B7-H3 Silencing Increases Paclitaxel Sensitivity by Abrogating Jak2/Stat3 Phosphorylation

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Abstract
In many types of cancer, the expression of the immunoregulatory protein B7-H3 has been associated with poor prognosis. Previously, we observed a link between B7-H3 and tumor cell migration and invasion, and in present study, we have investigated the role of B7-H3 in chemoresistance in breast cancer. We observed that silencing of B7-H3, via stable short hairpin RNA or transient short interfering RNA transfection, increased the sensitivity of multiple human breast cancer cell lines to paclitaxel as a result of enhanced drug-induced apoptosis. Overexpression of B7-H3 made the cancer cells more resistant to the drug. Next, we investigated the mechanisms behind B7-H3–mediated paclitaxel resistance and found that the level of Stat3 Tyr705 phosphorylation was decreased in B7-H3 knockdown cells along with the expression of its direct downstream targets Mcl-1 and survivin. The phosphorylation of Janus kinase 2 (Jak2), an upstream molecule of Stat3, was also significantly decreased. In contrast, reexpression of B7-H3 in B7-H3 knockdown and low B7-H3 expressing cells increased the phosphorylation of Jak2 and Stat3. In vivo animal experiments showed that B7-H3 knockdown tumors displayed a slower growth rate than the control xenografts. Importantly, paclitaxel treatment showed a strong antitumor activity in the mice with B7-H3 knockdown tumors, but only a marginal effect in the control group. Taken together, our data show that in breast cancer cells, B7-H3 induces paclitaxel resistance, at least partially by interfering with Jak2/Stat3 pathway. These results provide novel insight into the function of B7-H3 and encourage the design and testing of approaches targeting this protein and its partners. Mol Cancer Ther; 10(6); 960–71. ©2011 AACR.

Introduction
Breast cancer is the most common type of cancer and the most common cause of cancer-related death in women. Once breast cancer becomes metastatic, it is difficult to cure because it is often resistant to commonly used chemotherapeutics, such as taxanes, which are among the most effective chemotherapeutic agents against this tumor type. Hence, if the sensitivity to taxane-based chemotherapy could be increased, it would represent an important step in improving the clinical management of this disease. B7-H3, a transmembrane protein with immunoglobulin-like structure (1), is known to have immunoregulatory properties with both inhibitory and stimulatory effects on the activation of T cells (2–5). Although the B7-H3 protein is found in many human tissues, it is overexpressed in several types of human cancers. Notably, immunohistochemical reports have shown a correlation between high expression of B7-H3 and poor outcome in patients with breast cancer (6), clear cell renal cell carcinoma (7), urothelial cell carcinoma (8), prostate cancer (9), non–small cell lung cancer (10), and pancreatic cancer (11). Whereas these studies have focused on the immunoregulatory function of B7-H3, our previous in vitro studies in a nonimmunological system showed that the knockdown of B7-H3 expression resulted in a robust inhibition of tumor cell migration and invasion, indicating a role of B7-H3 in these processes (12). Since metastasis is closely related to chemoresistance, we focused the present study on the role of B7-H3 in the sensitivity of metastatic breast cancer cells to paclitaxel and the possible underlying mechanisms. We found that...
silencing of B7-H3 sensitized the MDA-MB-231, MDA-MB-435, and MDA-MB-436 tumor cell lines to paclitaxel, whereas B7-H3 overexpression led to increased resistance to this drug. Moreover, we discovered that knockdown of B7-H3 abrogated the phosphorylation of Stat3 on Tyr705 through inactivation of Janus kinase 2 (Jak2), and led to downregulation of the direct target genes of Stat3: Mcl-1 and to a lesser extent survivin. In contrast, overexpression of B7-H3 increased the phosphorylation of Jak2 and Stat3, indicating that Jak2/Stat3 pathway contributes to B7-H3-mediated paclitaxel resistance. These novel findings have important implications for the therapeutic strategy for treating paclitaxel-refractory cancers.

Materials and Methods

Reagents

Antihuman B7-H3 antibody and anti-survivin were purchased from R&D Systems, Inc. Antibodies against Stat3, phospho (p)-Stat3(Tyr705) p-Stat5(pSer727), Jak2, p-Jak1(Tyr1022/1023), p-Jak2(Tyr1007/1008), p-Src(Tyr416), Mcl-1, and PARP were from Cell Signaling Technology. Antihuman B7-H3 antibody and anti-survivin were purchased from R & D Systems, Inc. Antigodies against B7-H3 (shB7-H3) and control plasmid pRS non-targeted TR30003 (TR33) were purchased from Origene Technologies, Inc. The sequences of shB7-H3 were as follows: shRNA-1, 5'-TTACAGCTCGACACGGCTCAA- CCTATCGT-3'; and shRNA-2, 5'-TCGTTGCTGGAGAAGATCAAAACGACG-3'. The nontargeted control sequence was: 5'-GCACCATCAAGGCTAATCTCAA- TACT-3'. Either B7-H3 shRNA construct or control vector were transfected into MDA-MB-231 and MDA-MB-435 cells by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocols, followed by selection with 1 μg/mL puromycin for 2 weeks. Antibiotic-resistant clones were isolated in medium with 0.5 μg/mL puromycin. Reverse transcription PCR and immunoblotting were carried out to confirm the knockdown of mRNA and protein of B7-H3 in those transfectants. To generate B7-H3 overexpression stable cell lines, cDNAs for full-length human B7-H3 (NM_001024736) were amplified by PCR by using the following primer sequences: forward (5’-TCACCTGAGCCCTGAGTCAGTCCGAGTCGG-GC-3’); reverse (5’-ACTGATTCCGTGTGGGTGTCGTCGTTT-CAT-3’); and then the full-length cDNA was cloned into xhol and EcoRI linearized plasmid vector pIRE2-EGFP (Clontech). The control vector pIRE2-EGFP and human B7-H3 expression vector pIRE2-B7-H3 were transfected in MDA-MB-231 cells by using Lipofectamine 2000 (Invitrogen). Stable clones were selected in medium containing 1.8 mg/mL Geneticin (Invitrogen). The expression of B7-H3 was determined by Western blot analysis.

Short interfering RNA experiments

Short interfering RNA (siRNA) targeting human B7-H3 and the control scrambled siRNA were purchased from Sigma-Aldrich. Transfection was done by Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cells treated with 20 nmol/L paclitaxel for another 48 hours were used for Western blot analysis and apoptosis-specific ELISA detection.

In vitro growth inhibition

Cells (1 x 10^4 cells) were initially plated in triplicate in 96-well culture plates. Twenty-four hours later, the medium was replaced with fresh medium with or without paclitaxel and incubated for indicated time. Cell viability was determined by CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega).

Annexin V-FITC staining

Cells (3 x 10^5 cells) were grown in triplicate in 60 mm dishes with exposure of 20 nmol/L paclitaxel for 0, 48, and 72 hours, respectively. And then cells were harvested and processed as described in the Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit I manual (BD Transduction Laboratories, BD Biosciences) and analyzed by flow cytometry (BD LSR II).

TUNEL assay

Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling (TUNEL) assay was performed by using recombinant terminal transferase (TdT) and biotin-16-dUTP (Roche Diagnostics GmbH). After treatment with 0, 10 and 20 nmol/L paclitaxel for 72 hours, cells were processed by following manufacturer’s protocol and analyzed by flow cytometry (BD Biosciences). Each experiment was repeated 3 times.

Quantification of apoptosis by ELISA kit

An apoptosis ELISA kit (Roche Diagnostics Co.) was used to quantitatively measure cytoplasmic histone-associated DNA fragments. After treatment with 0 and

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20 nmol/L paclitaxel for 72 hours, cells were analyzed by following manufacturer's protocol. Each experiment was repeated 3 times.

**Western blot analysis**

Western blotting was done on whole-cell extracts prepared by lysing cells in 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% Triton-X 100, 10 mmol/L EDTA, 5 mmol/L NaF, 5 mmol/L sodium pyrophosphate, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na3VO4, and protease inhibitor cocktail (Sigma-Aldrich) for 20 minutes on ice. The proteins were separated by SDS-PAGE and then electrotransferred onto nitrocellulose membrane (Bio-rad). Membranes were probed with indicated antibodies by following the manufacturer's protocol, and immunoreactive bands were visualized by using ECL Western Blotting Substrate (Pierce Biotechnology, Inc.).

**In vivo studies**

Four groups of 5 to 6 women Balb/c nude mice, bred at the nude rodent facility at the Norwegian Radium Hospital, were used. The animals were maintained under specific pathogen-free conditions, and food and water were supplied **ad libitum**. Animal experiments were done according to protocols approved by the animal care and use committee and were in compliance with the guidelines on animal welfare of the Norwegian National Committee for Animal Experiments. When the animals were 6 to 8 weeks of age, 5 x 10⁶ cells (MDA-MB-435 shB7-H3 or MDA-MB-435 TR33 cells) in 0.2 mL PBS were injected s.c. in both flanks of nude mice. For therapy experiments, a stock solution of paclitaxel in ethanol (6 mg/mL) was dissolved in PBS and a single dose of 10 mg/kg of the drug was injected i.v. into the tail vein when the mean tumor diameter was 5 to 6 mm (day 0). The control mice received only the solvent. Tumor volumes were measured 1 to 2 times per week. Tumor diameters were measured 1 to 2 times per week. Tumor volume was calculated by the formula 0.5 x length x width² and growth curves constructed, and the data presented as mean of 2 independent experiments ± SEM.

**Immunohistochemistry**

The tumor xenografts were removed on day 7 after treatment and used for immunohistochemical studies. A total of 4 μm sections were stained by the Dako EnVision + System (K4007 and K4011; Dako A/S). The sections were stained toward B7-H3 by using a goat antihuman polyclonal antibody (R&D System). For the clinical breast carcinoma samples, 5 micron sections were cut and incubated with the antibody against B7-H3 (R&D System) using the Dako EnVision system (K4007; Dako). Positive and negative controls were included. The slides were observed under an Olympus BX51 microscope at 10x and 40x magnification.

**Statistical analysis**

Statistical evaluation for experimental data analysis was determined with an unpaired student’s *t*-test. A statistical difference of *P* < 0.05 was considered significant.

**Results**

**B7-H3 expression in breast cancer cell lines and clinical specimen**

The expression of B7-H3 in human breast cancer tissues from primary tumors and lymph node metastasis has recently been shown (6). Here, we tested a panel of breast cell lines, including immortalized normal HMECs, transformed luminal cells BT474, MCF7, and SKBR3, basal A cells BT20, and basal B cells MDA-MB-231, MDA-MB-435, and MDA-MB-436 (Fig. 1A). As assessed by Western blotting, the B7-H3 protein was present in all the tested breast cancer cell lines except SKBR3 and compared with the nontransformed breast epithelial HMEC cells, the protein levels were elevated. We also included samples from breast cancer patients, and as shown in Fig. 1B (panels 1–4), the immunohistochemical evaluation revealed that all tumor cells in ductal and lobular carcinomas and in a lymph node metastasis showed strong membrane and cytoplasmatic staining. Tumor near stromal cells were also stained, but generally much weaker than the tumor cells, both in primary tumors and metastases.

**Silencing of B7-H3 enhances paclitaxel-induced cytotoxicity in cancer cells**

To study the possible role of B7-H3 in affecting the sensitivity of breast cancer cells to paclitaxel, we used shRNA to create 2 stable B7-H3 knockdown cell variants derived from the MDA-MB-231 and MDA-MB-435 cell lines. As compared with the parental and the nontarget shRNA–transfected control cell variants MDA-MB-231-TR33 (231-TR33) and MDA-MB-435-TR33 (435-TR33), their corresponding B7-H3 knockdown cell variants MDA-MB-231-shB7-H3 (231-shB7-H3) and MDA-MB-435-shB7-H3 (435-shB7-H3) expressed very low levels of B7-H3, showing an effective knockdown of the B7-H3 protein (Fig. 2A).

On treatment with various concentrations of paclitaxel for 72 hours, a dose-dependent inhibition of cell growth was observed in both MDA-MB-231 and MDA-MB-435 cells (Fig. 2B), and the B7-H3 knockdown cells were about 2-fold more sensitive to paclitaxel than parental and control cells. In MDA-MB-231 cell variants, the inhibition of cell growth was 52% after exposure to 20 nmol/L paclitaxel in the B7-H3 knockdown cells compared with 18% and 25% in parental and control cells. In the MDA-MB-435 variants, 15 nmol/L paclitaxel induced inhibition of cell growth by 55%, 31%, and 38% in the B7-H3 knockdown, parental, and control cells, respectively. Statistical analysis shows that the differences between B7-H3 knockdown and control cells were significant in both the MDA-MB-231 and MDA-MB-435 cell lines. These results indicate that B7-H3 plays a role in tumor cell resistance to paclitaxel. There were no clear differences...
between the parental and vector control cells with respect to paclitaxel responsiveness; hence, we did not include the parental cell lines in the further biochemical and molecular studies.

**B7-H3 plays a critical role in cancer cell resistance to paclitaxel-induced apoptosis**

Paclitaxel is known to exert its cytotoxic effect through induction of apoptosis (14), and hence, we investigated whether the increased paclitaxel cytotoxicity observed in B7-H3 knockdown cells could be related to effects on apoptosis. The extent of apoptosis was investigated by measuring the amount of Annexin V stained cells, a marker for early stage apoptosis, in MDA-MB-231 and MDA-MB-435 cells, and in MDA-MB-231 cells also for the amount of TUNEL-positive cells, which reflects late stage apoptosis. In the Annexin V assay, the response to 20 nmol/L paclitaxel was time-dependent with an increase in the amount of Annexin V-positive cells detected at 48 and 72 hours. The MDA-MB-231 B7-H3 knockdown cells were more sensitive to paclitaxel-induced apoptosis than the control cells, with approximately 2-fold difference in the percentage of apoptotic cells at both 48 and 72 hours (Fig. 3A). Similar results were observed for the MDA-MB-435 cell variants (Supplementary Fig. S1A). As shown in Fig. 3B, the late stage apoptotic response was dose-dependent in MDA-MB-231 cells, showing increased DNA fragmentation with drug concentration, and the 231-shB7-H3 cells were significantly more susceptible to paclitaxel-induced apoptosis than the vector control cells, as the amount of TUNEL positive cells was 11% versus 2% at 48 hours and 18% versus 6% at 72 hours. These results were confirmed by investigating the cleavage of PARP, another marker of apoptosis. Both MDA-MB-231 and MDA-MB-435 B7-H3 knockdown cells had increased PARP cleavage compared with the control cells on treatment with 20 nmol/L paclitaxel for 72 hours (Fig. 3C and Supplementary Fig. S1B). To validate the specificity of the effects observed in B7-H3 knockdown cells, we confirmed the results with another stable cell clone 231-sh1-b9 (Supplementary Fig. S1C and D), which was constructed by a different B7-H3-targeting sequence shRNA-1 (see Materials and Methods). In addition, we transiently transfected B7-H3 siRNA, targeting a different

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**Figure 1.** B7-H3 expression in breast cancer cell lines and clinical specimen. A, protein expression of B7-H3 was analyzed by Western blotting in a panel of human breast cell lines as indicated. β-Actin levels served as loading control. B, immunostaining of B7-H3 in primary breast carcinomas. 1, ductal carcinoma with strong membranous and cytoplasmic staining. 2, lobular carcinoma. Single cells in a collagen rich stroma showed diffuse cytoplasmic staining. Note the normal glandular structures in the upper left corner being moderately stained. 3, ductal breast carcinoma and lymphoid infiltrate. Note the intense cytoplasmic staining of the epithelial cells. 4, lymph node metastasis from ductal carcinoma. All lymphoid tissue was replaced with carcinoma cells with diffuse cytoplasmic staining. (magnification: panel 1, ×400; panels 2–4, ×100).
sequence than the shRNAs, into another breast cancer cell line MDA-MB-231 (left) and MDA-MB-435 cells (right), expressing either nontarget vector control TR33 or shB7-H3 and their corresponding parental cells. β-Actin levels served as loading control. B, cell viability of 231, 231-TR33, 231-shB7-H3 (left), 435, 435-TR33, and 435-shB7-H3 cell variants (right) following treatment with paclitaxel at various concentrations for 72 hours was assessed by MTS assay. Data are presented as the percentage of cell growth inhibition measured in paclitaxel-treated cells compared with untreated cells. The P value shows the difference between paclitaxel-treated B7-H3 knockdown cells and parental cells. Columns, mean of 3 independent experiments done in triplicates; bars, SE; **, P < 0.01; ***, P < 0.001.

B7-H3 regulates the activation of Jak2/Stat3 pathway and its downstream antiapoptotic molecules

Since we observed chemosensitization accompanied by an increase in apoptosis in paclitaxel-treated B7-H3 knockdown cells, we studied whether the effects of B7-H3 could be related to molecules and signaling pathways known to be involved in the apoptotic response. Stat3, a transcription factor often constitutively activated in breast cancer cells, has previously been reported to lead to drug resistance (15) through the upregulation of antiapoptotic factors such as Mcl-1, Bcl-xL, Bcl-2, and survivin (16). As seen in Fig. 4A, the silencing of B7-H3
induced a dramatic reduction in the phosphorylation level of Stat3, an indicator of Stat3 activation, both in untreated and paclitaxel-treated cells. Comparable results were obtained in transient shRNA B7-H3–transfected MDA-MB-436 cells (Supplementary Fig. S2A), targeting a different sequence than the shRNAs. Furthermore, Mcl-1 was dramatically repressed in B7-H3 silenced cells with reduced Stat3 phosphorylation (Fig. 4B). Survivin, another direct target of Stat3, was also, to a lesser extent, reduced in B7-H3 silenced cells (Supplementary Fig. S3). To elucidate whether the effects of B7-H3 on Stat3 was direct or indirect, we examined whether molecular kinases upstream of Stat3, such as Jak1, Jak2, and Src, were affected by the silencing of B7-H3. We observed a significant decline in the phosphorylation of Jak2 in both 231- and 435-shB7-H3 cells (Fig. 4C) and similar results were also observed in the B7-H3 shRNA–transfected 231-sh1-h9 and 231-sh1-g10 cell variants (Supplementary Fig. S2B). In addition, the phosphorylation of Src seemed to be reduced in B7-H3 knockdown cells (Supplementary Fig. S4), indicating the possibility that Src may be partially involved in the B7-H3–mediated effects, whereas there was no obvious change in Jak1 phosphorylation in these cells. When we treated the cells with the Jak2-selective inhibitor AG490 for 24 hours, at concentrations similar to what has been used in previous studies (17), the phosphorylation level of Jak2 was almost abolished followed by a dramatic inhibition of tyrosine phosphorylation of Stat3 in both B7-H3 knockdown cells and control cells (Fig. 5A). This indicates that the effect of B7-H3 on Stat3 occurs through Jak2. To investigate the involvement of Jak2/Stat3 pathway in the observed antiapoptotic function of B7-H3, we tested the effect of AG490 in combination with paclitaxel on B7-H3 expressing cells, 231-TR33 and 435-TR33 (Fig. 5B and C). Interestingly, we observed the combination showed a synergistic effect in enhancement of paclitaxel-induced apoptosis, compared with each agent alone. In summary, these results indicate that the silencing of B7-H3 reduces the phosphorylation of Jak2, which leads to reduced phosphorylation of Stat3, which, in turn, leads to decreased expression of the antiapoptotic proteins, Mcl-1 and survivin.

To validate this conclusion, we examined overexpression of B7-H3 is sufficient to cause Jak2/Stat3 activation. We transiently transfected B7-H3 into B7-H3 knockdown cells 231-shB7-H3 (Fig. 5D, left) and B7-H3 low expressing HMEC cells (Fig. 5D, right). Noteworthy, the results show that the phosphorylation level of both Jak2 and Stat3 increased with the B7-H3 expression, further confirming an important role of B7-H3 in regulating the Jak2/Stat3 signaling pathway.

Silencing of B7-H3 enhances cancer cell sensitivity to paclitaxel in a xenograft mouse model

The in vitro experiments with the MDA-MB-231 and MDA-MB-435 cells showed that the cytotoxic effect of paclitaxel was enhanced in cells with silenced B7-H3 and reduced in cells with overexpression of B7-H3. Hence, we examined whether this could be observed also in vivo. MDA-MB-435 B7-H3 knockdown and control cells were injected s.c. in nude mice, and the animals were treated with paclitaxel when the tumors had reached a mean diameter of 5 to 6 mm. As shown in Fig. 6A, the growth rate was reduced by the knockdown in B7-H3 alone, and whereas paclitaxel had a marginal effect on the growth of MDA-MB-435 TR33 tumors, it showed a strong antitumor effect in the mice carrying MDA-MB-435 shB7-H3 xenografts. Thus, the difference in tumor volume assessed at each time point became statistically significant from day 22 (all the P values at each time point after day 22 were lower than 0.03). The knockdown of the shB7-H3 xenografts was confirmed by immunohistochemical staining 7-day posttreatment, and whereas the level of B7-H3 expression retained low in the shB7-H3 tumors, the TR33 control tumors showed strong staining (Fig. 6B, panels 1–2). Clearly, these in vivo results strongly support the effects observed in vitro that B7-H3 plays a critical role in paclitaxel responsiveness of breast cancer cells.

Discussion

In this study, we examined the role of B7-H3 in paclitaxel resistance in several metastatic breast cancer cell lines. B7-H3 shRNA induced knockdown of the B7-H3 protein in these cells resulted in increased sensitivity to the drug, whereas B7-H3 overexpressing cells were less sensitive to paclitaxel-induced apoptosis. Our findings show that targeting B7-H3 could counteract cellular resistance to paclitaxel. Furthermore, in attempts to elucidate the mechanisms underlying the observed effects, we obtained the evidence for B7-H3 regulating key genes in the Jak2/Stat3 pathway.

Stat3 is a cytoplasmic transcription factor that regulates cellular differentiation, proliferation, and survival (18–20). It is activated through phosphorylation by various tyrosine kinases, such as Jak and Src family kinases, and high activity has been shown to predict intrinsic chemotherapy drug resistance due to the upregulation of the antiapoptotic factors Mcl-1, Bcl-xL, Bcl-2, and survivin (15, 21, 22). Interestingly, here, we found that downregulation of B7-H3 reduced phosphorylation of both Stat3 and its upstream activator Jak2, whereas overexpression of B7-H3 activated Jak2/Stat3 signaling. This may explain why the B7-H3 knockdown cells became more prone to paclitaxel-induced apoptosis, and the findings are in accordance with reports showing induction of apoptosis following a blockade of Stat3 signaling in multiple malignancies (23–31). On the basis of these findings, we investigated the effects of B7-H3 knockdown on Stat3-regulated genes involved in apoptosis. Mcl-1 is a direct target gene transcriptionally activated by Stat3 (32, 33) and its downregulation has been shown to promote apoptosis in numerous human cancer cells (34–36). In our study, reduced expression of Mcl-1 occurred in parallel
A

![Graph showing apoptotic cell percentage over time](image)

**B**

![Graph showing TUNEL-positive cell percentage](image)

**C**

![Western blot images](image)

**D**

![Graph showing absorbance](image)
with increased apoptosis in B7-H3 knockdown cells treated with paclitaxel. The expression of survivin, a member of the IAP family of antiapoptotic proteins, was also reduced on silencing of B7-H3. In a previous study, it was found that overexpression of survivin is associated with resistance to paclitaxel-induced apoptosis in breast cancer cells (37). Blockage of phosphorylation of Jak2 by its specific inhibitor AG490 resulted in more dramatic reduction on Stat3 phosphorylation in B7-H3 expressing cells than B7-H3 knockdown cells, indicating that Jak2

Figure 3. B7-H3 silencing sensitizes breast cancer cells to paclitaxel-induced apoptosis. A, the percentage of Annexin V-FITC stained cells was increased in B7-H3 knockdown population. The 231-TR33 and 231-shB7-H3 cells were treated with 20 nmol/L paclitaxel for 0, 48, and 72 hours, respectively, and apoptosis was examined by Annexin V-FITC staining and flow cytometry. Left, the percentage of apoptotic cells. Right, flow cytometry diagram at 48 hours. B, the percentage of TUNEL-positive cells was increased in B7-H3 knockdown population. Cells were treated with 0, 10, and 20 nmol/L paclitaxel for 72 hours, and apoptosis was examined by TUNEL assay and flow cytometry. C, PARP cleavage was increased in B7-H3 knockdown cells as detected by Western blotting following treatment with 20 nmol/L paclitaxel for 72 hours. β-Actin was used as a loading control. D, B7-H3 overexpressing MDA-MB-231 cell variants were more resistance to paclitaxel-induced apoptosis. Left, B7-H3 overexpressing 231-B7-H3 cells expressed higher level of B7-H3 than the 231-EGFP control cells as detected by Western blot analysis. Middle, B7-H3 overexpressing cells showed decreased level of C-PARP compared with the control cells on treatment with 20 nmol/L paclitaxel for 72 hours. Right, apoptosis-specific ELISA detection revealed that the level of cytoplasmic histone–associated DNA fragments in paclitaxel-treated 231-B7H3 cells was significantly lower than in control 231-EGFP cells. Bars, SE; *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Figure 4. B7-H3 regulated the phosphorylation of Stat3 and the expression of Mcl-1 at least partially through Jak2 in breast cancer cells. Cells were treated with 20 nmol/L paclitaxel (72 hours) or left untreated. Whole cell lysates were probed with indicated antibodies with β-actin as a loading control. A, B7-H3 silencing suppressed Stat3 phosphorylation in 231 (left) or 435 (right) cells. B, B7-H3 silencing downregulated Mcl-1 expression in 231 (left) or 435 (right) cells. C, the levels of p-Jak2 were decreased in B7-H3 knockdown cells.

with resistance to paclitaxel-induced apoptosis in breast cancer cells (37). Blockage of phosphorylation of Jak2 by its specific inhibitor AG490 resulted in more dramatic reduction on Stat3 phosphorylation in B7-H3 expressing cells than B7-H3 knockdown cells, indicating that Jak2
plays a major role in mediating the effect of B7-H3 on Stat3.

Importantly, the results on paclitaxel sensitivity in vitro were confirmed in our animal model. The growth rate of established B7-H3 knockdown xenografts was slower than that of vector control tumors, but still these tumors were significantly inhibited on paclitaxel treatment, whereas the growth of vector control tumors was
only marginally affected. This is an interesting observation as it is well established that fast-growing tumors generally respond better to chemotherapy than more slowly growing counterparts (38, 39). The data indicate that silencing of B7-H3 induces in parallel reduced proliferation and enhances the apoptosis induced by paclitaxel. Immunohistochemical analysis of the xenograft tissue confirmed that the tumors originating from shB7-H3 cells retained low expression levels of the protein, whereas the transfection control tumors showed strong B7-H3 staining.

The human MDA-MB-435 cell line used in our study was originally described as of breast cancer origin, whereas gene expression array studies indicated the cells to originate from malignant melanoma (40). However, subsequent evidence suggests that MDA-MB-435 is, in fact, a breast cancer cell line (41, 42). Importantly, in our study, the results obtained with MDA-MB-435 cells were closely similar to those with MDA-MB-231 and MDA-MB-436 breast cancer cells.

In summary, our study investigating the role of B7-H3 in drug resistance shows that the protein confers resistance to paclitaxel both in vitro and in vivo by reducing the sensitivity of breast cancer cells to apoptosis, mediated via the Jak2/Stat3 pathway. Furthermore, in contrast to previous reports focusing on the immunoregulatory effects of B7-H3, our data show that it plays an important role in determining the resistance to paclitaxel via
nonimmunomechanisms. These findings provide new insight into the role of B7-H3 in cancer and may have important implications in the development of targeted therapeutics for overcoming paclitaxel resistance. The clinical relevance of our results in breast cancer is illustrated by the recent findings of Ariaghi and colleagues on high levels of B7-H3 expression in primary tumors and metastases (6). In separate experiments, we have showed that knocking down B7-H3 also increased breast cancer cell sensitivity to cisplatin (data not shown); thus, the effects may be extrapolated to other chemotherapeutic compounds a possibility being investigated in ongoing studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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