Gynecologic Oncology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Gynecologic Oncology

journal homepage: www.elsevier.com/locate/ygyno

BAG3 upregulates Mcl-1 through downregulation of miR-29b to induce anticancer drug resistance in ovarian cancer

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HIGHLIGHTS

- BAG3 knockdown downregulates expression of Mcl-1 through upregulation of miR-29b.
- BAG3 knockdown increases the chemosensitivity of ovarian cancer cells, especially clear cell carcinoma, to paclitaxel.
- BAG3 may be a useful therapeutic target for the treatment of ovarian cancer.

ARTICLE INFO

Article history:
Received 3 February 2014
Accepted 24 June 2014
Available online xxxx

Keywords:
BAG3
Mcl-1
miR-29b
Ovarian cancer
Chemoresistance

ABSTRACT

Objective. Ovarian cancer is the leading cause of death from gynecologic cancer, reflecting its often late diagnosis and its chemoresistance. We identified a set of microRNAs whose expression is altered upon BAG3 knockdown. Our primary objective was to examine the relationships between BAG3, miR-29b and Mcl-1, an antiapoptotic Bcl-2 family protein, in ovarian cancer cells.

Methods. Ovarian cancer cells were cultured and their responsiveness to paclitaxel was tested. Microarray analysis was performed to identify microRNAs differentially expressed in ES2 BAG3 knockdown ovarian cancer cells and their control cells. Primary ovarian cancer tissues were obtained from 56 patients operated on for ovarian cancer. The patients' clinical and pathological data were obtained from their medical records.

Results. BAG3 knockdown increased the chemosensitivity to paclitaxel of ES2 ovarian clear cell carcinoma cells to a greater degree than AMOC2 serous adenocarcinoma cells. qRT-PCR analysis showed that miR-29b expression was significantly upregulated in primary cancer tissue expressing low levels of BAG3, as compared to tissue expressing high levels. Moreover, levels of miR-29b correlated significantly with progression-free survival. Upregulation of miR-29b also reduced levels of Mcl-1 and sensitized ES2 cells to low-dose paclitaxel.

Conclusions. BAG3 knockdown appears to downregulate expression of Mcl-1 through upregulation of miR-29b, thereby increasing the chemosensitivity of ovarian clear cell carcinoma cells. This suggests that BAG3 is a key determinant of the responsiveness of ovarian cancer cells, especially clear cell carcinoma, to paclitaxel and that BAG3 may be a useful therapeutic target for the treatment of ovarian cancer.

Introduction

Ovarian cancer is the leading cause of gynecologic cancer death, reflecting its often late diagnosis and its chemoresistance. Standard treatment includes debulking surgery followed by chemotherapy. Despite good initial response rates, frequent recurrence and acquired chemoresistance typically result in therapeutic failure; consequently, the overall 5-year survival rate for ovarian cancer is only about 30% [1]. Although the process by which chemoresistance develops appears to be multifactorial, progression toward drug resistance can be achieved through the inactivation of apoptosis, generally considered a hallmark of cancer. This suggests that a fuller knowledge of the mechanisms underlying drug resistance could potentially enable identification of ways to reactivate the apoptotic response [2,3]. Paclitaxel is one of the taxane classes of anti-neoplastic, microtubule-damaging agents and exhibits activity against a wide range of human malignancies. It is one of the key drugs used to treat patients with ovarian cancer [4,5].

Bcl2-associated athanogene (BAG) proteins are a family of co-chaperones that interact with the ATPase domain of heat shock protein (Hsp) 70 through a specific structural domain known as the BAG domain (110–124 amino acids) [6,7]. BAG3 also contains a WW and a proline-rich repeat (PXXP) domain, which mediate interactions with

http://dx.doi.org/10.1016/j.ygyno.2014.06.024
0090-8258/

other proteins. BAG3 participates in a wide variety of cellular processes including those contributing to cell survival, cellular stress response, proliferation, migration and apoptosis [8].

The Bcl-2 family proteins are key regulators of apoptosis that include both proapoptotic and antiapoptotic members, and alterations in their expression and function are associated with cancer development [9,10]. Oligomerization of the multidomain apoptotic family members, Bax and Bak, results in mitochondrial outer membrane permeabilization, which leads to release of proapoptogenic proteins, such as cytochrome *c*, and subsequent activation of caspases and induction of cell death. The antiapoptotic family members, Bcl-2, Bcl-xL, Mcl-1 and Bcl-w, bind to proapoptotic members, preventing their oligomerization and thus inhibiting programmed cell death [11]. Notably, overexpression of Mcl-1 has been seen in a wide range of cancer tissues as well as in various cancer cell lines [12–15], and increased expression of Mcl-1 is associated with a poor prognosis in breast cancer [16]. Mcl-1 also appears to be an important factor involved in resistance to cancer therapies, and its downregulation has proved effective for inducing apoptosis [14]. RNA silencing of BAG3 leads to a marked reduction in Mcl-1 levels and increased apoptosis in several cell lines [17].

MicroRNAs (miRNAs) are phylogenetically conserved short RNAs that suppress protein expression through base-pairing with the 3′-untranslated region (3′-UTR) of target mRNAs. Growing evidence suggests that miRNAs play important roles in diverse biological processes, and their dysfunction is involved in the development of cancer. Some miRNAs are involved in growth control or apoptosis, providing a mechanistic underpinning for the relation between miRNA and cancer [18–20]. Moreover, miRNAs (e.g., let-7 family members, miR-15a, -16, -29, etc.) involved in specific networks, such as apoptotic, proliferation or receptor-driven pathways, could potentially influence the response to targeted therapies or to chemotherapy [18,19]. Recent studies have shown that miRNA-29b regulates expression of Mcl-1 in cancer cells, thereby affecting apoptosis and susceptibility to chemotherapy [20–22].

In the present study, we found that knocking down BAG3 expression upregulates expression of miR-29b, which prompted us to examine the activity of miR-29b in ovarian cancer cells and its effect on susceptibility to anticancer therapy.

Materials and methods

Cell culture and treatment

The ES2 (clear cell carcinoma) and AMOC2 (serous adenocarcinoma) ovarian cancer cell lines were cultured in DMEM (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and 1% penicillin/streptomycin (Gibco BRL). Cells were maintained in a CO_2 incubator (5% CO_2) at 37 °C.

Gene silencing using a short hairpin RNA vector

A gene silencing vector (pLTRH1) specific for BAG3 was used for transfection. This vector contains a RNA polymerase promoter producing shRNA. Oligonucleotides [5′-GATCCCGTACCTGATGATCGAAGAGTTTCAAGAGAACTCTTCGATCATCAGGTATTTTTGGAG-3′ (sense) and 5′-TCGACTTCCAAAAAATACCTGATGATCGAAGAGTTCTCTTGAAACTCTTCGATCATCAGGTACGG-3′ (antisense)] specific for mouse bag3 were synthesized and subcloned into the vector's Bgl II and Sal I sites, downstream of the H1 promoter [23]. G3T-hi amphotropic packaging cells (Takara Bio) were transfected with pLTRH1bag3 puro or pLTRH1 puro vector according to the manufacturer's instructions to obtain a retroviral supernatant, which was added at a 1:5 ratio to DMEM supplemented with 10% FBS and used to infect ES2 cells. Infected cells were selected by incubation with 0.5 μg/ml puromycin for 48 h after infection. High-responder clones showing BAG3 knockdown were selected for subsequent experiments.

Cell viability assay

To test the responsiveness of the cells to paclitaxel under our culture conditions, cells were plated in 96-well plates (2500 cells/well) and incubated at 37 °C under a 5% CO_2 atmosphere. After 24 h, the medium was replaced with fresh medium containing 5% serum and paclitaxel (Nippon Kayaku Co., Ltd.). Cell viability assays were then performed after 24 h, 48 h and 72 h. After the desired incubation period, 50 μl of XTT labeling mixture was added to each well and the cells were incubated for an additional 5 h, after which absorbance at 492 nm was recorded using an ELISA plate reader.

MicroRNA microarray analysis

Microarray profiles were obtained using a Human miRNA Microarray V3 8x15K (miRbase release 12.0) (Agilent Technologies) and used to identify miRNAs differentially expressed in ES2-shBAG3 and ES2-LTRH1 control cells. Increases by more than 3-fold and decreases by more than 2-fold in ES2-shBAG3 cells were selected as thresholds for significant differential expression.

Real time quantitative RT-PCR (qRT-PCR) for mRNA

Total RNA was prepared from cells using TRIzol reagent (Invitrogen), after which cDNA was synthesized from the collected RNA using a SuperScript VILO™ cDNA Synthesis Kit (Invitrogen). Real-time quantitative PCR was carried out using Fast SYBR Green Master Mix (Applied Biosystems) in a StepOnePlus™ Real-Time PCR System (Applied Biosystems). mRNA levels were standardized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. The PCR protocol entailed denaturation at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The following primers were designed and used for real-time PCR: for BAG3, 5′-TGAGAAGTTTAACCCCGTTGCTTGT-3′ (forward) and 5′-CCCCATCTACCCCTCCAGTCCAG-3′ (reverse); for MCL-1, 5′-TGCTGGAGTAGGAGCTGGTT-3′ (forward) and 5′-CCTCTTGCCACTTGCTTTTC-3′ (reverse); for GAPDH, 5′-TGAACGGGAAGCTCACTGG-3′ (forward) and 5′-TCCACCACCCTGTTGCTGTA-3′ (reverse).

Real time quantitative RT-PCR (qRT-PCR) for microRNA

Total RNA was extracted using TRIzol reagent (Invitrogen) and precipitated with 75% ethanol. Reverse transcription was performed with 10 ng of total RNA, using a TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems) and sequence-specific RT primers from the TaqMan® MicroRNA Assays (Applied Biosystems) according to the manufacturer's instructions. Separate reverse transcription reactions were run for each TaqMan® MicroRNA Assay on every RNA sample. Real-time PCR was performed with cDNA using inventoried TaqMan® MicroRNA Assays and TaqMan® Universal Master Mix II (Applied Biosystems). The assay was performed in triplicate, and the PCR amplification was performed using a StepOnePlus™ Real-Time PCR System (Applied Biosystems).

Western immunoblotting

Cells were washed in PBS and then lysed in RIPA buffer (25 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 0.1% sodium deoxycholate and 0.1% SDS) containing a protease inhibitor cocktail (1:100 dilution; Thermo SCIENTIFIC, U.S.A.) for protein quantification. Thereafter, sample buffer was added, and the proteins were heat denatured at 100 °C for 5 min. Proteins in the lysate were then separated using 12% SDS-polyacrylamide gel electrophoresis (PAGE) and electrophoretically transferred onto PVDF membranes. The membranes were then blocked for 2 h with PBS containing 10% dried milk powder and incubated overnight at 4 °C with rabbit anti-Mcl-1 (1:100, Santa Cruz Biotechnology, Inc.), rabbit anti-BAG3 (1:1000) or mouse anti-MMP2 (1:200, Daiichi

Please cite this article as: Sugio A, et al, BAG3 upregulates Mcl-1 through downregulation of miR-29b to induce anticancer drug resistance in ovarian cancer, Gynecol Oncol (2014), http://dx.doi.org/10.1016/j.ygyno.2014.06.024