

Translocation of BBAP from the cytoplasm to the nucleus reduces the metastatic ability of vemurafenib-resistant SKMEL28 cells

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Abstract. To the best of our knowledge, the present study is the first to demonstrate that treatment of vemurafenib-resistant SKMEL28 (SKMEL28-R) cells with paclitaxel leads to a shift in localization of the E3-ligase BBAP from the cytoplasm to the nucleus, consequently decreasing the metastatic ability of this cell line. The present study revealed that the movement of BBAP from the cytoplasm to nucleus initiated a change in cell morphology. In addition, the translocation of BBAP led to a decrease of metastatic characteristics in SKMEL28-R cells, including migration and invasion via downregulation of the phosphorylated form of focal adhesion kinase and N-cadherin, as well as an upregulation of p21 and E-cadherin. The results of the present study suggested that BBAP may not only be a novel biomarker for melanoma, but also a novel therapeutic target for treatment of metastatic melanoma.

Introduction

BBAP is an E3 ubiquitin ligase protein and is a member of the Deltex family. The total protein is 740 amino acids in length, containing, two potential nuclear localization signals (amino acids 20-26 and 462-478), a possible nuclear export signal (amino acids 325-334), a classic RING finger domain (amino acids 561-599) and a C-terminus (amino acids 555-740), and is highly homologous to the conserved C-termini of other Deltex family members. BBAP was originally identified as a binding partner of the B-aggressive lymphoma 1 (BAL1) protein, present in diffuse large B-cell lymphoma (1,2). BBAP has been previously revealed as being highly expressed in the thymus of mice (3). BBAP expression has also been detected in the

telencephalic vesicles, hypothalamus, anterior pituitary, olfactory bulb, nasal cavity, mouth cavity, urogenital sinus, midgut loops and rectum (3). Histone H4 undergoes monoubiquitylation by BBAP and selectively modulates the DNA damage response. In addition, the increased expression of BBAP in lymphoma is resistant to DNA-damaging chemotherapeutic agents (4). Previous reports have demonstrated the functions of BBAP in the development of melanoma and lymphoma (2,5).

Metastasis of a primary tumor to a secondary site is the major cause of mortality from solid tumor types (4-6). The progression to metastasis involves a series of discrete steps, commonly known as the metastatic cascade. Tumor cells must first invade the primary tumor, dissociate from the tumor mass and be transported to nearby or distant secondary sites in the cascade (4). Invasion is a hallmark for the malignancy of cancer cells.

N-cadherin serves a pivotal role in promoting metastasis through differential regulation of extracellular signal-regulated kinases. N-cadherin-dependent adhesion impairs the upregulation of the two cyclin-dependent kinase inhibitors, p21 and p27 (7,8). Ectopic expression of N-cadherin increases tumor cell motility, leading to cadherin switching in the regulation of cell behavior (9,10). In addition, a direct relationship between N-cadherin and E-cadherin exists, whereby downregulation of E-cadherin is negatively correlated with upregulation of N-cadherin (11-14). p21 (WAF1/CIP1) serves an important role in controlling cell cycle arrest by regulating the activity of cyclins and cyclin-dependent kinases (15-18). p21 is able to inhibit cell growth through cell cycle arrest of skin cancer cells, including melanoma (7,19-21).

Previous reports have demonstrated that localization sites of certain molecules define their roles and regulate the development of cancer (22-24). Our previous study indicated that *BRAF*^{V600E}-harboring melanoma cell lines were resistant to the B-Raf enzyme inhibitor, vemurafenib, through various mechanisms (25). It has also been revealed that treatment of vemurafenib-resistant SKMEL28 cells (SKMEL28-R) with paclitaxel decreased the metastatic characteristics of the cells via downregulation of the epidermal growth factor receptor/AKT pathway. Furthermore, a previous study demonstrated that BBAP may serve an important role in melanoma development and progression (5); however, previous studies have demonstrated that BBAP predominantly exists in the

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cytoplasm of the cells (1,2). In the present study, the role of BBAP in melanoma *in vitro* was investigated when it was translocated from the cytoplasm to the nucleus of SKMEL28-R cells.

Materials and methods

Cell culture. SKMEL-28 cells were purchased from American Type Culture Collection (Manassas, VA, USA). An SKMEL28-R cell line was established, as previously described (25). SKMEL28-R cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in 5% CO₂.

Immunocytochemistry. Immunocytochemistry was performed to investigate the protein expression of molecules, according to a previous study (25). SKMEL28-R cells were fixed with 1% paraformaldehyde and blocked with 5% FBS for 30 min at room temperature. The cells were subsequently stained with 10 µg/ml rabbit anti-BBAP antibody (cat. no. A300-833; Bethyl Laboratories, Inc., Montgomery, USA; 1:50) conjugated with Alexa Fluor® 594 (Thermo Fisher Scientific, Inc.) with ratio 1/3 of anti-BBAP antibody/Alexa Fluor® 594 at room temperature for 3 h in the dark. The nuclei were counterstained with DAPI (blue). Images were captured with a x40 objective.

Immunoblotting. Immunoblotting was performed to investigate protein, as previously described (25). The cells were washed twice with ice-cold phosphate-buffered saline and lysed in 0.3 ml lysis buffer containing HEPES (20 mM, pH 7.4), NaCl (150 mM), b-glycerophosphate (12.5 mM), MgCl₂ (1.5 mM), EDTA (2 mM), NaF (10 mM), DTT (2 mM), Na₃VO₄ (1 mM), phenylmethylsulfonyl fluoride (1 mM), aprotinin (20 mM) and Triton X-100 (0.5%). Whole cell lysates were resolved by SDS-PAGE and transferred onto Hybond-P membranes (GE Healthcare Life Sciences, Chalfont, UK). The membranes were incubated with the following various antibodies: Rabbit polyclonal anti-BBAP (cat. no. A300-833; Bethyl Laboratories Inc., Montgomery, USA), rabbit polyclonal anti-phosphorylated FAK (tyrosine 397; cat. no. 44-624G; Invitrogen; Thermo Fisher Scientific, Inc.), mouse monoclonal anti-E-cadherin (cat. no. 610181; BD Biosciences, Franklin Lakes, NJ, USA), mouse monoclonal anti-α-FAK (cat. no. 05-537; Merck-Millipore, Darmstadt, Germany), mouse monoclonal anti-N-cadherin (cat. no. 610921; BD Biosciences) and goat polyclonal anti-p21 (WAF1/CIP1; cat. no. sc-397G; Santa Cruz Biotechnology, Inc), all used at 1:1,000. Following incubation with primary antibody, the membranes were probed with horseradish peroxidase-conjugated antibodies against goat anti-rabbit (cat. no. A16110; Invitrogen; Thermo Fisher Scientific, Inc.), goat anti-mouse IgG (cat. no. 31430; Invitrogen; Thermo Fisher Scientific, Inc.), donkey anti-goat IgG (cat. no. 31402; Invitrogen; Thermo Fisher Scientific, Inc.) or goat anti-mouse IgG (cat. no. 12-3491 Merck-Millipore, Darmstadt, Germany) with dilution rates of 1:5,000. The proteins were visualized using an Enhanced Chemiluminescence Western Blotting system or ECL Advance (GE Healthcare Life Sciences). Mouse monoclonal anti-β-tubulin antibody (cat. no. T4026;

Sigma-Aldrich, St. Louis, MO, USA; 1:2,000) was used as a loading control for the immunoblotting.

Scratch wound healing assay. Cell wound healing was performed, as described previously (25). Six-well plates (BD Biosciences, Franklin Lakes, NJ, USA) were incubated overnight in 1 ml RPMI-1640 medium, containing 40 µg/ml collagen. The cells were grown to 100% confluence on the collagen-coated plates in RPMI-1640 medium supplemented with 10% FBS. Scratch wounds were created in confluent monolayers using a sterile p200 pipette tip. A total of four perpendicular semi-opaque marks were placed across each scratch on the external surface of the well to standardize quantitative analysis. Following washing, the suspended cells were washed three times, and the wounded monolayers were again cultured in RPMI-1640 medium. Following incubation for 12 and 24 h, repopulation of the wounded areas was observed under phase-contrast microscopy (Olympus Corporation, Tokyo, Japan). Using the NIH ImageJ image-processing program (National Institutes of Health, Bethesda, MD, USA), the size of the scratch wound area was determined at each time point from the digital images.

Invasion assay. Cell invasion ability was evaluated using an invasion assay, according to a previous study (5). A total of 2x10⁵ cells in either 300 µl normal culture medium (10% FBS) or starving culture medium (0.5% FBS) were applied to a matrigel-coated upper chamber (8 µm in pore size). The upper chambers were subsequently placed in 24-well culture plates containing 600 µl conditioned medium with 0.5% FBS to trigger invasion activity and were incubated for 12 h. Invading cells were stained with hematoxylin/eosin and counted under a microscope.

Statistical analysis. Statistical analysis in was performed according to a previous study (5). Results from three independent experiments in each group were statistically analyzed by Student's *t*-test. The data are presented as the mean ± standard deviation. SPSS (version 18.0) software package (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis, *P*<0.05 was considered to indicate a statistically significant difference.

Results

Translocation of BBAP causes morphological changes of SKMEL28-R cells. SKMEL28-R cells were treated with 100 nM paclitaxel. Notably, treatment with paclitaxel resulted in presentation of several colonies with abnormal morphology consisting of elongated and dendritic shapes, compared with the oval shapes of control cell populations (Fig. 1A and B). These colonies were isolated and cultured for further experiments. The role of BBAP in melanoma development and progression was previously established (5). Therefore, the present study examined the expression levels and localizations of BBAP in untreated and paclitaxel-treated SKMEL28-R cells. Of note, although the total expression level of BBAP was almost unchanged in paclitaxel-treated SKMEL-28R cells (Fig. 1C, lane 1) compared with that in the untreated SKMEL-28R (Fig. 1C, lane 2), localization of BBAP was shifted from the cytoplasm in untreated cells (Fig. 1B) to the

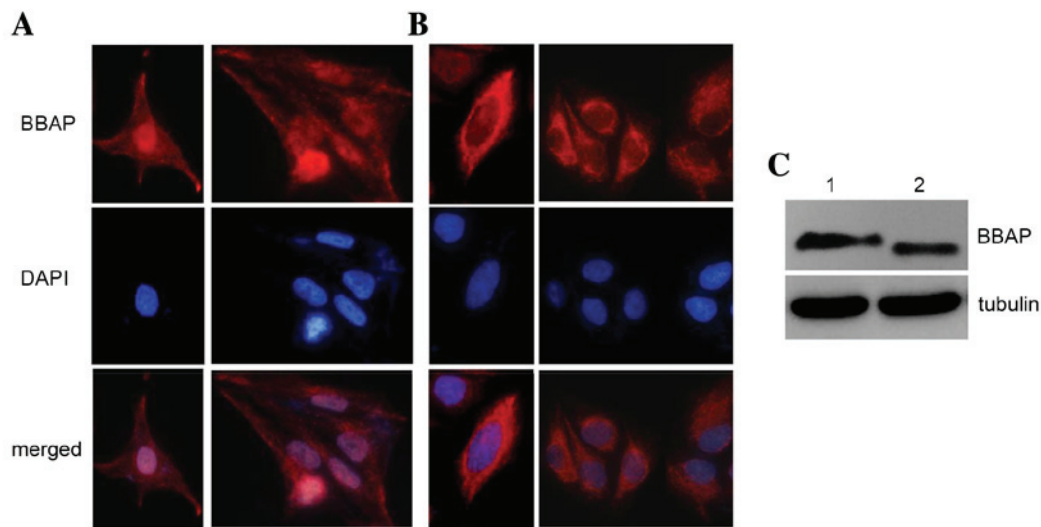


Figure 1. Localization sites and expression levels of BBAP in SKMEL28-R cells. BBAP localizes to the nucleus of (A) paclitaxel-treated SKMEL28-R cells and the cytoplasm of (B) untreated SKMEL28-R cells. (C) The total protein expressions of BBAP in paclitaxel-treated SKMEL-28R cells (lane 1) and in untreated SKMEL-28R cells (lane 2) were measured by immunoblotting. Tubulin was used as an internal control.

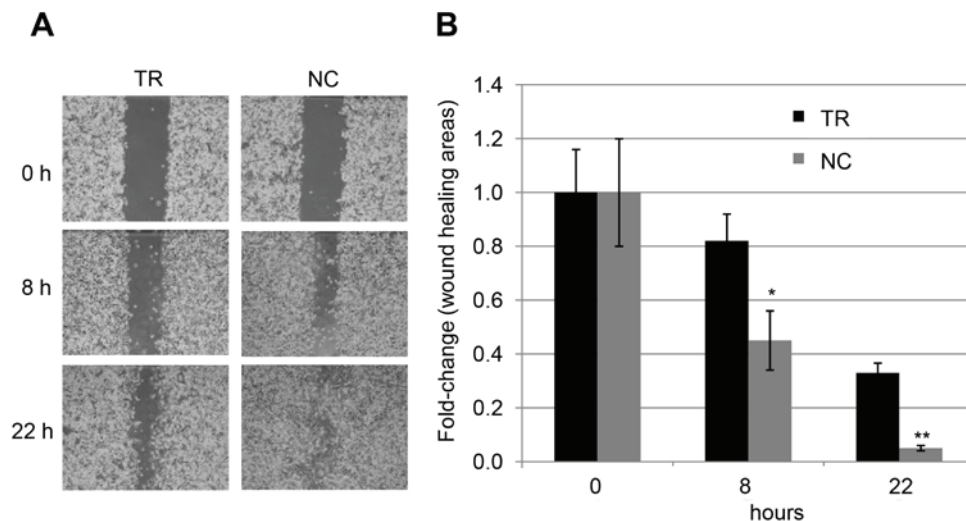


Figure 2. Effect of localization sites of BBAP on migration in SKMEL28-R cells. The migratory ability of SKMEL28-R cells was evaluated using a scratch wound healing assay. Wound areas healed by untreated SKMEL28-R cells and treated SKMEL28-R cells are presented as (A) images and (B) fold-change. The data are presented as the mean \pm standard deviation (* P <0.05 and ** P <0.01 compared with the NC group, as determined by Student's t -test. NC, negative control; TR, treatment group).

nucleus in paclitaxel-treated cells (Fig. 1A). This movement resulted in the expression level of BBAP changing in the cytoplasm and nucleus of treated cells compared with that in untreated cells. The immunohistochemistry results using anti-BBAP antibodies demonstrated that the expression of BBAP in cytoplasm of untreated cells (Fig. 1B) was increased compared with that of the treated cells (Fig. 1A). Conversely, the expression of BBAP in the nucleus of untreated cells (Fig. 1B) was decreased compared with that of the treated cells (Fig. 1B).

Localization of BBAP in the nucleus decreases metastatic ability of SKMEL28-R cells. Scratch wound healing and invasion assays were performed to investigate the metastatic and invasive ability of the SKMEL28-R cell line.

Paclitaxel-treated SKMEL28-R and untreated SKMEL28-R cells were pre-cultured in starving conditions (0.5% FBS) for 8 h prior to use in both assays. The results demonstrated that untreated SKMEL28-R cells had 2.2- and 4.1-fold higher metastatic abilities, respectively, in scratch wound healing (Fig. 2) and invasion (Fig. 3) assays when compared with treated SKMEL28-R cells. This suggested that the metastatic ability of SKMEL28-R cell line may be associated with localization sites of BBAP.

Localization of BBAP in the nucleus downregulates the expression levels of N-cadherin and pFAK, and upregulates the expression levels of E-cadherin and p21 in SKMEL28-R cells. Western blotting was used to examine the expression levels of BBAP and other molecules associated with metastatic

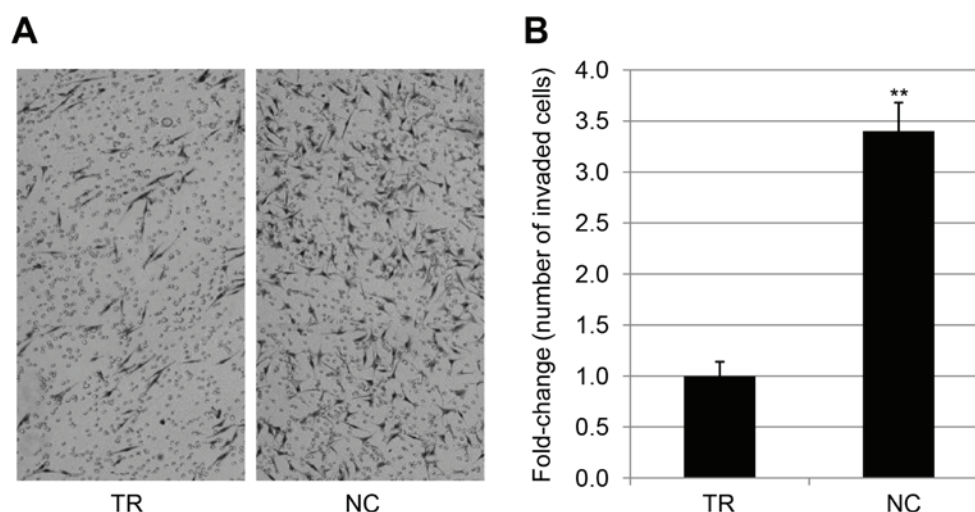


Figure 3. Effect of BBAP localization sites on the invasion of SKMEL28-R cells. The invasive ability of SKMEL28-R cells was evaluated using an invasion assay. The number of invading untreated SKMEL28-R cells and treated SKMEL28-R cells are presented as (A) images and (B) the fold-change. The data are presented as the mean \pm standard deviation (** $P < 0.01$ compared with the NC group, as determined by Student's *t*-test). NC, negative control; TR, treatment group.

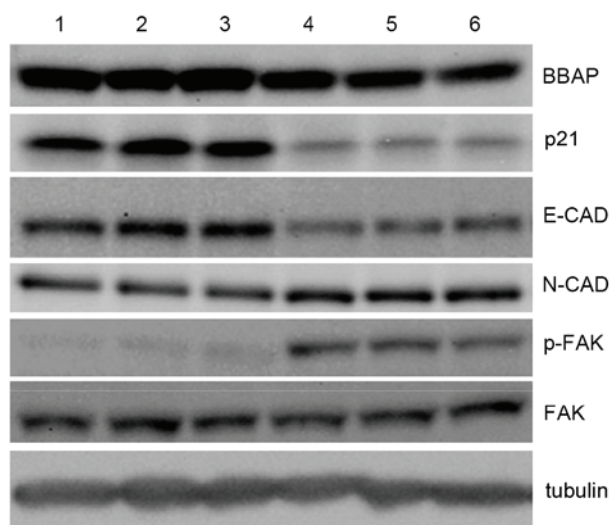


Figure 4. Protein expression levels in SKMEL28-R cells. Protein and phosphorylation levels of BBAP, p21, E-CAD, N-CAD, p-FAK and FAK in paclitaxel-treated SKMEL28-R cells (lanes 1-3) and untreated SKMEL28-R cells (lanes 4-6) were measured by immunoblotting. Tubulin was used as an internal control. E-CAD, E-cadherin; N-CAD, N-cadherin; p-, phosphorylated; FAK, focal adhesion kinase.

ability, including N-cadherin, E-cadherin, FAK, phosphorylated (p-)FAK and p21 in untreated and paclitaxel-treated SKMEL28-R cells (Fig. 4). The results revealed that while the expression levels of BBAP were marginally decreased, the expression levels of N-cadherin and p-FAK in treated cells (Fig. 4, lanes 1-3) were markedly decreased compared with those in the untreated cells (Fig. 4, lanes 4-6). In addition, the expression levels of E-cadherin and p21 were higher in treated cells (Fig. 4, lanes 1-3) compared with those in the untreated cells (Fig. 4, lanes 4-6). These results suggested that BBAP localization to the nucleus downregulated the expression of several molecules, which, in turn, reduced the metastatic ability of the cells.

Discussion

Previous studies identified that the expression of BBAP in skin tissue, normal epithelial cells and fibroblasts is low (3,26). Our previous study identified that SKMEL-28 cells expressed BBAP more highly compared with that of other melanoma cell lines (5); therefore the present study examined this particular cell line to further investigate the role of BBAP, since expression levels may be associated with the development of melanoma (5). This is the first study, to the best of our knowledge, to indicate that the localization site of the BBAP may contribute to its role in regulating the metastatic activities of SKMEL28-R cells. The results of the *in vitro* scratch wound healing and invasion assays demonstrated that migration and invasion abilities were decreased in paclitaxel-treated SKMEL28-R cells compared with those of untreated SKMEL28-R cells. The process of metastasis has been recorded as follows: Cell invasion of the primary solid tumor, dissociation from the tumor mass and transportation to nearby or distant secondary sites (4). Various signaling molecules have been reported to control the process. In the present study, it was revealed that the translocation of BBAP from the cytoplasm to the nucleus led to the downregulation of N-cadherin and p-FAK, which acted as cancer-promoting molecules, and upregulation of E-cadherin and p21, which were reported as cancer-inhibiting molecules (7-14).

Currently, few studies have investigated the role of BBAP in the development of leukemia (1,3,4,26-29). In addition, a limited number of publications examine the association between BBAP and solid tumor cancer types, including melanoma. Bachmann *et al* (29) demonstrated that BBAP regulated IRF-1 via STAT1 signaling and consequently affected metastasis in prostate cancer. The inhibition of BBAP resulted in decreased metastasis in prostate cancer cells (30). The association and interactions between STAT1 and FAK and/or N-cadherin and/or p21 have been previously well-established (30-36).

Paclitaxel has been reported as a drug that regulates the development of cancer via promotion of the expression of p21 (37-38) and E-cadherin (39), and inhibition of FAK expression in certain type of cancer cells (40). However, the molecular mechanism of this process remains unclear. The present study is the first, to the best of our knowledge, to demonstrate that treatment with paclitaxel leads to morphological changes of cancer cells via translocation of BBAP and consequent upregulation of p21 and E-cadherin, and downregulation of N-cadherin and p-FAK. The results suggested that BBAP may be a novel molecule that can significantly contribute in the paclitaxel-mediated FAK/STAT1/p21 signaling pathway. In another recently published study, retardation of DNA damage and enhanced cellular viability was dependent on the localization of the BAL1-BBAP complex (27). This may further support the importance of BBAP localization in cancer development.

Previous studies have exhibited that vemurafenib had potential to decrease the potential for metastasis of *BRAF*^{V600E}-carrying melanoma via the inhibition of p-AKT (41,42). As a result, the Food and Drug Administration approved vemurafenib for clinical application to treat metastatic melanoma (43). Unfortunately, the drug is only effective to inhibit melanoma for ~1 year. The resistance of the melanoma to vemurafenib is caused by the reactivation of p-AKT (44). Based on the present results, the current study involved production of a vemurafenib-resistant SKMEL-28 (named SKMEL-28R) cell line for further studies, with the hope that the combination treatment of vemurafenib and other novel approaches will boost the effectiveness on *BRAF*^{V600E}-harboring melanomas. This preliminary study focuses only on SKMEL-28R cell line, and therefore, one finite conclusion cannot be drawn for other cancer cells. Therefore further experiments are necessary to examine the localization sites of BBAP and cellular metastasis in various melanoma cells that highly express BBAP.

Taken together, the present results suggested that localization of BBAP to the nucleus of the cells may reduce the metastatic abilities of cells via the inhibition of N-cadherin and p-FAK, and activation of E-cadherin and p21 molecules. In conclusion, the analysis of BBAP demonstrated not only a role as a potential biomarker, but also a role as a potential therapeutic target in melanoma.

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