PMBE inducing cell cycle arrest and apoptosis. However, we have found few studies on cellular senescence by PMBE. Based on these findings, we hypothesized that PMBE may induce senescent cells to apoptosis by up-regulating the expression of P53 or P21 genes. Therefore, this present study aimed to evaluate the effect of PMBE on cell senescence of hepatoma with HepG2 as cell model.

2 Materials and methods

2.1 Chemicals and cell line

PMBE powder was purchased from N.B.C.Biological Material Co. Ltd. (Huzhou, Zhejiang, China) and contained more than 95% PAs, which consist of 24.18% monomers, 50.11% dimers, 20.32% trimers, and 5.39% polymers as determined by HPLC[20].

Sorafenib-naive human hepatocellular carcinoma cell line (HepG2) and normal hepatic cell line (L02) was purchased from the cell banks of Chinese Academy of Sciences Committee Type Culture Collection. They were cultured in Dulbecco's modified Eagle's medium (DMEM; Solarbio, Beijing, China) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), 100 units/ml penicillin, and 100 μg/ml streptomycin. The penicillin/streptomycin mixture was purchased from Beyotime (Shanghai, China). Cells were maintained at 37°C in a humidified incubator containing 5% CO_2. All other chemicals were of analytical grade. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT), dimethylsulfoxide (DMSO), trypsin, Senescence β-Galactosidase Staining Kit were purchased from Beyotime, Shanghai, China.

2.2 Preparation of PMBE stock and working solutions

PMBE powder was dissolved in dimethyl sulphoxide (DMSO; Beyotime, Shanghai, China) to make a 10 mg/ml stock solution, which was then sterilized by being passed through a 0.22 μm micro-filter (Beyotime, Shanghai, China). Finally stored at -20°C until required. DMSO was not expected to exhibit any effect on cells under its final concentration in the working solution lower than 0.25%[20].

2.3 The cell growth curve

HepG2 and L02 cells cultured in 10% FBS–DMEM media were digested with 0.25% trypsin (Gibico) and counted, then seeded into 24-well plates at a density of 1×10^4 cells per well, respectively. The average cell density of the next three wells was calculated on each subsequent day. The data were collected to plot the cell growth curve, and the logarithmic growth period of HepG2 and L02 cells was found for the following experiments.

2.4 Determination of suitable concentration of PMBE by MTT assay

HepG2 and L02 cells were cultured in 10% FBS–DMEM media till to logarithmic growth further above 90% confluency, then digested with 0.25% trypsin (Gibico) in sterile 1×phosphate buffered saline (PBS; HyClone), and finally suspended in complete culture media. Then, cells were seeded into a 96-well plate at a density of 2×10^3 cells per well. When the cells had grown into the logarithmic growth phase, they were divided into several groups. Different concentrations of PMBE (0-130 μg/ml) were added into each well. After 48h, the growth inhibition rate of each concentration was obtained by 2-(3,5-diphenyltetrazol-2-ium-2-yl)-4,5-dimethyl-1,3-thiazole bromide (MTT) assay (Beyotime, Shanghai, China).

2.5 Senescence β-Galactosidase Staining

Two suitable concentrations of PMBE (70 μg/ml and 40 μg/ml) were obtained through the above experiments. HepG2 and L02 cells were seeded in 6-well plates at a density of 1×10^4 cells per well and cultured for 3 days and entered the logarithmic growth phase. Then cells were treated with 70 μg/ml and 40 μg/ml PMBE for 48 h, respectively. Then, senescence assay was performed according to the protocol of senescence β-galactosidase staining kit from Beyotime, Shanghai, China[21]. Observed under the ordinary optical microscope, the percentage of blue-stained cells was calculated from ten randomly selected viewing fields according to the following equation: senescence rate (%) = $N_{\text{senescent cells}}/N_{\text{all cells}}\times100$, where N stood for the total cell number in all viewed fields.

2.6 Statistical analysis

Statistical analyses were performed with Microsoft Excel 2010 and SPSS 20.0 using the t test for linear regression analysis. Results were expressed as the mean $\pm$ standard deviation (SD) of n independent experiments. "*" indicates that the calculated p value was less than 0.05 ($p< 0.05$) versus the corresponding control, which was regarded as significant.

3 Results

3.1 The cell growth curve

HepG2 and L02 cells were seeded into 24-well plates at a density of 1×10^4 cells per well and counted using blood

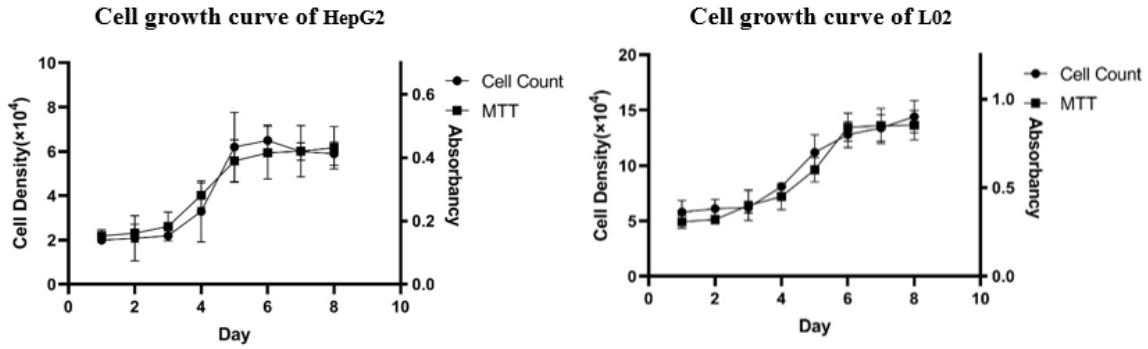


Figure 1. Growth curve of HepG2 and L02 cells. Both HepG2 and L02 cells entered the logarithmic growth phase on the fourth day. In terms of mechanism, cell counting method is more reliable than MTT method

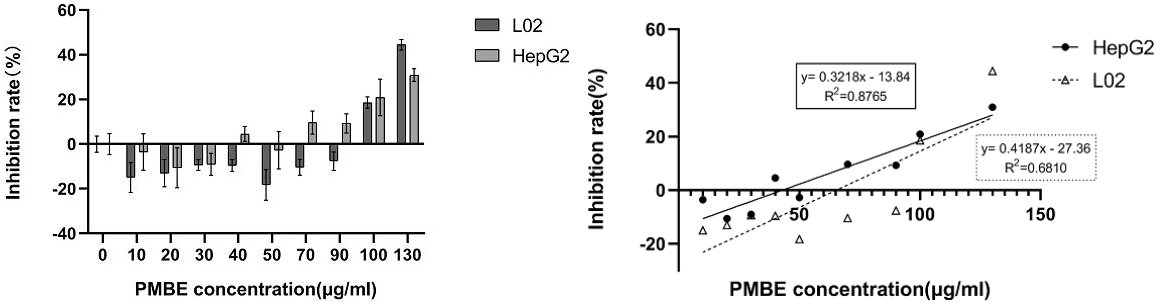


Figure 2. Effect of PMBE on the growth of L02 and HepG2 cells. (a) Inhibition rate and standard deviation of L02 and HepG2 cells at different concentrations. Mean ± standard deviation (n = 6); (b) Statistical analysis of inhibition rate. Results showed obvious concentration-dependent effect, however, no obvious regularity was found between adjacent concentration gradients. Under concentrations of 40 μg/ml and 70 μg/ml, PMBE inhibited the growth of HepG2 cells rather than L02 cells

counting chamber and MTT method, respectively. The results showed that both HepG2 and L02 cells entered the logarithmic growth phase on the fourth day (Figure 1).

3.2 Determination of suitable selective concentration of PMBE by MTT assay

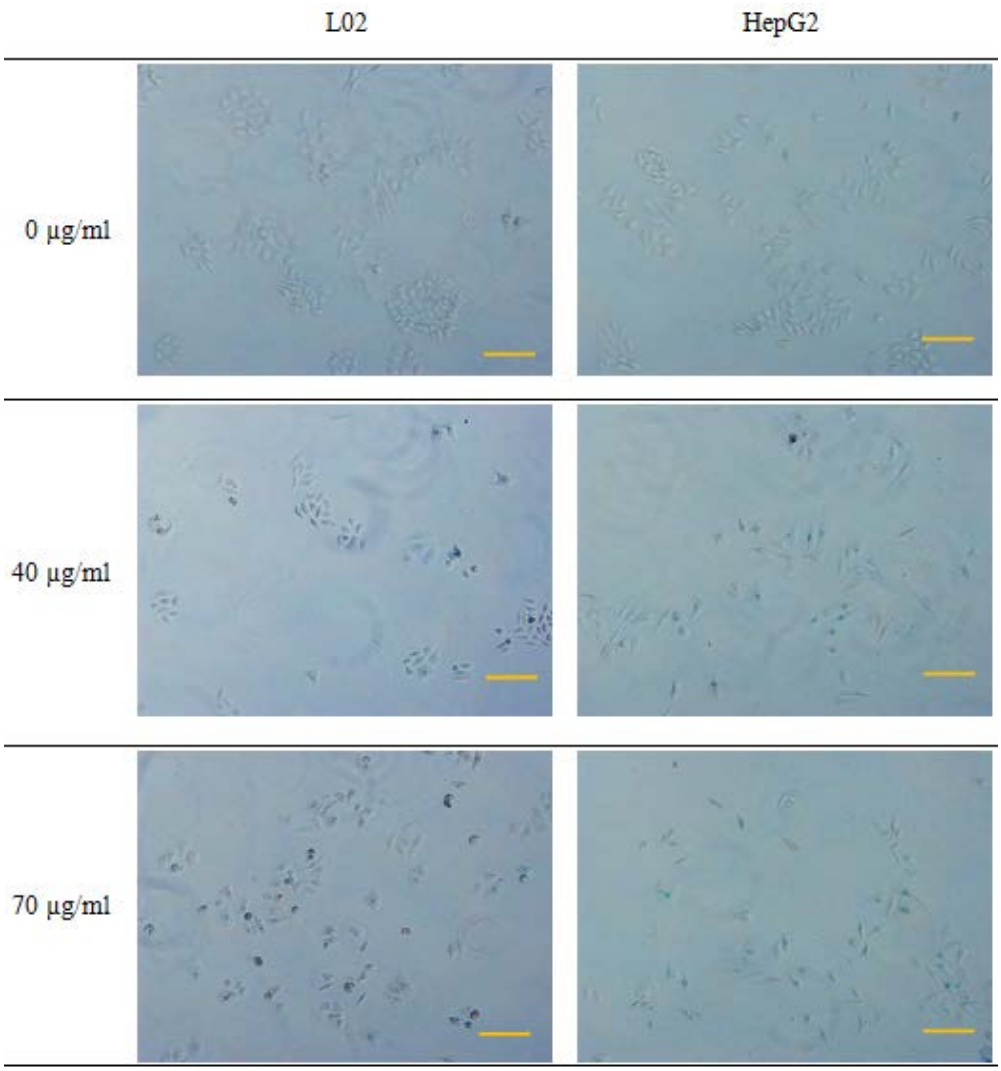
HepG2 and L02 cells with logarithmic growth were treated with different concentrations of PMBE for 48 h. The inhibition rate for cell growth was detected by MTT assay and calculated by the following equation: inhibition rate (%) = (1 - $A_{Treatment}/A_{Control}$) × 100, where A was the absorbance at 570 nm. By calculating the inhibition rate, two concentrations (40 μg/ml and 70 μg/ml) only inhibiting HepG2 cells were found (Figure 2).

3.3 Senescence β-Galactosidase Staining assay

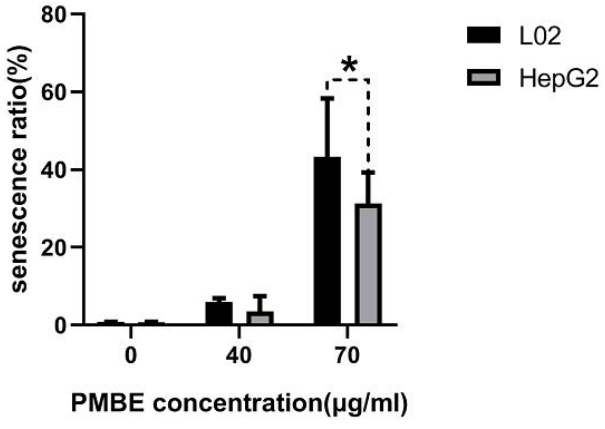
The present concept of cellular senescence stems from the fact that it is an arrest of cellular cycle resulting in a permanent loss of proliferative potential in spite of viable signals (which fails to reinitiate cell cycling). Using the classical senescence-associated-β-galactosidase (SA-β-gal) staining in the HepG2 and L02 cells, we observed that PMBE induced senescence up to 3.5± 4%, 31.3 ± 8% for HepG2 at dose of 40 μg/ml and 70 μg/ml, respectively, in contrast to 6.0 ± 1%, 43.3 ±15% for the control group (L02). Besides SA-β-gal staining, the small and wizened morphology which is marked as an important hallmark of apoptotic cell was also prominently observed in the treated cells with 70 μg/ml PMBE. We inferred that with the increase of the concentration of PMBE, the percentage of stained cells gradually increased, showing an obvious concentration-dependent effect. PMBE was a highly potent therapeutic agent which diminishes the cancer cells' proliferative potential. However, under 40 μg/ml there was no difference in the senescence-inducing effect of PMBE on HepG2 and L02 cells ($p = 0.56$, >0.05), and under 70 μg/ml the percentage of stained cells of L02 was significantly greater than that of HepG2 ($p = 0.043$, <0.05, Figure 3).

4 Discussion

Pinus massoniana is a tree species native to Southern China[14] and *P. massoniana* bark extract (PMBE) is an established traditional Chinese medicine used for the treatment of rheumatism, arthralgia, inflammation and cancer[22,23]. HPLC analysis indicated that PMBE contains several polyphenolic compounds, such as taxifolin



(a)



(b)

Figure 3. PMBE's effect on cell senescence of HepG2 cells. (a) The senescence induced by PMBE was quantified by counting SA-β-Gal positive cells (scale bar 10 μm); (b) Both L02 and HepG2 cells showed different degrees of senescence at 40 μg/ml concentration, while apoptotic cells were even observed at 70 μg/ml concentration. PMBE induced senescence up to 3.5$\pm$ 4%, 31.3 $\pm$ 8% for HepG2 at dose of 40 μg/ml and 70 μg/ml respectively in contrast to 6.0 $\pm$ 1%, 43.3 $\pm$15% for the control group (L02). Mean $\pm$ standard deviation (n = 3); $^{\star}p < 0.05$

epicatechin and epigallocatechin galloate[14]. PMBE is reported to have many biomedical properties, such as antioxidant, anticarcinogenic, antimutagenic, antimicrobial, anti-inflammatory, and cardio-cerebrovascular protective, *etc*.[1].

Even though, the anticancer effect has always been a hot topic in PMBE research. PMBE can inhibit the growth of eight different tumor cells *in vitro*[12] and selectively inhibit the proliferation of several cancer cells, such as human hepatoma BEL-7402 cells and HepG2 cells. But PMBE seldom influences normal cells[2,14]. Previous studies have demonstrated that PMBE selectively induce the apoptosis of hepatoma cells without impacting the growth of normal liver cells[14], the investigation of the mechanism indicated that activation of extrinsic and intrinsic Caspases, inhibition of NF-κB activation and decrease of the antiapoptotic protein Bcl-2 and the intact Bid protein were involved[14]. In addition, PMBE was found to have obvious antiproliferation and apoptosis-inducing effects on colon cancer LoVo cells, mainly by inhibiting the growth of cancer cells by activating $p53$ to up-regulate the expression of $p21$, and/or promoting the apoptosis of cancer cells by down-regulating the expression of *bcl*-2 gene[24].

The P53/P21 signaling pathway is one of the main mechanisms to regulate cell senescence, various stimulators of cell senescence function mainly by activating this pathway[15]. Cell senescence refers to the irreversible cell cycle stagnation caused by the activation of a series of intracellular signaling pathways under the stimulation of various internal and external factors[25]. Its characteristics mainly include chromatin changes, cell metabolism and morphological changes, and β-galactosidase activity enhancement[16]. Inducing cancer cell senescence can inhibit the proliferation of cancer cells, activate the body's immune response, and promote the clearance of cancer cells[26]. The present study investigated the senescence-inducing effect of PMBE on hepatoma HepG2 cells and the potential underlying mechanisms.

Firstly, the results of the current study indicated that PMBE was able to significantly affect the growth of hepatoma HepG2 cells with dose-dependent manner. The growth of HepG2 was promoted when PMBE concentration is below 50 μg/ml, while when PMBE concentration is above 50 μg/ml, the growth of HepG2 cells was inhibited by PMBE at dose-dependent manner. As for normal liver L02 cell, PMBE exhibit no inhibitory effect until the concentration was $>$ 90 μg/ml, which is discrepant with previous studies, Ma *et al.* reported that PMBE exhibited little growth inhibition of L02 cells when its concentration is within 200 μg/ml[14]. In addition, another study revealed that PMBE have hepatoprotective activity against hydrogen peroxide ($H_2O_{2)}$-induced damage in normal human liver L02 cells[27]. These discrepancies may result from different sources of PMBE and different status of cell lines.

Secondly, the present study demonstrated that PMBE could induce HepG2 cells into senescence at concentration of 40 μg/ml.

To the best of our knowledge, the current study is the first report to indicate that PMBE can induce cell senescence of hepatoma cells and further inhibit its growth, and the latter is similar to previous studies' report. However, the controversial result is that PMBE also exhibit cell senescence-inducing and cell growth-inhibiting effect on normal liver cells. Cui *et al.* ever reported that PMBE could inhibit the growth of human liver cancer cells BEL-7402 while slightly promote that of normal liver cells L02 under 200 μg/ml[2]. Here may be some possible reasons. Firstly, the biological characteristics of L02 cell may change during cell passage, which resulted in increased sensitivity to PMBE; secondly, different sources of PMBE and storage conditions of PMBE stock solution may cause changes in its biological activity[2]; furthermore, DMSO in PMBE working solutions may exhibit cytotoxic effect, but previous studies demonstrated that DMSO was not expected to exhibit any effect on cultured cells when its concentration in the working solution was lower than 0.25%[20]. Therefore, in order to solve this dispute, we need to optimize the experimental scheme and repeat the experiments to obtain more accurate results. In addition, there are some limitations in this study. First, we used HepG2 cells as our target cell line, meaning our conclusions are based on *in vitro* data and the effects have not been examined *in vivo*; second, the conclusions of this study are just at the cellular level, to further understand the comprehensive effect of PMBE on cancer cells, the corresponding specific molecular mechanisms should be explored.

5 Conclusion

Summarily, the current study indicated PMBE could induce hepatoma cells into senescence and then inhibit their growth, but more studies should be performed to identify the potential mechanism and its safety in normal cells before its application in treatment of cancer in clinic.

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Conflict of Interest

The authors declare no conflict of interest.

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Initial Hypothesis

Editor's Note

Since the 2nd half of the 16th century, over more than 300 hundred years of exploration and accumulation, research has entered the stage of revealing the nature of objects from observing the surface phenomena. Meanwhile, hypothesis-driven research (HDR) has gradually become the main form of research in many fields of modern science.

HDR begins with familiarizing the background knowledge, according to the direction of the problem, using rational thinking methods to generalize the known relevant scientific phenomena and laws and build scientific hypotheses, then carrying out theoretical deduction and prediction, and then designing corresponding research programs including scientific observation, scientific experiment or scientific investigation, so as to test hypotheses and further obtain the answers.

Usually, the formation of scientific hypothesis includes two stages: the initial hypothesis and the complete hypothesis. The former is only based on a small number of factual materials and existing relevant theories, and puts forward a tentative hypothesis for the answer of questions through thinking processing, which has not yet constituted a systematic discussion. Further, the initial hypothesis mainly includes the following four basic links: accumulating facts, mastering knowledge; analyzing facts, sorting out data; guessing, drawing conclusions; constructing concepts, expressing hypotheses. After putting forward the initial hypothesis, researchers should take this as the center, use various existing scientific theories and as many relevant background knowledge or conditions as possible to carry out extensive argumentation, explain the known facts, predict the unknown facts and potential rules or laws, and strive to theorize and systematize them, so as to expand the initial hypothesis into a relatively complete and stable theoretical system.

In brief, the formation of scientific hypothesis is a process of describing facts, explaining facts, and finding causal links and objective laws. Therefore, it requires objective analysis of problems, comprehensive collection of materials and in-depth processing, and on this basis, a comprehensive grasp of the links between things.

Considering the fact that the ability to propose hypotheses is an important feature of innovative talents, budding researchers should be trained from endowing them with capabilities for putting forward hypothesis. Just in this sense, CCR plans to select some hypothesis papers for publication to encourage more graduates or even undergraduates to express their special observation perspectives and innovative thinking on carcinogenesis, cancer prevention and treatment, further help them develop into mature researchers as soon as possible. The subsequent hypothesis is a good case in point.

Effects of *Pinus massoniana* bark extract on the size of HeLa cells via Nesprin-2 pathway

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Abstract: Proanthocyanidins (PAs) is the main constituent of *Pinus massoniana* bark extract (PMBE). PMBE was reported to induce cell cycle arrest and apoptosis in HeLa cells. During cell division, cells synthesize protein in G1 and G2 phases and replicate chromatin in S phase during interphase, which increases cell mass. Nesprins, a kind of protein encoded by *syne* gene, is a vital part of cytoskeleton and plays a role in cell cycle progress and cell division. HeLa cells were used as a model to examine effects of PMBE on cell growth and Nesprins expression with MTT assay and RT- PCR analysis, respectively. The cell size was evaluated by counting the cell number in a fixed area under microscope. The results showed that the size of survival HeLa cells in PMBE-treated group was obviously larger than that of those in control group ($p = 0.00223$, < 0.01), while the mRNA expression level of Nesprin-2 decreased significantly in PMBE-treated group ($p = 0.0201$, < 0.05). On this account, we put forward a hypothesis that PMBE inhibits the expression of *syne-2*, which leads to the decrease of Nesprin-2 and further results in the size increase of HeLa cells.

Keywords: Proanthocyanidins (PAs), *Pinus massoniana* bark extract (PMBE), cell size, HeLa cells, Nesprins

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1 Introduction

Pinus massoniana Lamb, a member of Pinaceae family, is mainly grown in the south region of Changjiang River of China[1]. In traditional Chinese medicine, its bark, needles, pollen and turpentine are usually used to treat diseases including rheumatism arthralgia, hypertension, chilblain, and inflammation[1,2]. In addition, *Pinus massoniana* Lamb is rich in proanthocyanidins (PAs), which is a kind of natural antioxidant flavonoid[3,4]. In 2005, the components of *Pinus massoniana* bark extract (PMBE) were firstly reported as a natural extract developed and researched independently in China[2]. PMBE is a polyphenolic compound, whose primary active compound is PAs, including monomeric, oligomeric, and polymeric flavonoids. The B-type PAs, especially proanthocyanidin B_3 is the main component of PMBE[5,6].

PMBE was demonstrated to exhibit various bioactivities such as antiviral, anti-allergic, antioxidant and anticancer[2,7,8]. It has been proven that PMBE mainly induces cancer cells cycle arrest at S and G2/M phase[9], further induces apoptosis of cancer cells via mitochondrial pathway and / or death receptor pathway[9–11]. While anthocyanins, metabolites of PAs heated in acidic media, can induce autophagy of human hepatocellular carcinoma (HCC) cells[11]. In addition, PMBE can also inhibit the migration and invasion of HeLa cell *in vitro*[12,13]. Obviously, the previous research of PMBE was focused on the inhibition of tumor cell growth and malignant behavior. The effect of PMBE on tumor cells' size has never been reported.

Nesprin was found in vascular smooth muscle cell in 2001, and there are 4 members of Nesprins encoded by *syne*-1∼4 genes respectively in mammal. Nesprins are located in the nuclear envelope (NE), and their basic structures include C-terminal, ANC-1 and Syne/Nesprin homology (KASH), N-terminal-actin binding domain (ABD) and spectrin repeat (SR)[14]. The C-terminal of KASH protein interacts with inner nuclear membrane SUN-domain family members and the N-ABD can connect with F-actin. All of these components take part in constituting LINC complex[15,16]. The LINC spans both nuclear membranes, forming the bridge between nuclear lamina and cytoskeleton. Any depletion of the components of LINC complex will lead to cell mechanical tension decrease[17]. Nesprin-2 is located in both inner and outer nuclear membranes[14]. It has higher isogeny with Nesprin-1, and works in a similarly way. Nesprins located in outer nuclear membrane can help the nuclear and cytoskeleton position, promote the material transportation between nuclear and cytoplasm, power conduction and adjust the cytoplasmic network. Nesprin-2 links the nucleus to actin filaments by interacting with SUN1 & 2[17,18], connects with centrosome via microtubule and plays a role in cell polarization, and helps centrosome dislodge from nuclear membrane in the prophase of mitosis[19].

Normally, cells have species specificity with a uniform size, which is regulated by nucleo-cytoplasmic ratio, ploidy, nutrition and so on[20]. Variability in cell size is affected by cell growth rate, cell-cycle length and asymmetry in cell division[21]. Increasing evidence indicates that cells can measure and regulate their size autonomously[21]. When the size of cell increases, its nucleo-cytoplasmic (N/C) ratio will decrease and gradually achieve the size threshold[21]. The cell enters S phase and starts to divide as soon as the N/C ratio reduces to a specific threshold value. If the growth is blocked, cell cannot divide any more. However, this is not always the case for tumor cells[20,22], namely, when tumor cell division is blocked, tumor cell can grow as usually, thus huge cell and/or polyploid will appear, so-called giant tumor cells (GTC). Owing to mitotic disorders, GTC are possible to show inheritable drug resistance compared with typical small tumor cells[23–25]. Furthermore, for some kinds of tumor cells, tetraploid cells can revert to diploid, and acquire drug resistance and stronger viability[26]. Thus, the drug-resistant giant cells could be potential reason for recurrence of tumor[27]. Therefore, the present study aims to explore the effects of PMBE on cell size in HeLa cells and potential mechanism, mainly focusing on the regulation of Nesprin-2.

2 Results

2.1 Hypothesis

Based on the results of our primary experiments: (1) the size of the cells in PMBE group was obviously larger than that of cells in blank group (Figure 1); (2) the transcription of gene *syne-2* was significantly inhibited (Figure 2), we put forward a hypothesis that PMBE can regulate HeLa cell size through inhibiting the transcription of *syne*-2 gene (Figure 3). This may lead to the reduction of Nesprin-2 on nuclear membrane, further interrupting their connection with centrosome via microtubule, and

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weakening the function of LINC complex. All these result in the decrease of cell mechanical tension, and finally, the cells become more oblate and bigger. In addition, centrosome cannot dislodge from NE successfully. However, the location of centrosome determines the polarity of cell. In other words, the failure of centrosome movement may lead to the failure of cell division, or results in cell's abnormal division and further aneuploid. Cell performs protein synthesis and chromosome replication during interphase before mitosis, if failure in division, cell will be larger in size and further becomes tetraploid. In tetraploid cell, the chromosome ploidy makes its nuclear volume and N/C ratio raise, respectively, and further influence the expression level of unknown genes[20]. All of these may make cell size larger and larger. Inferior to diploid cell in stability, aneuploid and tetraploid cells are easier to die and / or in a few cases, become immortalization. Usually it is hard for aneuploid cell to grow or divide in normal tissue, however, more aneuploid cells are observed in tumor tissue, probably because gene mutation endows them more superiority to withstand the negative effect of aneuploidy. Finally, the tetraploid and aneuploidy of HeLa cells with larger size survive and be observed.

2.2 Figures

Cell size can be reflected indirectly via cell numbers in a fixed field under microscope, and the cell size is negative correlation to the cell number.

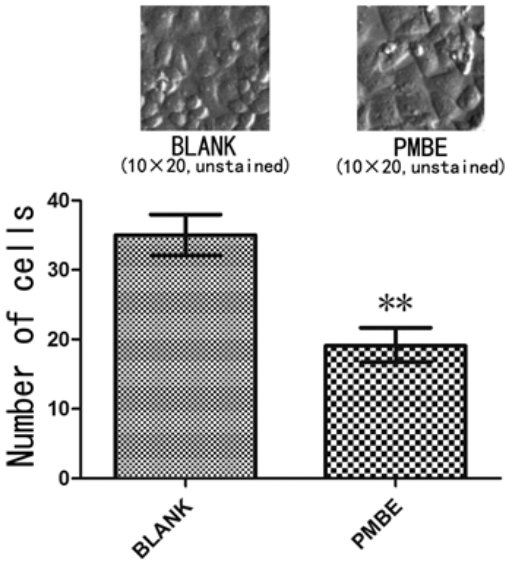

Figure 1. Effect of PMBE on the size of HeLa cells cultured *in vitro* (Mean ± SD; $^{**}p = 0.002$, < 0.01 versus blank group; n = 3)

The transcription level of *syne-2* is evaluated by the brightness value of bands of PCR products. The influence of PMBE on the transcription of *syne-2* can be reflected via the variation of the ratio of the brightness value of *syne-2* PCR band to the brightness value of *GAPDH* PCR band. The inset shows a representative result of the electrophoresis of respective PCR products.

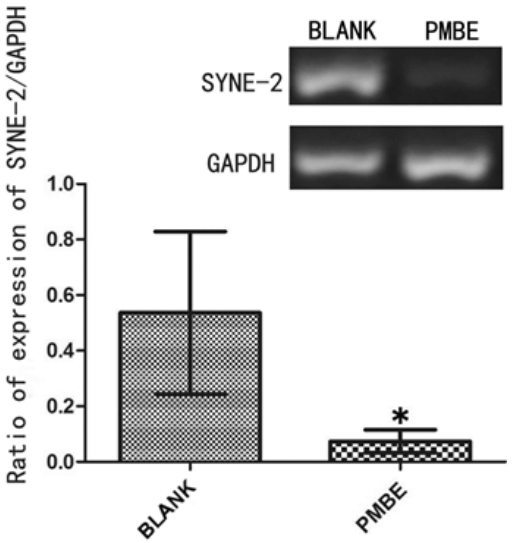

Figure 2. Impact of PMBE on the expression of *syne-2* at the transcriptional level in HeLa cells (Mean ± SD; $^{*}p = 0.02$, < 0.05 versus blank group; n = 5)

PMBE inhibits the transcription of *syne-2* and leads to the reduction of Nesprin-2, whose location is across nuclear membranes. The polarization of centrosome is inhibited, and mitosis cannot continue. The giant tetraploid tumor cells grow and further divide into aneuploidy and/or diploid, which may help them acquire potential drug resistance.

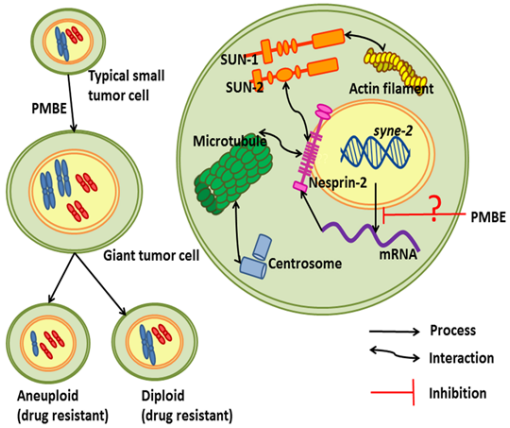

Figure 3. Hypothesis of PMBE affecting the size of HeLa cells via inhibiting *syne-2* transcription

3 Discussion

There are more aneuploidy cells in tumor tissue. Research has shown that appearance of tetraploid cells is related to the initialization of tumor, for tetraploid is in-

stable and tends to become aneuploidy, which may give cell immortalization[33]. This hints that it is possible for PMBE to induce chromosome abberation of HeLa cells by blocking its division via abnormal expression of Nesprin-2. Nesprin-2 is an important composition of LINC, and plays a role in gene expression and cell movement. In *syne*-2 knock-down cells, perinuclear F-actin filaments were largely absent. In *syne*-2 depletion cells, keratin bundles adjacent to NE were reduced significantly or absent. The loss of Nesprin-2 or kinesin light chain-1 (KLC1), or the uncoupling of Nesprin-2 with SUN domain all will hinder centrosome move away from nuclear. Lack of Nesprin-2 will also affect the expression of KLC1 and then affects cytoskeleton and Golgi apparatus. Mice with *syne*-2 knockout have an increased epidermal thickness, and harbor cell polarity defects[19]. In *syne*-1 & 2 depletion endothelial cells, the cellular protrusions are longer, nuclear area increase, cell migration and angiogenic loop formation decrease[17]. All of these prove that Nesprins are crucial to nuclear shape and behavior, as well as cell polarity.

Nesprins are nuclear envelope protein. And nuclear, as the center of cell, its change will certainly influence the whole cell. As the important member of LINC complex, change of Nesprins can also affect cell shape and behaviors. For tetraploid cell, when it divides, redundant centrosomes will appear, and exactly results in cell instability. So it is reasonable to hypothesize that Nesprin-2 is the key reason for HeLa cell's size increase. In studies of inducing drug resistant tumor cells using antineoplastic agents, several kinds of resistant tumor cells are observed increase in size, including ovarian cancer cells[28], lung cancer cells[29,30] and murine lymphoma cells[23], or abnormal shape of nuclear, such as gastric cancer cells[31]. Some resistant tumor cells, such as breast cancer cells, cell cycle arrest is observed[32]. These results signify that the linkage between tumor cell size and antineoplastic drug resistance may exist. GTCs show inheritable drug resistant and stronger viability[24–26], and can be the potential reason for tumor relapse[27].

Our primary experiment results illuminated that PMBE inhibited the expression of *syne*-2 at transcriptional level. This is the basis of our hypothesis. However, whether PMBE influences the translation or the activity of Nesprin-2 is still unknown. Nesprin-1 & 2 have higher isogeny, both of them link the nucleus to actin filaments by interacting with SUN1 & 2. It has been confirmed that *syne*-1 knock-down has no effect on other LINC protein located in NE. As the member of LINC, location of SUN-1 & 2 and Nesprin-2 is not influenced by the lack of Lamin A/C. If *syne*-1 is knocked down, the SUN-1 & 2 and Lamin A/C will locate on NE; while if Lamin A/C is knocked down, the SUN-1 & 2 and Nesprin-2 will still locate on NE[15]. It means members of LINC can compensate each other in some way. When Nesprin-2 decreased, as Nesprin-1 has the similar function with Nesprin-2, Nesprin-1 is possible to be increased. Whether cellular morphology is affected by the decrease of Nesprin-2 alone is uncertain. In addition, as a kind of malignant tumor cell, HeLa cell has chromosome abnormality itself, whether this abnormality will be changed by PMBE needs further validation.

Furthermore, it is necessary to measure the expression and activity of Nesprin-2 at translational and post-translational level after treatment with PMBE. To perform karyotype analysis of HeLa cells in both PMBE group and blank group, and calculate the proportion of abnormal karyotype, respectively, is a way to verify whether the abnormality change is correlated with PMBE treatment.

Previous peer researches prove that the LINC plays an important role in cell polarization and the location of centrosome, but the underlying molecular mechanisms stand still unclear. If the hypothesis is verified, the influence of cytoskeleton members, *e.g.* Nesprin-2, on cell polarization and cell size will become more clarity. In our experiments, we used 154 μg/mL PMBE (IC_{50}) to treat HeLa cells, 50% cells were inhibited, and the other escaped from drug were highly possible to get drug resistance. Drug resistance is a vital reason for the failure in chemotherapy of malignant tumor in clinic[34], and also a problem urgent to resolve. For over a century, clinical cancer treatment research has focused on drug development. Of note, recently, the theory of evolution by Charles Darwin has been suggested to help direct cancer treatment[35], or so-called Darwinian Cancer Drug Program. Taking the tumor microenvironment alteration into consideration, the program suggests the existing drugs should be used more effectively than the standard continuous administration at maximum tolerated dose until the remaining cancer cells progress to a certain extent[36]. And this treatment strategy can not only maintain the control of tumor for a longer time, but also reduce the toxicity due to the significant reduction of drug dosage, patients will reduce the harm caused by toxic side effects[36]. This novel program and strategy seems to support the correctness of our preliminary findings and the potential application value of further research. Furthermore, it still needs verifying whether drug resistance is related to cell size, centrosome and chromosome karyotype. If true, it may offer novel targets for the R&D of antineoplastic drugs.

4 Conclusion

In summary, we provided the primary evidence that PMBE treatment increases the size of HeLa cells and

inhibits the expression of *syne-2* in them. Based on these results, we put forward a hypothesis that PMBE regulates the size of HeLa cells via Nesprin-2 pathway. Targeting Nesprin-2 might serve as a novel promising regimen to control tumor size and antagonize the formation of drug-resistance in tumors.

5 Materials and methods

5.1 Cells and reagents

HeLa cells were obtained from American Type Culture Collection. PMBE powder (Lot.1116) was purchased from N.B.C Biological Material Co.Ltd (Huzhou, Zhejiang, China). HeLa cells were cultured in Dulbecco's modified Eagle's medium (HyClone, GE Healthcare, Logan, UT, USA) added 10% heat-inactivated fetal bovine serum (HyClone, GE Healthcare, Logan, UT, USA), 100 units/mL penicillin and 100 μg/mL streptomycin (Beyotime Biotechnology, Shanghai, China), and maintained at 37°C in a humidified incubator containing 5% CO_2.

5.2 MTT assay and determinate the 50% inhibition concentration (IC_{50}) of PMBE

HeLa cells were seeded into 96-well plate and then treated with different concentrations of PMBE (40-200 μg/mL) for 48 hours. The MTT Cell Proliferation and Cytotoxicity Assay Kit was purchased from Beyotime Biotechnology (Shanghai, China). MTT assay and IC_{50} software were used to obtain the IC_{50}[13].

5.3 Cell size evaluation

HeLa cells were divided into control group (blank) and PMBE group (treated with PMBE of IC_{50} = 154 μg/mL for 48 h). The cell size was evaluated indirectly by counting the total cell number in a fixed area in three different fields. Photos were taken under microscope at 10×20 magnification.

5.4 RT-PCR

Extract total RNA from cells using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and transcribe them into complementary DNA (cDNA) using an PrimeScript RT Reagent kit (TaKaRa Biotechnology, Dalian, China). PCR was used to measure the transcription level of *syne-2* and *GAPDH* (Internal control) using PCR Amplification Kit (TaKaRa Biotechnology, Dalian, China). The PCR results were analyzed by Image J2x software and Adobe Photoshop. Measure the brightness value of band of *syne-2* and *GAPDH* of each group, respectively, and then calculate the ratio of *syne-2/GAPDH*.

5.5 Statistical analysis

Results are presented as mean ± S.D. of n independent experiments. Statistical significance was assessed using analysis of variance. Subsequent pair-wise comparisons of specific means were assessed using the Student's t test. $p < 0.05$ was considered statistically significant.

Author Contributions

Conceptualization, Y.Y. Cui; methodology, X.L. Zhang, Y.Y. Li and J. Feng; validation, X.L. Zhang, M.Q. Li, Y.Y. Li and J. Feng; formal analysis, X.L. Zhang. and M.Q. Li; data curation, Y.Y. Cui; writing—original draft preparation, X.L. Zhang; writing—review and editing, M.Q. Li, Y.Y. Li and J. Feng.; supervision, Y.Y. Cui; project administration, Y.Y. Cui; funding acquisition, Y.Y. Cui.

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Conflicts of interest

There was no conflict of interest.

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RESEARCH ARTICLE

Financial distress among breast cancer survivors

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Abstract: Aims: there has been an increasing awareness of the potential for oncology care to result in long-term financial burdens and financial toxicity. Patients who report cancer-related financial problems or high costs are more likely to forgo or delay prescription medications and medical care. Materials and Methods: we examined financial distress using data from a survey of 164 breast cancer survivors who had completed primary therapy for the disease. Key Findings: among respondents, 8.6% (13 of 151) reported that "being less able to provide for the financial needs of their family" was as a severe problem; 14.4% (22 of 153) reported "difficulty in meeting medical expenses" was a severe problem; and 8.4% (13 of 154) reported that "no money for cost of or co-payment for medical visits" was a severe problem. About 8.4% (13 of 154) of the respondents reported that "no money for cost of or co-payment for medicine" was a severe problem. In logistic regression analysis, younger age and lower household income were significant predictors of financial distress. In multiple linear regression analysis, younger age and lower household income were significant predictors of financial distress. Significance: financial toxicity remains a major issue in breast cancer care. Efforts are needed to ensure patients experiencing high levels of financial toxicity are able to access recommended care. In addition, patients should talk with their providers about the costs of oncology care and about opportunities to reduce costs while maintaining high quality of care.

Keywords: breast cancer survivors, costs, financial distress

1 Introduction

There has been increasing awareness of the potential for oncology care to result in long-term financial burdens and financial toxicity to patients and their families[1–3]. About 28% to 48% of cancer survivors experience financial toxicity based upon monetary measures and 16% to 73% experience financial toxicity based upon subjective measures[4]. Sources of financial distress include costs associated with cancer care services (*e.g.*, medications, supplies, co-payments, transportation, parking) and reduced income because of missing work, loss of employment, or unplanned retirement[5]. Cancer survivors may be vulnerable to out-of-pocket expenses due to unemployment, medical debt, and diminished consumer credit[6,7]. This is especially true for women, young patients, racial and ethnic minorities, persons who have low income or financial illiteracy, and those without health insurance[1,4,8]. Cancer survivors with public insurance experience greater economic burden than those with private insurance[9], although having health insurance does not fully protect against financial distress associated with cancer[8]. Among low-income women, the costs of breast cancer care has been reported to represent up to 98% of an individual's annual earnings[3,10]. High cancer-related financial burden affects treatment choice, treatment compliance, and cancer outcomes[8]. Patients who report cancer-related financial problems or high costs may be more likely to forgo or delay prescription medications or medical care[4,11]. Financial distress due to financial obligations, debt and diminished wealth may interfere with the ability of cancer patients to cope with physical symptoms and follow-up care, and lead to poorer health-related quality of life and health outcomes[4].

We examined financial distress using data from a survey of 164 breast cancer survivors who had completed primary therapy for the disease. The overall objective was to determine the prevalence of financial strain and financial distress, and to identify predictors of financial distress.

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Table 1 Characteristics of study participants (n=164)

Characteristic	Frequency (%)
Age (years) mean (SD) (N=163)	67 (41.1)
Race (N = 156)	
White, Non-Hispanic	104 (66.7)
African American, Non-Hispanic	46 (29.5)
Other[1]	6 (3.9)
Annual Income (N = 46)	
<$20,000	17 (10.4)
$20,000 - $34,999	17 (10.4)
$35,000 - $49,999	17 (10.4)
$50,000 - $64,999	14 (8.5)
$65,000 - $79,999	8 (4.9)
$80,000 +	38 (23.2)
Missing[2]	53 (32.3)
Number of people in household (N = 160)	
1	48 (30.0)
2	83 (51.9)
3 +	29 (18.1)
Employment status (N = 163)	
Retired	99 (60.7)
Employed	34 (20.9)
On disability	16 (9.8)
Homemaker	9 (5.5)
Temporarily unemployed	4 (2.5)
Marital status (N = 163)	
Married/Partner	84 (51.5)
Single	24 (14.7)
Widowed	32 (19.6)
Separated/Divorced	23 (14.1)
Education (N = 157)	
Less than HS	5 (3.2)
HS or equivalent	42 (26.8)
Some college	27 (17.2)
Associate degree	22 (14.0)
Bachelor degree	27 (17.2)
Graduate degree	34 (21.7)
Health Insurance (N = 161)	
Medicare	94 (58.4)
Private insurance	47 (29.2)
Other[3]	20 (12.4)
Perceived general health (N = 162)	
Excellent	16 (9.9)
Very good	58 (35.8)
Good	59 (36.4)
Fair	24 (14.8)
Poor	5 (3.1)
Breast cancer stage at diagnosis (N = 157)	
Ductal carcinoma in situ	31 (19.8)
Stage I	42 (26.8)
Stage II	33 (21.0)
Stage III	14 (8.9)
Stage IV	8 (5.1)
Don't know	29 (18.5)
Time since diagnosis (in years) mean (SD) (N = 155)	9.4 (8.8)
Type of treatment received[4] (N = 164)	
None	2 (1.2)
Surgery	161 (98.2)
Radiation	111 (67.7)
Chemotherapy	90 (54.9)
Hormone therapy	74 (45.1)
Biologic/Targeted therapy	8 (4.9)

2 Methods

The Cardiovascular Disease Outcomes among Breast Cancer Survivors Study (CVDBCS) was a postal survey of a multiethnic cohort of breast cancer survivors who reside in Augusta, GA and who had been treated at Augusta University Health or the Georgia Cancer Center. Non-institutionalized women were eligible to take part in the study if they resided in Augusta-Richmond County and Columbia County, GA, or Aiken County, SC and had been diagnosed with stage I-IV breast cancer and completed primary therapy for the disease (chemotherapy, radiation, surgery).

Table 2 Self-reported history of financial distress among breast cancer survivors (n = 164)

Being less able to provide for the financial needs of my family	
Not a problem	116 (76.82%)
Somewhat a problem	22 (14.57%)
A severe problem	13 (8.61%)
Difficulty in meeting my medical expenses	
Not a problem	108 (70.59%)
Somewhat a problem	23 (15.03%)
A severe problem	22 (14.38%)
No money for cost of or co-payment for medical visits	
Not a problem	125 (81.17%)
Somewhat a problem	16 (10.39%)
A severe problem	13 (8.44%)
No money for cost of or co-payment for medicine	
Not a problem	128 (83.12%)
Somewhat a problem	13 (8.44%)
A severe problem	13 (8.44%)

Data were collected using postal survey questionnaires and via abstraction of electronic medical records. The mailings were sent to 1,000 randomly sampled potential research participants who had been treated at Augusta University Health or the Georgia Cancer Center after July 2019. A sequential mailing protocol was followed using a modified Dillman method. An advance letter was mailed to the women by the study principal investigator. The letter provided information about the study (purpose, potential benefits, and risks) and informed them that they could opt out and not receive further mailings about the study. Three weeks later, an informed consent letter was mailed to women who had not opted out along with a copy of the questionnaire and a pre-addressed, stamped return envelope. Women who had not opted out or returned a completed questionnaire were sent a reminder postcard four weeks later. Survey responses were checked for completeness and then coded and entered into an electronic database. Survey questions about breast cancer diagnosis were obtained from a previous study of breast cancer survivors[12]. Respondents were asked about a variety of

symptoms and problems in living: "Tell us about your experience as a survivor. Below is a list of problems that people may have after cancer treatment. Please check the box to show how much this has been a problem for you during the past month (Not a problem, Somewhat a problem, A severe problem)." The inventory of symptoms and challenges in living included "Being less able to provide for the financial needs of my family", "Difficulty in meeting my medical expenses", "No money for cost of or co-payment for medical visits", and "No money for cost or co-payment for medicine".

After crosstabulations and exploratory analyses of the survey data were completed, logistic regression methods were used to compare groups of breast cancer survivors who did or did not report financial strain or financial distress according to age, race, education, household income, stage-at-diagnosis, years since breast cancer diagnosis, cancer treatment, hypercholesterolemia, hypertension, diabetes, congestive heart failure, and smoking status. The dependent variable in these analyses was whether or not the respondent answered at least one of the four questions about financial distress as somewhat/severe distress.

Potential confounding factors were controlled for in these analyses. Ninety-five percent confidence intervals were obtained for adjusted odds ratios. Levels of statistical significance were determined using Wald chi-square tests and Log-likelihood ratio tests. The goodness-of-fit of each model was examined using the Log-likelihood ratio tests.

Following the logistic regression analysis, multiple linear regression techniques were used to examine predictors of financial strain or distress according to age, race, education, household income, stage-at diagnosis, years since breast cancer diagnosis, cancer treatment, hypercholesterolemia, hypertension, diabetes, congestive heart failure, and smoking status. The dependent variable in these analyses was a quantitative score defined as follows: No stress was scored as zero, somewhat/severe distress was scored as 1, and the total distress was defined as the total score over the four questions.

3 Results

A total of 164 women completed the study questions (response rate 16.4%). The mean age of the women was 67 years (SD: 41.1) (Table 1). Among all participants, 66.7% were white, 29.5% were African-American, and the remainder were of other races. More than half (58.4%) of the women were insured through Medicare and 29.2% held private insurance. With respect to breast cancer stage at diagnosis, 19.8% of the women had ductal carcinoma in situ, 26.8% had stage I disease, 21.0% had stage II disease, 8.9% had stage III disease, and 5.1% had stage IV disease. The mean number of years since diagnosis was 9.4 years (SD: 8.8). About 54.9% of the women reported receiving chemotherapy; 45.1% reported receiving hormonal therapy; and only 4.9% reported biologic/targeted therapy.

About 8.6% (13 of 151) of the respondents reported that "being less able to provide for the financial needs of their family" was a severe problem (Table 2). Nearly 14.4% (22 of 153) of the respondents reported "difficulty in meeting medical expenses" was a severe problem (Table 2). Approximately 8.4% (13 of 154) of the respondents reported that "no money for cost of or co-payment for medical visits" was a severe problem (Table 2). About 8.4% (13 of 154) of the respondents reported that "no money for cost of or co-payment for medicine(s)" was a severe problem (Table 2). About 65.81% of the respondents (102 of 155) answered at least one of the four questions about financial distress as somewhat/severe problem (results not shown).

In logistic regression analysis (Table 3), younger age and lower household income were significant predictors of financial distress. Having an associate degree was of borderline significance. In multiple linear regression analysis (Table 4), younger age, and lower household income were significant predictors of financial distress.

4 Discussion

The results of this survey indicate that over 8% of the breast cancer survivors in this sample are having severe difficulty paying for medical visits or for the cost of medications. Over 8% reported that providing for the financial needs of their family was a severe problem. A total of 65.81% of the respondents (102 of 155) answered at least one of the four questions about financial distress as a somewhat/severe problem. Not being able to afford household expenses is one of the most commonly reported reasons for delayed medical care among cancer patients[13].

In multivariate analysis with logistic regression or multivariate linear regression, younger age and lower household income were significant predictors of financial distress in the current study. Our results are consistent with previous studies that indicate that younger age and low income are associated with financial toxicity among cancer patients[4, 14, 15]. Younger women are less likely to have Medicare insurance and they may also have expenses associated with childrearing.

The number of cancer treatment options has significantly increased over the past two decades, leading to improvement in patients outcomes for many malignancy types[14]. These advancements, however, are costly and

Table 3 Predictors of financial distress among breast cancer survivors from logistic regression analysis (n = 164)

Covariate	Odds Ratio	OR Confidence Interval	p-value
Age	0.823	0.7127 – 0.9112	0.0013
Race - White	Referent		
Race - African American	0.9134	0.0877 – 9.699	0.9377
Race - Other	32.2569	0.4334 – 4497.288	0.1227
Stage - DCIS	Referent		
Stage I	0.3031	0.0107 – 6.435	0.4507
Stage II	0.5921	0.0161 – 20.0556	0.7659
Stage III	0.2622	0.0027 – 13.8554	0.5307
Stage IV	37.5132	0.3774 – 9668.54	0.1442
Unknown Stage	0.1153	0.0014 – 4.1836	0.2674
Education - Less than High School	Referent		
Education - Some college	2.8747	0.0796 – 141.7124	0.5657
Education - Associate Degree	29.3424	1.362 – 2580.691	0.0636
Education Bachelor's Degree	0.1015	2e-04 – 10.651	0.3923
Education - Graduate Degree	1.2416	0.0175 – 80.3945	0.917
Income - < $20,000	Referent		
Income - $20,000 – $34,999	0.0081	0 – 0.4612	0.0361
Income - $35,000 – $49,999	9.00E-04	0 – 0.0564	0.005
Income - $50,000 – $64,999	1.94E-05	0 – 0.0045	0.0018
Income - $65,000 – $79,999	2.01E-05	0 – 0.0179	0.0133
Income - > $80,000	9.00E-04	0 – 0.0609	0.0063
Time since diagnosis	1.0305	0.9022 – 1.1899	0.6605
Treatment - Radiation	8.1986	0.6816 – 205.3786	0.1357
Treatment - Chemotherapy	2.5642	0.1885 – 48.1497	0.4916
Treatment - Hormone	0.7243	0.068 – 6.4841	0.7703
Treatment - Targeted	73.6146	0.2043 – 87784.25	0.168
High blood pressure	5.3151	0.3477 – 213.9699	0.2885
High cholesterol	0.5336	0.0229 – 7.0406	0.6498
Diabetes	0.6015	0.021 – 16.3123	0.7565
Congestive Heart Failure	1.6094	0.0082 – 267.5163	0.8556
Smoking - Never	Referent		
Smoking - Current	2.5995	0.0751 – 173.4794	0.6042
Smoking - Former	1.0864	0.1218 – 9.1872	0.9376

cancer is now the second most expensive disease in the United States, after heart disease[14]. As the cost of oncology care escalates, financial concerns of patients, families, physicians, and health care systems are increasingly common[15,16].

The causes of financial distress among cancer patients is multifactorial and includes patient demographics and socioeconomic characteristics, disease characteristics, treatment characteristics, and healthcare system factors[14]. In the current study, younger patients and those with a low-income or less educational attainment were more likely to report financial distress. Because we lacked information about healthcare system factors[14], the current study does not provide any information about the contribution of healthcare system factors to financial distress among breast cancer survivors. We did not observe any statistically significant associations with disease characteristics (*e.g.*, stage at diagnosis) or type of treatment received.

As noted by Lentz *et al.*[14], health care providers can take several steps to reduce financial toxicity including: 1) consider cost in addition to adverse effects if multiple treatment regimens have similar efficacy; 2) provide patients with estimates of cancer care costs; 3) consider whether an intervention will provide meaningful improvement; 4) incorporate financial toxicity screening into clinic evaluation and workflow; and 5) educate and provide assistance to patients about insurance benefits, other financial aid that may be available to them, etc. Providers such as medical, surgical, and radiation oncologists have a critical role to play in addressing financial toxicity. Early referral to a financial navigator should be considered when a need is identified[14]. A multidisciplinary approach involving nurses, social workers, and financial navigators provides additional expertise. Financial navigators can assess any risk of financial toxicity at time of cancer diagnosis and provide financial education and counseling[14].

With respect to other limitations, misclassification bias is a possibility due to the use of self-reported information. The current study was cross-sectional in nature which precludes conclusions on financial impact over a period of time. An additional limitation is the results of this study may not be generalizable to other populations of breast cancer survivors. However, the sample was somewhat diverse by race, socioeconomic factors, and history of breast cancer diagnosis and treatment.

Financial toxicity remains a major issue in breast cancer care. A significant number of cancer patients and families struggle with financial difficulty[15]. Financial distress

Table 4 Predictors of financial distress among breast cancer survivors from multiple regression analysis

Covariate	Beta coefficient	Confidence interval	p-value
(Intercept)	4.6323	2.39 – 6.8748	1.00E-04
Age	-0.04613	-0.073 – -0.0192	0.0011
Race - White	Referent		
Race - African American	0.4245	-0.2542 – 1.1033	0.2162
Race - Other	0.6946	-0.7433 – 2.1326	0.3384
Stage - DCIS	Referent		
Stage I	-0.4663	-1.28 – 0.3474	0.2567
Stage II	0.2067	-0.708 – 1.1215	0.6533
Stage III	-0.1379	-1.2347 – 0.9588	0.8025
Stage IV	0.1291	-1.1416 – 1.3998	0.8399
Unknown Stage	-0.6052	-1.4943 – 0.284	0.1789
Education - Less than HS	Referent		
Education - Some college	0.5293	-0.4183 – 1.4769	0.2689
Education - Associate Degree	0.8975	-0.0135 – 1.8084	0.0534
Education Bachelor's Degree	0.0108	-0.9813 – 1.0028	0.9828
Education - Graduate Degree	0.1326	-0.7972 – 1.0623	0.7768
Income - < $20,000	Referent		
Income - $20,000 – $34,999	-1.6202	-2.6414 – -0.5991	0.0023
Income - $35,000 – $49,999	-2.2768	-3.3355 – -1.2181	1.00E-04
Income - $50,000 – $64,999	-2.6749	-3.7687 – -1.5811	0
Income - $65,000 – $79,999	-2.3341	-3.7195 – -0.9487	0.0013
Income - > $80,000	-1.801	-2.8544 – -0.7477	0.0011
Time since diagnosis	0.0174	-0.0184 – 0.0532	0.3359
Treatment - Radiation	0.4533	-0.2331 – 1.1397	0.1919
Treatment - Chemotherapy	-0.0344	-0.678 – 0.6093	0.9155
Treatment - Hormone	0.1802	-0.3688 – 0.7292	0.5146
Treatment - Targeted	0.1565	-1.049 – 1.3621	0.7963
High blood pressure	0.3042	-0.3237 – 0.9321	0.3371
High cholesterol	0.1525	-0.4805 – 0.7854	0.6322
Diabetes	0.2215	-0.4892 – 0.9322	0.5361
CHF	-0.1209	-1.4663 – 1.2245	0.8582
Smoking - Never	Referent		
Smoking - Current	0.4856	-0.4319 – 1.4031	0.2946
Smoking - Former	0.1589	-0.4274 – 0.7453	0.5902

may be seen as distinct from, but not isolated from, the overall anxiety and discomfort experienced as a result of the cancer diagnosis and resulting treatment(s)[4]. Financial distress from mounting financial obligations and debt may interfere with the patient's ability to cope effectively with cancer and its treatment, thereby adversely affecting health outcomes[4]. Identifying those patients most at risk of facing financial difficulty is an important measure to ensure safety nets are available for these targeted populations[15]. Further studies are needed that include a larger percentage of minority patients as well as rural and underserved patients experiencing cancer and its resulting treatment(s). Efforts are needed to ensure all patients experiencing high levels of financial toxicity are able to access recommended care.

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Conflict of interest

The authors declare they have no conflicts of interest.

Ethical approval

This study was approved by the Augusta University Institutional Review Board.

Informed consent

The informed consent of research participants was obtained.

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RESEARCH ARTICLE

HIF and COX-2 expression in triple negative breast cancer cells with hypoxia and 5-fluorouracil

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Abstract: Our purpose was to understand the effects of normoxia or hypoxia on 5-fluorouracil (5-FU) treatment in triple negative breast cancer (TNBC) cells, and characterize the molecular changes in hypoxia inducible factors (HIFs) and cyclooxygenase-2 (COX-2) following treatment. Cell viability and protein levels of HIFs and COX-2 were determined after wild type and HIF silenced MDA-MB-231 cells, and wild type SUM-149 cells, were treated with 5-FU under normoxia or hypoxia. 5-FU reduced cell viability to the same levels irrespective of normoxia or hypoxia. HIF silenced MDA-MB-231 cells showed comparable changes in cell viability, supporting observations that hypoxia and the HIF pathways did not significantly influence cell viability reduction by 5-FU. Our data suggest that HIF-2α accumulation may predispose cancer cells to cell death under hypoxia. SUM-149 cells that have higher COX-2 and HIF-2α following 24 h of hypoxia, were more sensitive to 96 h of hypoxia compared to MDA-MB-231 cells, and were more sensitive to 5-FU than MDA-MB-231 cells. COX-2 levels changed with hypoxia and with 5-FU treatment but patterns were different between the two cell lines. At 96 h, COX-2 increased in both untreated and 5-FU treated cells under hypoxia in MDA-MB-231 cells. In SUM-149 cells, only treatment with 5-FU increased COX-2 at 96 h of hypoxia. Cells that survive hypoxia and 5-FU treatment may exhibit a more aggressive phenotype. Our results support understanding interactions between HIF and COX-2 with chemotherapeutic agents under normoxia and hypoxia, and investigating the use of COX-2 inhibitors in these settings.

Keywords: 5-FU, COX-2, HIF, Hypoxia, TNBC

1 Introduction

Although surgery is the primary form of treatment for breast cancer, some form of systemic neoadjuvant therapy is increasingly given prior to surgery[1]. Understanding the impact of these systemic treatments and the molecular changes induced by these treatments, especially within the abnormal microenvironments of tumors, therefore becomes important within the context of relapse. 10-30 % of all breast cancer cases are triple-negative breast cancer (TNBC)[2]. TNBCs, so defined because of the lack of expression of estrogen receptor (ER), progesterone receptor, and human epidermal growth factor receptor 2 (HER2)[3], typically have early relapse and poor overall survival[4]. Identifying mechanisms that may contribute to relapse is of significant importance in developing treatment strategies to overcome the poor prognosis of TNBC.

The abnormal vasculature that exists in tumors results in poor drug delivery as well as in the formation of hypoxic areas[5]. Hypoxia results in the stabilization of hypoxia inducible factors (HIFs) that activate the transcription of numerous genes involved in angiogenesis, metabolism, invasion, metastasis, the immune system, and resistance to chemo- and radiation therapy[6–8]. There are three isoforms of the α subunit, HIF-1α, HIF-2α, and HIF-3α. HIF-1α is frequently elevated in TNBC[9]. HIF-2α has been shown to correlate to distant recurrence and poor outcome in breast cancer[10]. Both play a major role in breast cancer cell metabolism[11], although they have been found to have opposing roles in hypoxic tumor growth[12] and metabolism[13]. Both HIF-1α

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and HIF-2α are important in the metastatic cascade[11]. Cyclooxygenase-2 (COX-2), a rate-limiting enzyme in prostaglandin synthesis, is overexpressed in various cancers, including breast cancers[14,15]. Hypoxia has been found to induce COX-2 expression in various cells and tissues including pulmonary artery smooth muscle, epithelial cells, and colorectal tumors[16,17]. HIF-1α or HIF-2α has been found to regulate COX-2 expression[16–20]. Inhibition of COX-2 was found to reduce metastasis formation in breast cancer cells and xenografts[21–23], and in women with breast cancer[24].

Because of the abnormal tumor vasculature and poor drug delivery, cancer cells *in vivo* are exposed to a range of drug concentrations under normoxic and hypoxic conditions. These drugs are metabolized or cleared through perfusion. The damage to these cells, as well as the molecular changes induced by the drug under these conditions, evolves over a period of time. We therefore treated two TNBC cell lines, MDA-MB-231 and SUM-149, and three sublines of MDA-MB-231 cells engineered to stably express shRNA against HIF-1α, HIF-2α and both HIF-1α and HIF-2α with varying doses of 5-FU. HIF-1α, HIF-2α and COX-2 levels were characterized at multiple time points after treatment with 5-FU. 5-FU, a synthetic fluorinated pyrimidine analogue that inhibits RNA synthesis[25,26], or its prodrug capecitabine, form an integral part of neoadjuvant therapy of TNBC[27]. Cells were maintained under normoxia or hypoxia during 5-FU treatment, as well as during multiple time points after treatment, to mimic conditions *in vivo*.

2 Materials and methods

2.1 Cell culture and treatment

Triple negative MDA-MB-231 human breast cancer cells were obtained from American Type Culture Collection (ATCC, Manassas, VA), and grown in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO). Triple negative SUM-149 inflammatory human breast cancer cells were obtained from Asterand (Detroit, MI), and were grown in DMEM/F12 (1:1) medium (Mediatech, Manassas, VA) supplemented with 5% FBS, 5 μg/ml insulin (Life Technologies, Grand island, NY) and 0.5 μg/ml hydrocortisone (Sigma-Aldrich). MDA-MB-231 cells stably expressing shRNA against HIF-1α (231-sh-HIF-1α), HIF-2α (231-sh-HIF-2α) and both HIF-1α and HIF-2α (231-sh-HIF-1/2α) were generated using lentiviral transduction as previously described[11,28]. These genetically engineered MDA-MB-231 sublines were maintained in RPMI-1640 medium supplemented with 10 % fetal bovine serum. All cell lines were maintained in a humidified atmosphere with 5 % CO_2 in air at 37^0C, and were tested routinely for mycoplasma contamination. Hypoxic treatment of cells was performed by placing the plates or dishes in a modular incubator chamber (Billups-Rothenberg, Del Mar, CA) flushed at 2 p.s.i. for 3 minutes with a gas mixture of 1% O_2, 5% CO_2 and N_2 for balance.

Cells were treated with 0.5 to 50 μg/ml 5-FU (Fresenius Kabi USA, LLC, Lake Zurich, IL) or Hanks' balanced salt solution (HBSS, Sigma-Aldrich) as control for 24 h under normoxia (N, 20% O_2) or hypoxia (H, 1% O_2). After 24 h treatment (N 24 h or H 24 h), cells were analyzed for proliferation or protein expression. In additional batches of cells, medium with 5-FU or HBSS was changed to fresh culture medium without 5-FU or HBSS under normoxia within 15 minutes and cells were continued to be cultured under normoxia or hypoxia for another 24 h (N 48 h or H 48 h), 48 h (N 72 h or H 72 h), or 72 h (N 96 h or H 96 h) after 5-FU treatment. Cells were analyzed for proliferation or protein expression at these time points.

2.2 Cell viability/proliferation assay by CCK-8

5000 cells were seeded in each well of a 96 well plate and cultured overnight. Twenty-four hours later, cells were treated with 5-FU for 24 h under normoxia or hypoxia. Cell viability was determined at various time points using cell counting kit-8 assays (CCK-8, Dojindo Molecular Technologies, Inc. MD), performed using the manufacturer's instructions. Cell viability was measured at 450 nm using a 1420 Multilabel counter (Perkin Elmer, Waltham, MA) after 2 h incubation under normoxia with the CCK-8 reagent. In the CCK-8 colorimetric assay the amount of the formazan dye, which is generated by the activities of dehydrogenases in cells, is directly proportional to the number of living cells. Untreated cells and HBSS treated cells were used as negative controls. Values from each group were normalized to the average of values obtained from untreated normoxic cells that was set to 100% viability. We made sure that the incubation time (2 h) after adding the CCK-8 reagent and blank absorbance was similar in samples from N and H conditions to allow for comparisons. At least 3 independent experiments were performed.

2.3 Immunoblot analysis

Whole-cell extracts were prepared by lysing cells with RIPA lysis buffer supplemented with a protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA). Protein concentrations were estimated using the Bradford's

method (Bio-Rad, Hercules, CA). Equal amounts of total protein were resolved on a 7.5% SDS-PAGE gels and transferred to a nitrocellulose membrane (Bio-Rad). After blocking in 5% milk-TBST (TBS Tween), the membrane was separately probed with HIF-1α antibody (BD Biosciences San Jose, CA), HIF-2α antibody (Novus Biologicals, Littleton, CO) and COX-2 antibody (Cayman Chemical, Ann Arbor, MI). Anti-GAPDH antibody (Sigma-Aldrich) was used for equal loading assessment. Secondary antibodies were horseradish peroxidase conjugated anti-mouse, anti-rabbit (GE Healthcare, Chicago, IL), or anti-goat IgG (Novus Biologicals). The signal was visualized using ECL Plus reagents (Thermo Scientific, Rockford, IL) and developed on a Blue Bio film (Denville Scientific, Metuchen, NJ). The films were scanned and densitometric analysis was performed using the ImageJ (NIH, Bethesda, MD). Relative density changes against GAPDH were analyzed and in some cases normalized to the immunoreactive bands in control cells (HBSS treated), which was set to 1 using data from 2-3 cell lysates.

2.4 Statistical analysis

Data are expressed as Mean $\pm$ Standard Error Mean (SEM). Statistical significance was evaluated using a one-tailed unpaired Student's t-test. P values $\leq$ 0.05 were considered to be significant.

3 Results

3.1 Effects of 5-FU under normoxia or hypoxia on cell viability in MDA-MB-231 cells

Cell viabilities (%) of the different groups compared to untreated cells under normoxia are shown in Figure 1. The three doses of 0.5, 2.5, and 50 μg/ml were selected to represent low toxicity ($\sim$80% cell viability at 96 h), medium toxicity ($\sim$40-50% cell viability at 96 h), and high toxicity ($\sim$15% cell viability at 96 h).

MDA-MB-231 cells, maintained under 96 h hypoxia, showed significantly lower cell viability (77.7%) compared to normoxic cells at the same time point. Treatment with the highest dose of 50 μg/ml 5-FU resulted in a significant decrease of viability at all the time points with a greater reduction of cell viability with increasing time, even though 5-FU was withdrawn at 24 h. This dose was equally effective under normoxic or hypoxic conditions.

Similar to the higher dose, treatment with 0.5 μg/ml and 2.5 μg/ml 5-FU resulted in a progressive reduction of cell viability at the later times, although the decrease of cell viability was less than that for the higher dose. The lower doses were also equally effective under normoxic or hypoxic conditions in these cells.

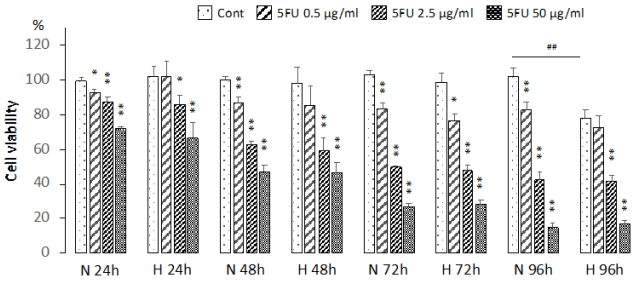

Figure 1. Cell viability (%) normalized to untreated cells under normoxia (100%) (n $\geq$ 3). MDA-MB-231 wild type (WT) cells were exposed to normoxia (N) or hypoxia (H) in the presence or absence of 0.5-50 μg/ml 5-FU for 24 h (N or H 24 h), following which medium was changed to fresh growth medium, and cells were cultured for another 24 h (N or H 48h), 48 h (N or H 72 h), and 72 h (N or H 96 h) under normoxia or hypoxia after which CCK-8 assays were performed. Values from each group were normalized to the average of values obtained from untreated normoxic cells that was set to 100% viability. Cont: HBSS treated cells. Values represent Mean $\pm$ SEM. ** P $\leq$ 0.01, * P $\leq$ 0.05, between Cont and 5-FU treatment at the same time point. $^{\#\#}$ P $\leq$ 0.01, between N and H.

3.2 Effects of 5-FU under normoxia or hypoxia on HIF-1α, HIF-2α and COX-2 in MDA-MB-231 cells

To understand the molecular changes in these cells, we characterized HIF-1α, HIF-2α, and COX-2 protein levels under different conditions. Since cell viability was most reduced with 5-FU treatment at N or H 96 h, we compared protein levels of HIF-1α, HIF-2α and COX-2 at N and H 24 h and N and H 96 h for 5-FU doses of 0.5 μg/ml and 2.5 μg/ml. Representative immunoblots from these studies are presented in Figure 2A. As anticipated, hypoxia increased HIF-1α and HIF-2α expression at H 24 h compared to N 24 h irrespective of the presence of absence of 5-FU. At N or H 24 h, COX-2 levels remained unchanged from control levels (Figure 2A).

At H 96 h, HIF-1α level remained unchanged irrespective of the presence or absence of 5-FU up to doses of 2.5 μg/ml (Figure 2A and 2B). However, at higher 5-FU doses of 25 and 50 μg/ml, HIF-1α decreased at H 96 h (Figure 2A). HIF-2α and COX-2 also decreased at these higher doses of 5-FU (data not shown). Unlike HIF-1α that required much higher doses to decrease, at H 96 h, HIF-2α significantly decreased following treatment with 2.5 μg/ml of 5-FU but not with the lower dose of 0.5 μg/ml (Figure 2A and 2C). At H 96 h, COX-2 levels significantly increased in untreated cells or cells treated with 0.5 μg/ml of 5-FU, suggesting that hypoxia drove up

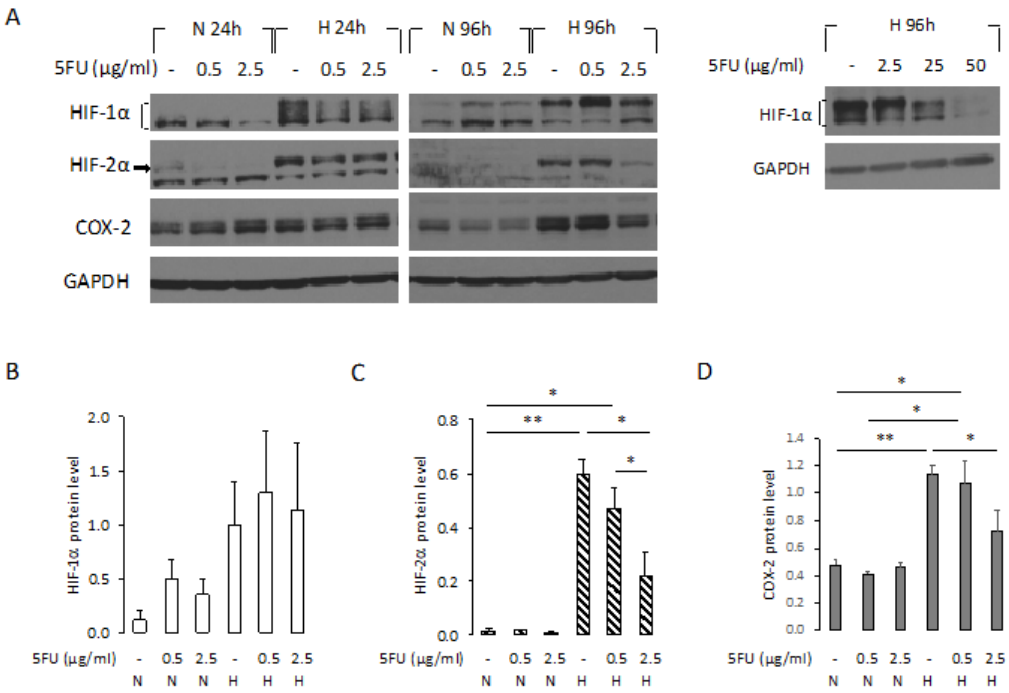

Figure 2. (A) Protein levels of HIF-1α, HIF-2α and COX-2 in MDA-MB-231 WT cells were determined by immunoblot analysis after treatment with HBSS (-) or 0.5 - 2.5 μg/ml 5-FU for 24 h (N 24h, H 24h) or after medium was changed to fresh growth medium, and cells were cultured another 72h (N 96h, H 96h). Also shown (right panel) are HIF-1α protein levels by immunoblot analysis after treatment with HBSS or 2.5 - 50 μg/ml 5-FU at H 96 h. GAPDH protein levels were used for equal loading assessment. Relative density changes in (B) HIF-1α, (C) HIF-2α, and (D) COX-2 protein levels, normalized to GAPDH protein levels, obtained using ImageJ at N 96 h (n = 3). Values represent Mean $\pm$ SEM. ** $P < 0.01$, * $P \leq 0.05$.

COX-2, but started to decrease at the 2.5 μg/ml dose of 5-FU suggesting that like HIF-2α, COX-2 also decreased with 5-FU treatment. Changes of COX-2 at 96 h are summarized in Figure 2D.

3.3 Effects of 5-FU under normoxia or hypoxia on cell viability in HIF-1α and HIF-2α silenced MDA-MB-231 cells

To further validate the cell viability data, we characterized the effects of hypoxia on cell viability following 5-FU treatment using HIF-1α, or HIF-2α, or HIF-1α and HIF-2α silenced MDA-MB-231 cells. As in the studies with MDA-MB-231 wild type cells, MDA-MB-231 sublines were treated with 2.5 μg/ml or 50 μg/ml 5-FU under normoxia or hypoxia and cell viability were determined. As shown in Figure 3A, downregulating HIF-1α, or HIF-2α, or HIF-1α and HIF-2α resulted in comparable decreases of cell viability between normoxic and hypoxic conditions with 5-FU treatment at 24 h, providing further evidence that 5-FU was almost equally effective in reducing cell viability, irrespective of the presence or absence of hypoxia and the silencing of HIF pathways. The same effectiveness was observed at 96 h (Figure 3B). The changes in cell viability were also comparable to the wild type MDA-MB-231 cells (compare to Figure 1). Control cells from HIF-1α silenced cells, and HIF-1α and HIF-2α silenced cells, but not HIF-2α silenced cells showed a decrease of cell viability at 96 h under hypoxia. Since wild type cells also showed a decrease of cell viability at 96 h under hypoxia, these data suggest that silencing of HIF-2α alone seemed to improve cell survival under hypoxic conditions, but not against the treatment with 5-FU.

3.4 Effects of 5-FU under normoxia or hypoxia on HIF-1α, HIF-2α and COX-2 in HIF-1α and HIF-2α silenced MDA-MB-231 cells

Immunoblots of HIF-1α, HIF-2α, and COX-2 expression in wild type, HIF-1α, HIF-2α, and HIF1α and HIF-2α silenced control cells at 96 h normoxia or hypoxia are shown in Figure 4A. As anticipated there was almost non-detectable expression of the corresponding HIF silenced protein under normoxia or hypoxia compared to the wild type cells. The regulation of COX-2 expression by HIF is evident from the increase of COX-2 under hypoxic conditions in the wild type cells and the decrease of COX-2 in the HIF silenced cells under normoxia at 96h (Figure 4A). Immunoblots of HIF-1α, HIF-2α, and COX-2 expression in HIF-1α, HIF-2α, and double silenced cells at N 96 h

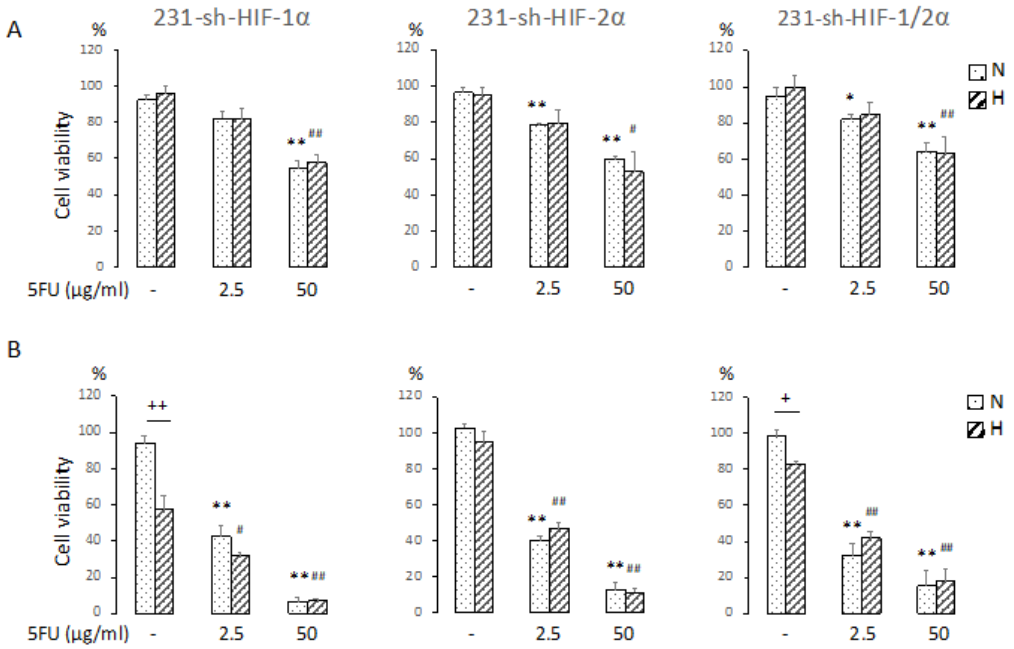

Figure 3. (A) Cell viability (%) at 24h and (B) Cell viability (%) at 96h (n $\geq$ 3). MDA-MB-231 sublines, 231-sh-HIF-1α, 231-sh-HIF-2α, 231-sh-HIF-1/2α cells were exposed to normoxia (N) or hypoxia (H) in the presence or absence of 2.5 or 50 μg/ml 5-FU for 24 h. For N and H 96 h, medium was changed to fresh growth medium after 24h 5-FU treatment, cells were cultured another 72 h under N or H, and cell viability assays were performed. Values from each group were normalized to the average of values obtained from untreated normoxic cells that was set to 100% viability. Values represent Mean $\pm$ SEM. **, $^{\#\#}$P $<$ 0.01, $^{\#}$ P $\leq$ 0.05, between Cont and 5-FU treatment in N or H. $^{+}$ P $\leq$ 0.05, between N and H.

and H 96 h with or without 2.5 μg/ml 5-FU treatment are shown in Figure 4B. As with wild type cells (Figure 2A), under hypoxic conditions, HIF-2α significantly decreased in the HIF-1α silenced cells following treatment with 5-FU (Figure 4B and 4C). HIF-1α tended to decrease in HIF-2α silenced cells following treatment with 5-FU, but the changes were not significant (Figure 4B and 4D).

Like the wild type cells, COX-2 significantly increased with hypoxia in the HIF-1α silenced cells, with the increase eliminated in 5-FU treated cells (Figure 4E). Although the increase of COX-2 under hypoxia was much less pronounced in HIF-2α silenced cells, 5-FU treatment resulted in a decrease. In double silenced cells we did not observe an increase of COX-2 with hypoxia; COX-2 levels did not change with 5-FU treatment in these cells (Figure 4E).

3.5 Effects of 5-FU under normoxia or hypoxia on cell viability, HIF-1α, HIF-2α and COX-2 in SUM-149 cells

We next used a second TNBC cell line, the inflammatory cell line SUM-149, to further characterize the role of HIF and COX-2 in the response of TNBC cells to 5-FU under normoxia and hypoxia. As shown in Figure 5A, these cells were significantly more sensitive to 5-FU compared to MDA-MB-231 cells, with a dose of 0.5 μg/ml resulting in a reduction of cell viability comparable or even more effective than a dose of 2.5 μg/ml in MDA-MB-231 cells. As shown in Figure 5A, hypoxia resulted in a significant decrease of cell viability in control cells and cells treated with 0.1 μg/ml compared to normoxic cells, suggesting that hypoxia played a dominant role in the reduction of cell viability at this very low dose. However, at 0.5 μg/ml there were no differences in viability between normoxic and hypoxic cells.

Immunoblot characterization of changes in HIF-1α, HIF-2α and COX-2 for N and H 24 h and 96 h are shown in Figure 5B and changes of HIF-1α, HIF-2α and COX-2 level for the 96 h time point are summarized in Figure 5C-5E. At H 24 h there was an increase of HIF-1α and HIF-2α but not COX-2. Treatment with 0.5 μg/ml of 5-FU decreased induction of HIF-1α protein level at H 24 h (Figure 5B). The levels of HIF-2α and COX-2 proteins were not modified by 5-FU treatment at H 24 h. At H 96 h, HIF-1α increased slightly following treatment with 0.1 and 0.5 μg/ml of 5-FU although this was not significant, whereas HIF-2α levels remained unchanged with treatment (Figure 5B, 5C and 5D). At N 96 h, HIF-2α levels decreased significantly following treatment with 0.1 and 0.5 μg/ml of 5-FU (Figure 5B and 5D). At H 96 h COX-2 level significantly increased with 5-FU treatment (Figure 5E). The patterns observed with SUM-149 cells

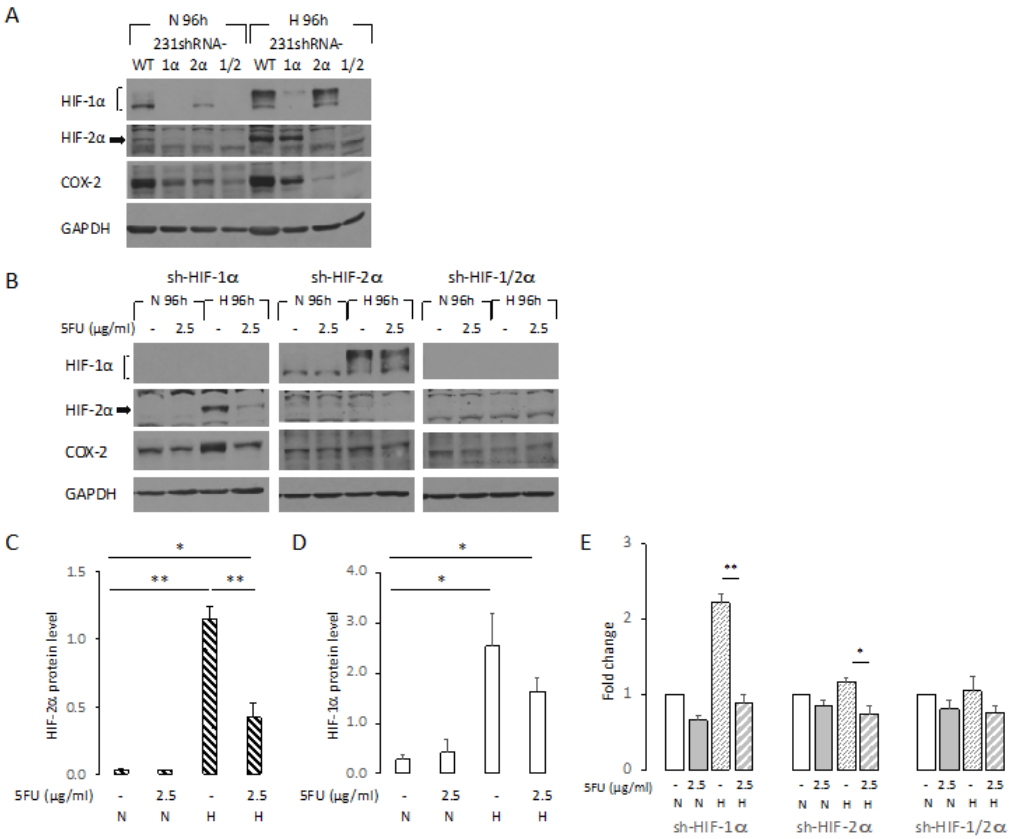

Figure 4. (A) Protein levels of HIF-1α, HIF-2α and COX-2 were determined by immunoblot analysis after culturing HBSS treated (control) MDA-MB-231 WT, 231-sh-HIF-1α (1α), 231-sh-HIF-2α (2α), and 231-sh-HIF-1/2α (1/2) cells for 96 h N or H. (B) Protein levels of HIF-1α, HIF-2α and COX-2 in 231-sh-HIF-1α (sh-HIF-1α), 231-sh-HIF-2α (sh-HIF-2α), and 231-sh-HIF-1/2α (sh-HIF-1/2α) cells were determined by immunoblot analysis after the treatment with HBSS (-) or 2.5 μg/ml 5-FU at N and H 24h, and N and H 96h. Relative density changes in (C) HIF-2α in 231-sh-HIF-1α cells (n = 4), and (D) HIF-1α (n = 3) in 231-sh-HIF-2α cells, normalized to GAPDH protein levels, were obtained using ImageJ at N 96 h. (E) Relative density changes in COX-2 protein level against GAPDH protein level obtained using ImageJ, and normalized to the value obtained in control cells at N 96 h (n $\geq$ 3). Values represent Mean $\pm$ SEM. ** P $<$ 0.01, * P $\leq$ 0.05.

were very different from those observed with MDA-MB-231 cells. A comparison of HIF-1α, HIF-2α and COX-2 displayed in Figure 5F shows the much higher levels of HIF-2α and COX-2 in the SUM-149 cells compared to the MDA-MB-231 cells at H 24 h; baseline levels of COX-2 under normoxic conditions were similar to those observed under hypoxia in SUM-149 cells, but hardly detectable in MDA-MB-231 cells.

4 Discussion and conclusion

We found that 5-FU decreased cell viability to similar levels irrespective of cells being maintained under normoxia or hypoxia (24 h to 96 h), in MDA-MB-231 and SUM-149 triple negative human breast cancer cells. This was further validated using HIF silenced MDA-MB-231 cells that showed comparable changes in cell viability supporting the observation that hypoxia and the HIF pathways did not significantly influence cell viability reduction by 5-FU at the doses used, at least in culture. Previous studies investigating the effects of hypoxia on sensitivity to 5-FU have shown a cell dependent effect[29]. Anemia in patients has been associated with relapse in breast cancer patients receiving cyclophosphamide/methotrexate/5-FU chemotherapy[30]. In general, 5-FU is thought to be less effective under hypoxia[31,32]. At 0.1% hypoxia, MDA-MB-231 cells showed an almost twofold increase of IC_{50} (14 μM *vs* 39 μM)[33]. In our studies with MDA-MB-231 and SUM-149 cells we found no differences in cell viability with 1% hypoxia following 24 h 5-FU treatment at doses of 0.5 μg/ml (3.8 μM) to 50 μg/ml (384 μM) that was additionally confirmed in HIF silenced cells. Our data suggest that the reduction of cell viability following 5-FU treatment was not dependent upon HIF regulated pathways in these two TNBC cell lines.

Consistent with its known role in mediating the adaptive response of cells to hypoxia[12], silencing HIF-1α resulted in a greater reduction of cell viability following 96 h of hypoxia. Interestingly, HIF-2α silencing improved MDA-MB-231 cell survival under hypoxic conditions,

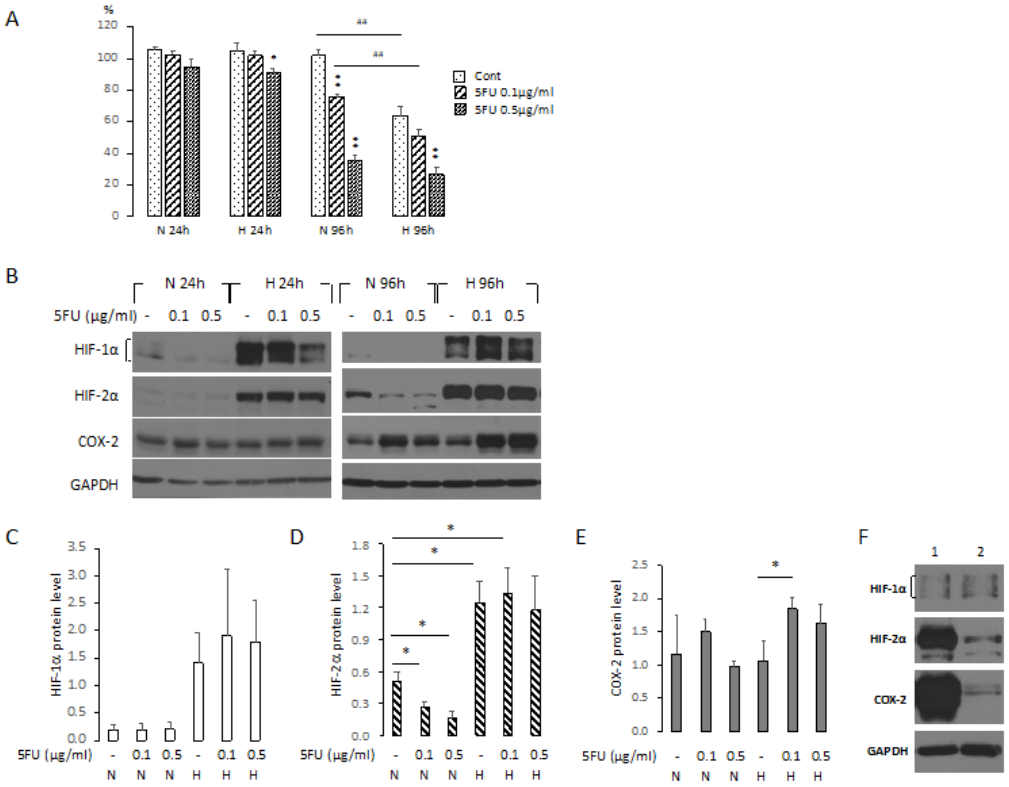

Figure 5. (A) Cell viability (%) compared to untreated cells under normoxia (100%) (n = 4). SUM-149 cells were exposed to normoxia (N) or hypoxia (H) in the presence or absence of 0.1 or 0.5 μg/ml 5-FU for 24h (N or H 24 h), following which medium was changed to fresh growth medium under normoxia, and cells were cultured another 72 h (N or H 96 h) under normoxia or hypoxia after which CCK-8 assays were performed. Values from each group were normalized to the average of values obtained from untreated normoxic cells that was set to 100% viability. Cont: HBSS treated cells. Values represent Mean $\pm$ SEM. ** $P < 0.01$, * $P \leq 0.05$, between Cont and 5-FU treatment at the same time point. $^{\#\#}$ $P < 0.01$, between N and H. (B) Protein levels of HIF-1α, HIF-2α and COX-2 in SUM-149 cells were determined by immunoblot analysis after treatment with HBSS (-) or 0.1 - 0.5 μg/ml 5-FU for 24 h (N 24h, H 24h) or after medium was changed to fresh growth medium, and cells were cultured another 72 h (N 96h, H 96h). Relative density changes in (C) HIF-1α (n = 2), (D) HIF-2α (n = 3), and (E) COX-2 protein levels (n = 3), normalized to GAPDH protein levels, obtained using ImageJ at N 96 h. Values represent Mean $\pm$ SEM. * $P \leq 0.05$. (F) Comparison of protein levels of HIF-1α, HIF-2α and COX-2 in (1) SUM-149 WT and (2) MDA-MB-231 WT at H 24 h.

suggesting that HIF-2α accumulation may predispose these cancer cells to cell death under hypoxia. Because of these opposing effects, silencing both HIF-1α and HIF-2α resulted in lesser reduction of cell viability than silencing HIF-1α alone. Our observations with HIF-2α are consistent with previous observations in neuroblastoma where HIF-2α was observed to be a key component in tumor response to azacytidine combined with retinoic acid, and a small molecule inhibitor of HIF-2α reduced tumor response[34]. Similarly, epigenetic reexpression of HIF-2α was found to suppress soft tissue sarcoma growth[35].

SUM-149 cells that were characterized by high basal COX-2 levels and significantly higher induction of HIF-2α following 24 h of hypoxia, compared to MDA-MB-231 cells, were more sensitive to a reduction of cell viability with 96 h of hypoxia (64%) compared to MDA-MB-231 cells (78%), further supporting the role of HIF-2α in predisposing cancer cells to death. SUM-149 cells were also more sensitive to 5-FU than MDA-MB-231 cells with a dose of 0.5 μg/ml resulting in a reduction of cell viability that was comparable or even more effective than a dose of 2.5 μg/ml in MDA-MB-231 cells.

The patterns of changes in HIF and COX-2 with hypoxia and with 5-FU treatment were different between the two cell lines. At 24 h, treatment with 5-FU did not induce COX-2 in either of the cell lines irrespective of normoxia or hypoxia. In MDA-MB-231 cells at 96 h, COX-2 increased in both control and in 5-FU treated cells under hypoxia but not under normoxia. This increase of COX-2 in control cells was also pronounced in HIF-1α silenced cells but was diminished in HIF-2α silenced cells, and eliminated in combined HIF-1 and HIF-2α silenced cells, suggesting that the increase of COX-2 under hypoxia may be mediated through HIF-2α. HIF-2α has been previously observed to regulate the COX2/mPGES-1/PGE2 pathway in colon cancer[18]. On the other hand, in SUM-149 cells at 96 h, COX-2 increased only with

combined hypoxia and 5-FU treatment, but not with hypoxia alone despite a robust increase of HIF-2α in the control cells. Factors such as cell-cell contact and acidic medium conditions that also exist in tumors may have also contributed to these differences.

In a study of biopsy samples from 14 esophageal carcinomas, COX-2 expression increased following 5-FU chemotherapy[36]. In the same study, cancer cells obtained from a panel of tumors including breast tumors exposed to 170 μM 5-FU for 6 days resulted in an upregulation of COX-2 mRNA. We used 5-FU doses of 0.1 μg/ml (0.76 μM) and 0.5 μg/ml (3.8 μM) for the SUM-149 cells, and 0.5 μg/ml (3.8 μM) and 2.5 μg/ml (19.2 μM) for the MDA-MB-231 cells treated over a 24 h period and observed a pronounced increase of COX-2 after 96 h hypoxia. Our results with the SUM-149 cells highlight how a combination of hypoxia and 5-FU treatment in tumors can increase COX-2 expression and its associated risks of increased invasion and metastasis[21,37].

It is possible that 5-FU mediated reduction of cell viability, and the molecular changes in COX-2 are independent outcomes. Indeed COX-2 downregulation with celecoxib in a high COX-2 expressing brain metastatic variant of MDA-MB-231 cells did little to alter resistance to 5-FU[38]. COX-2 may not play a role in the viability response but in itself may have a multitude of phenotypic effects. Cells that survive hypoxia and 5-FU treatment may exhibit a more aggressive phenotype through the formation of HIF and COX-2. This becomes increasingly relevant in the neoadjuvant setting. In the neoadjuvant setting, celecoxib has been found to increase the chemotherapy response rate by 20%[39]. In a Phase II study, a combination of celecoxib and capecitabine increased time to progression and overall survival in patients with high COX-2 expressing metastatic breast cancers[40]. Our data support further investigation of the effects of chemotherapy and hypoxia on COX-2 expression, and the role of COX-2 in the subsequent phenotypic characteristics of these cells such as invasion and metastasis. Our data also support investigating the role of HIF-2α in modifying cell survival following hypoxia or therapy.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

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All members of Current Cancer Reports (CCR) editorial office would like to extend greetings and sincere thanks to the peer reviewers. Thanks for their invaluable professional service to CCR. Without their help, it would not be possible for the journal to finish this full year of existence with 5 high-quality articles published by 20 different authors.

For next issue, CCR will continue to make efforts to improve the quality and service level of manuscript submission. We look forward to their continuous cooperation with us to improve the quality of manuscripts and contribute to the development of CCR.

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