Expression level of *beta protein 1* mRNA in Chinese breast cancer patients: A potential molecular marker for poor prognosis

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Recent studies revealed high ectopic beta protein 1 (BP1) expression in breast cancer. Remarkably, up to 100% (18/18) of estrogen receptor (ER)-negative tumors and 89% (25/28) of tumors from African American women were BP1-positive. However, the role of BP1 in breast cancer development and its clinical significance still has not been well defined. In the present study, we analyzed the quantitative level of *BP1* mRNA in breast carcinomas using real-time polymerase chain reaction and aimed to elucidate its association with tumor characteristics and patient prognosis. Our data showed that *BP1* mRNA was expressed at significantly higher levels in tumors with lymph node metastasis, with a high histological grade, and in those that were of ER-negative status. Furthermore, overexpression of BP1 was significantly associated with poor outcome of patients harboring tumors with a high histological grade and negative ER. Using both *in vitro* and *in vivo* systems, we also showed that the transcript level of BP1 was positively correlated to the growth rate of breast tumor cells. Taken together, our results support the notion that BP1 might contribute to breast neoplastic transformation or tumor progression and suggest for the first time that *BP1* mRNA level has potential as a prognostic predictor for breast cancer. (*Cancer Sci* 2008; 99: 173–178)

Breast cancer is the most common female malignancy in the developed world.[1,2] In China, it is the second most frequent cause of tumor-related deaths among women, but the incidence has dramatically increased in the past decade.[3] More importantly, despite the major progress achieved in early detection as well as therapeutic strategies with surgery, chemotherapy, and radiotherapy, the outcome for some patients with breast tumors remains poor.[4] For instance, approximately 30% of patients with negative lymph nodes undergo relapse and eventually die from the disease within 10 years of diagnosis.[5] Therefore, identification of novel molecular events underpinning breast tumor development and relapse is still crucial for providing potential targets of clinical intervention.

Homeobox genes encode a group of transcription factors regulating cell proliferation, differentiation, and commitment during embryonic development.[6] Although no direct link has been precisely established, accumulating evidence shows that some homeobox genes are aberrantly expressed in a variety of tumors including breast cancer.[7,8] HOXA5, which is expressed at high levels in normal epithelium, was found to be lost in nearly 70% of all breast tumors.[9] Moreover, loss of HOXA5 was correlated with loss of p53 expression, and it might activate the promoter of p53 by binding to an antiapoptotic protein, Twist.[10,11] Homeobox genes might also be inappropriately reactivated in cancer cells. HSIX1, whose expression is low in normal mammary gland, was elevated in 44% of primary tumors and in 90% of metastatic lesions.[12] In MCF-7 cells, HSIX1 overexpression abolishes the G_2 cell cycle checkpoint in response to irradiation, causing inappropriate entry into mitosis.[12,13]

Beta protein 1 (*BP1*), belonging to the distal-less (DLX) family of homeobox genes, is normally expressed in fetal liver, as well as in the placenta and kidney.[14,15] In normal erythroid lineage, it functions as a repressor of the β-globin gene through two upstream silencers.[16] Recent studies revealed high ectopic BP1 expression in breast cancer. Compared to estrogen receptor (ER)-positive tumors and those from Caucasian American women, up to 100% (18/18) of ER-negative tumors and 89% (25/28) of tumors from African American women were BP1-positive, distinguishing it as the first DLX gene strongly correlated with breast cancer.[17] BP1 might have more potential clinical significance, if its exclusive expression in breast tumors can be confirmed in a larger population group and its association with patient outcome and tumor cell growth further investigated. As ER-negative tumors usually have more aggressive clinical behavior and are unresponsive to anti-estrogen therapy,[5,18] a significantly high percentage of BP1 expression in these tumors might be linked to the poor prognosis. Additionally, in leukemia cells, BP1 appears to be specifically involved in blocking apoptosis and facilitating the expansion of a biologically more aggressive cell clone,[19] indicating that higher level of BP1 expression in breast tumors might also contribute to cell proliferation or invasion by way of this antiapoptotic pathway.

In agreement with the findings in Caucasian American and African American women, we previously reported that BP1 is highly expressed (53/82, 64.63%) in Chinese breast cancer patients.[20] To further clarify the role of BP1 in breast cancer, we therefore examined the quantitative level of *BP1* mRNA in 142 cases of invasive breast tumor tissues. Considering that breast cancer progression is a multiple-step procedure, sequentially progressing from normal, to hyperplastic, to *in situ*, and to invasive stages, and most of invasive tumors are likely to contain all four different tissue components,[21,22] in the present study, we applied laser capture microdissection to retrieve pure tumor cells for analysis. The copy number of BP1 was then determined by real-time polymerase chain reaction (PCR) and its correlations with tumor characteristics, as well as patient outcome, were evaluated. In addition, using breast cancer cell lines, we also tested the effect of BP1 overexpression on tumor cell growth both *in vitro* and *in vivo*. To our knowledge, this is the first report addressing the quantitative level of *BP1* mRNA in breast cancer and its potential value for prognosis.

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Table 1. Correlations between *beta protein 1* (*BP1*) mRNA levels and various clinicopathological factors in 142 cases of invasive breast tumors

Parameters	Group	Cases (*n*)	*BP1* mRNA level§	*P*-value
Age (years)	≤50	63	529 ± 578	0.271
	>50	79	561 ± 525	—
Tumor size (cm)	≤2	48	505 ± 493	0.190
	>2	94	553 ± 457	—
Menopausal status	Pre	61	571 ± 524	0.352
	Post	81	548 ± 419	—
Histological grade	I	34	456 ± 462	0.009
	II	75	542 ± 437	—
	III	33	619 ± 406	—
Lymph node metastasis	Negative	82	449 ± 435	0.036
	Positive	60	581 ± 540	—
Adjuvant therapy	Hormone	80	557 ± 429	0.408
	Chemo	19	573 ± 441	—
	Combination	31	530 ± 494	—
	None	12	502 ± 518	—
ER status†	Negative	55	607 ± 426	0.025
	Positive	87	474 ± 511	—
PR status†	Negative	65	596 ± 483	0.057
	Positive	77	504 ± 460	—
HER2 status‡	Negative	52	568 ± 492	0.213
	Positive	90	535 ± 453	—

†Positive when more than 15% of the membrane showed staining by immunohistochemical staining. ‡Positive when more than 10% of the nuclei showed staining by immunohistochemical staining. §The *BP1* mRNA levels are shown as the mean ± standard deviation. —, not applicable; ER, estrogen receptor; PR, progesterone receptor.

Materials and Methods

Clinical samples. With informed consent, tumor specimens were collected from 142 female breast cancer patients (stage I: 41; stage II: 80; stage III: 21) treated at Tianjin Cancer Hospital (Tianjin, China) during the period April 1996–January 2000. The median age of patients was 51.8 years (range 41–73 years). All tumors were histologically diagnosed as invasive breast tumor: 111 invasive ductal carcinomas and 31 invasive lobular carcinomas. Tissue samples were snap-frozen in liquid nitrogen after surgery and stored at –80°C. The detailed characteristics of these patients, including age, tumor size, menopausal status, histological grade, the presence of lymph node metastasis, and hormonal receptor status, were available for all participants (Table 1). Histological grade was determined according to the modified Bloom–Richardson criteria,[23] whereas lymph nodal status was assessed histopathologically. ER and progesterone receptor (PR) expression were evaluated using immunohistochemical staining (positive when >15% of the nuclei showed staining). Tumor-associated protein HER2 was defined as positive when >10% of the membrane showed staining using immunohistochemical assay.

As adjuvant hormone therapy, 80 patients were treated with tamoxifen (20 mg/day) ($n = 56$), or tamoxifen + goserelin ($n = 24$). As adjuvant chemotherapy, six cycles of CMF (cyclophosphamide 100 mg/day p.o., days 1–14; methotrexate 40 mg/m^2 i.v. days 1 and 8; 5-fluorouracil 600 mg/m^2 i.v. days 1 and 8) were given to 11 patients, and cycles of EC (epirubicin 60 mg/m^2 i.v. day 1 + cyclophosphamide 600 mg/m^2 i.v. day 1) were applied to eight patients. Thirty-one patients were treated with a combination of chemotherapy (CMF, $n = 5$; EC, $n = 22$) or other chemotherapies ($n = 4$) and hormone therapy (tamoxifen, $n = 25$ or tamoxifen + goserelin, $n = 6$). Twelve patients received no adjuvant therapy. Indication for adjuvant treatment was decided according to the St Gallen guidelines. All patients underwent a close follow-up study for disease recurrence by clinical and biological studies every 3 months for the first 2 years and every 6 months thereafter. Radiological examinations were carried out every 6 months or when considered necessary. The median follow-up period was 67 months (range 40–92 months). Of the 142 patients, 46 relapsed including 27 with local relapse and 19 with distant metastasis (five to lung; four to liver; three to bone; three to brain; two to the other breast; two to soft tissue). All experiments were approved by the Institutional ethics committee.

Laser capture microdissection and RNA extraction. For microdissection, frozen tissues were embedded in Shandon Cryomatrix medium (Thermo Fisher Scientific, Pittsburgh, PA) and cut into 8 µm sections on a Leica CM1850 standard cryostat (Leica Microsystems, Nussloch, Germany). The sections were fixed in acetone, stained by hematoxylin, and dehydrated in 100% ethanol and xylene. At least 1000 infiltrating ductal or lobular tumor cells per sample were collected using the Veritas Laser Capture Microdissection System (Arcturus Bioscience, Mountain View, CA) according to the manufacturer's protocol. The total RNA was extracted using an RNeasy Micro Kit (Qiagen, Hilden, Germany) and quantified on a spectrophotometer. All solutions were prepared with diethylpyrocarbonate (DEPC)-treated water. RNase-free instruments and RNaseZap (Ambion, Austin, TX) were used during all procedures.

cDNA preparation and real-time PCR. Briefly, 3 µg of total RNA was reverse-transcribed for single-strand cDNA using oligo(dT)15 and Superscript II reverse transcriptase (Invitrogen, Gaithersburg, MD). Real-time PCR for BP1 amplification was carried out on an ABI Prism 5700 Sequence Detection System (Applied Biosystems, Foster City, CA) with a 50 µL reaction mixture containing 4 µL of cDNA template, 25 µL QuantiTect SYBR Green PCR Master Mix (Qiagen), and 20 pmol of each primer. The primers for *BP1* (AF_254115) were: forward, 5′-GCT GAA AGA GGC TCA GAG AGA-3′; reverse, 5′-AGG TCT GGG AAG ACA GCT TTG-3′ (224 bp). To precisely quantify the transcript content, we detected the mRNA expression of *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) as an internal control. The primers for *GAPDH* (NM_002046) were: forward, 5′-GAA GGT GAA GGT GGG AGT C-3′; reverse, 5′-GAA GAT GGT

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GAT GGG ATT TC-3′ (226 bp). *BP1* and *GAPDH* mRNA was amplified in separate tubes using the following protocol: initial ‘Hot Start’ activation step for 15 min at 95°C and 40 cycles of 20 s at 95°C, 20 s at 56°C, and 30 s at 72°C.

For each PCR reaction, standard curves for BP1 and GAPDH were generated for quantifying mRNA copy numbers using serially diluted solutions of PCR product as templates. The copy number of *BP1* mRNA was normalized against that of *GAPDH* to give the relative value of *BP1* expression. Relative *BP1* level was calculated by the formula: $BP1/GAPDH \times 1000$ and shown as mean ± standard deviation. Real-time PCR was repeated at least in duplicate for each sample and a non-template control was included in each experiment.

Cell culture. Human breast cancer cell line T47D was originally purchased from American Type Culture Collection (ATCC) and was maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL of penicillin, and 100 µg/mL of streptomycin at 37°C under a 5% CO_2 atmosphere. All cell culture medium and supplements were purchased from Gibco/Invitrogen.

Isolation of breast tumor cells stably overexpressing BP1. A BP1 cDNA of 1030 bp containing the complete open reading frame was synthesized by reverse transcription–PCR from breast tumor T47D cell total RNA. This cDNA fragment was inserted into the plasmid pGEM7, sequenced, then subcloned into a pRc/RSV eukaryotic expression vector (Invitrogen) containing the Rous sarcoma virus (RSV) as the promoter and the neomycin gene as a selectable marker.[19] Subsequently, the construct was transfected into cultured T47D cells by applying the Lipofectamine method (Invitrogen) based on the provided instructions, and the empty pRC/RSV vector was used as a negative control. Briefly, following transfection, cells were incubated in the presence of G418 (800 µg/mL; Sigma). After 2 weeks of selection, the resistant transformants were picked, individually expanded, and finally screened for BP1 stable overexpression using real-time PCR.

***In vitro* cell proliferation assay.** In brief, cells from two BP1-overexpressing cell lines (BP1-OS1 and BP1-OS2), as well as T47D cells with pRC/RSV empty vector, were seeded at an equal density of 1×10^4 cells/dish and the numbers of viable cells were counted every 24 h for a total of 6 days using the 0.2% Trypan blue exclusion method. Fresh media was added every 24 h in order to maintain optimal growth conditions. At each time point, the experiments were repeated in triplicate.

Colony formation in soft agar. Two percent gum agar (Sigma) was mixed with RMPI-1640 and FBS to give 0.8% agar and 10% FBS. Then 1 mL of 0.8% agar was added to a 6-well plate and allowed to set as the base layer. The top layer of agar was similarly prepared to give 0.4% agar and 10% FBS. Then 0.9 mL of 0.4% agar was mixed with 0.1 mL of cell suspension (containing 2000 cells from either BP1-overexpressing or control cell lines) by vortexing vigorously until the cells were evenly suspended. The cell-containing mixture was plated onto the base layer agar, then was overlaid with 1 mL of culture medium. After 15 days of incubation, colonies >0.1 mm were counted using an ocular micrometer on an inverted microscope (Olympus, Tokyo, Japan).

***In vivo* tumorigenicity study.** To investigate the *in vivo* tumor growth rate in correspondence to BP1 expression levels, 5×10^6 cells from the two BP1-overexpressing T47D cell lines and the negative control cells were subcutaneously inoculated into both flanks of 6-week-old female SCID mice ($n = 3$ for each group; purchased from the Animal Research Center, Beijing University) as previously described.[24] Tumor growth was monitored assuming spherical growth of tumor volumes. When a tumor mass was visually detectable, its maximum (a) and minimum (b) diameters were recorded every 3 days till 42 days using a caliper. The tumor volume (V) was calculated according to the formula: $V = 1/2 \times (a^2 \times b)$. All animal experiments were approved by the Institutional Animal Care and Use Committee.

Statistical analysis. The non-parametric Mann–Whitney *U*-test and the Kruskal–Wallis test (for histological grade and adjuvant therapy) were used to compare *BP1* mRNA expression differences between/among different clinicopathological groups. Disease-free survival time was defined as the time interval from surgery to local relapse/first distant organ metastases or to the last follow-up visit. Survival analyses were carried out according to the Kaplan–Meier method and compared by the log–rank test. Cox's proportional hazards regression model was carried out to calculate the independent hazard ratio of each variable by both univariate and multivariate analysis. Comparisons of quantitative measures between BP1-overexpressing and control cells were done using a two-tailed unpaired Student's *t*-test. $P < 0.05$ in all cases was considered statistically significant.

Results

Expression level of *BP1* mRNA in tested tumors. The mRNA level of *BP1* in 142 cases of examined tumor cells ranged from 0 to 1287 with a median value of 551 relative copy numbers. Clinicopathological parameters of all these tumor samples are summarized in Table 1.

Level of *BP1* mRNA correlates with histological grade and lymph node metastasis. Using the real-time PCR assay, we identified that the level of *BP1* mRNA in tumors with a high histological grade (619 ± 406) was significantly higher than in those with low and intermediate histological grades (456 ± 462 and 542 ± 437, $P = 0.009$, Table 1). In addition, the amount of *BP1* mRNA expression was also significantly higher in the lymph node metastasis-positive group (581 ± 540) than in the corresponding negative group (449 ± 435, $P = 0.036$; Table 1). However, no significant differences were found between the mRNA level of *BP1* and other clinicopathological factors, including age, tumor size, menopausal status, and adjuvant therapeutic regimens ($P > 0.05$; Table 1).

Level of *BP1* mRNA is associated with ER, but not PR or HER2 status. Estrogen and progesterone, which modulate the growth of breast epithelium in a delicate balance, are two crucial hormones involved not only in normal breast development but also in tumor progression.[25] In the present set of samples, we found that the level of *BP1* mRNA expression was significantly higher in the ER-negative tumors (607 ± 426) than in the ER-positive group (474 ± 511, $P = 0.025$; Table 1). Moreover, although tumor cells in the PR-negative group showed a certain degree of upregulation in *BP1* mRNA levels (596 ± 483) compared to that in the PR-positive group (504 ± 460), the difference between groups was not statistically significant ($P = 0.057$; Table 1). We also analyzed the amount of BP1 transcript in the subgroups divided by the expression of well-defined tumor-associated protein HER2, which was found to be immunohistologically colocalized with BP1,[26] but no significant association was identified in this study ($P = 0.213$; Table 1).

Prediction of disease-free survival (DFS) based on the mRNA level of *BP1*. The *BP1* mRNA level correlated with a higher histological grade, positive lymph nodes, and ER-negative status, raising a possibility that the amount of BP1 might affect the prognosis of breast cancer patients. Thus, based on different values of BP1 relative expression, we next categorized the 142 patients into two similarly-sized subgroups: a high BP1 expression group ($n = 71$) and a low expression group ($n = 71$), using a cutoff value of 551, the median level of BP1 transcript in all tumor specimens. As shown in Fig. 1a, the DFS of patients with high-level BP1 expression (745 ± 278) was significantly shorter than the patients in the BP1 low expression group (361 ± 192, $P = 0.012$). In order to determine whether BP1 is an independent risk factor for poor prognosis, conventional clinicopathological

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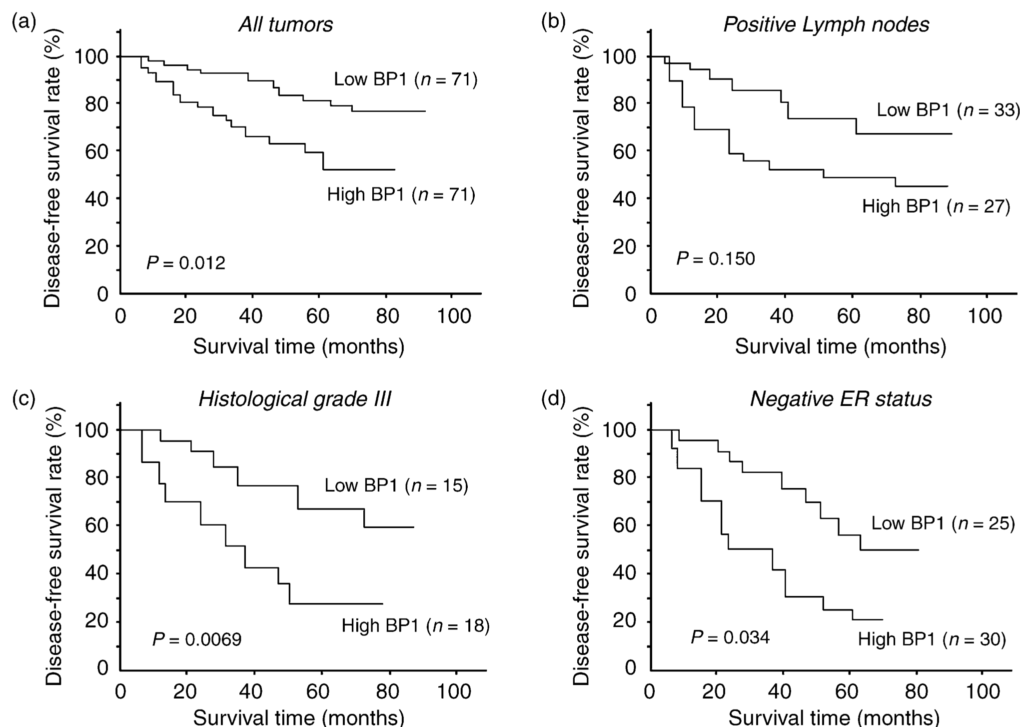

Fig. 1. Kaplan–Meier survival plots showing a correlation of *beta protein 1* (*BP1*) mRNA levels with disease-free survival (DFS) among examined patients. (a) Patients with tumors expressing higher level of *BP1* mRNA had poorer DFS rates than those with lower levels of expression ($P = 0.012$). (b–d) DFS curves in poor-prognosis patient subpopulations [positive lymph nodes metastasis (B), histological grade III (C), and estrogen receptor-negative status (D)] according to the *BP1* mRNA levels.

Table 2. Univariate and multivariate analyses of disease-free survival rates in 142 Chinese female breast cancer patients

Parameters	Categories	Univariate	Multivariate	
		P-value	*P*-value	HR (95% CI)
Age	≤50/>50	0.216	NS	—
Tumor size	≤2/>2	0.037	0.046	1.31 (0.73–2.04)
Menopausal status	Pre-/post-	0.408	NS	—
Histological grade	I, II, III	0.002	0.008	3.01 (1.26–7.94)
Lymph node metastasis	+/–	0.011	0.023	2.20 (1.04–5.95)
ER status	+/–	0.040	NS	—
PR status	+/–	0.034	0.047	0.74 (0.43–1.91)
HER2 status	+/–	0.179	NS	—
BP1 mRNA	Low/high	0.025	0.039	2.73 (0.97–6.89)

—, not applicable; +, positive; –, negative. BP1, beta protein 1; CI, confidence interval; ER, estrogen receptor; HR, hazard ratio; NS, not significant; PR, progesterone receptor.

parameters and BP1 were assessed using Cox's univariate and multivariate regression analysis. As a result, tumor size greater than 2 cm, higher histological grade, positive lymph nodes, negative PR, and high *BP1* mRNA level were independent factors in predicting the outcome of breast cancer (Table 2). More importantly, compared to the BP1 low expression group, the outcome of patients with BP1 high expressing tumors was still worse when the analysis focused on three well-established specific subsets of poor prognosis breast cancer patients, that is, those with positive lymph node metastasis ($P = 0.150$; Fig. 1b), those with histological grade III tumor ($P = 0.0069$; Fig. 1c), and those of negative ER status ($P = 0.034$; Fig. 1d).

BP1 overexpression is linked to increased cell proliferation both *in vitro* and *in vivo*. To investigate the potential mechanism by which BP1 upregulation might be playing a role in breast cancer, we observed the effects of enforced BP1 overexpression on the proliferative ability of breast tumor T47D cells. Two independent clones stably overexpressing BP1, designated as BP1-OS1 and BP1-OS2, were isolated. The *BP1* mRNA levels in these two stable cell lines compared to the empty vector controls were 8.7 ± 0.4-fold and 12.1 ± 0.7-fold increase, respectively, as quantified by real-time PCR (data not shown). *In vitro* cell proliferation assay showed that the cell growth rate was greatly increased in BP1-OS1 and BP1-OS2 cells, compared to that of the control group (Fig. 2a). Cells were also evaluated for anchorage-independent colony formation ability, a well-defined indicator of oncogenic potential. Two thousand cells were plated in soft agar and observed for growth characteristics over 15 days. As expected, there was a significant difference between the numbers of colonies found in BP1-overexpressing cells and in T47D cells

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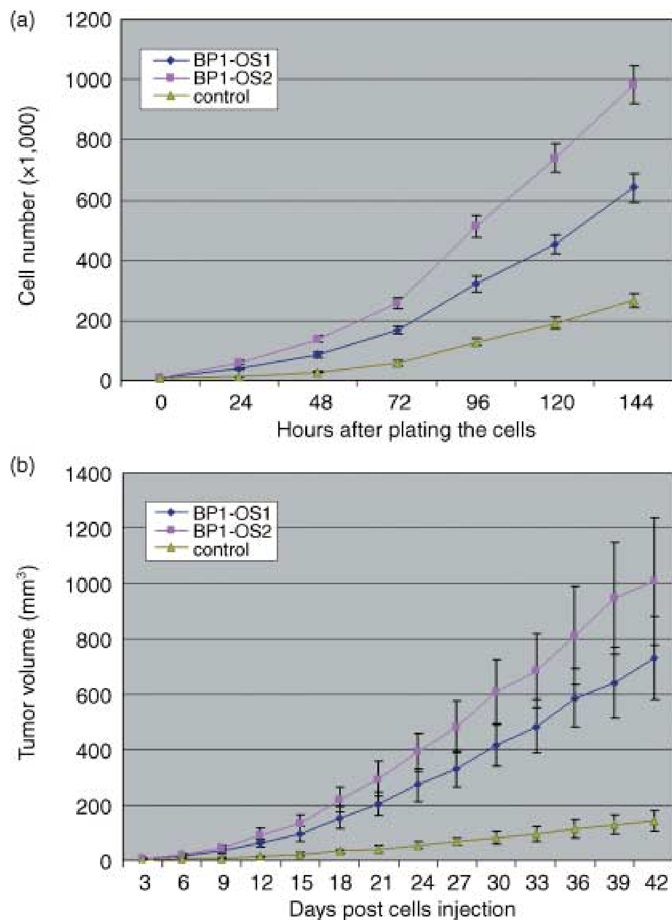

Fig. 2. (a) Increase of breast tumor T47D cell growth *in vitro* by beta protein 1 (BP1) overexpression. The graph shows the growth curves of BP1-transfected cells (BP1-OS1 and BP1-OS2) and T47D cells with empty vector (control). The equal quantities of 1×10^4 cells were seeded and cultured for 6 days. At each time point, the number of cells was counted. Each value represents the mean ± standard deviation (SD) ($n = 3$). (b) *In vivo* growth characteristics of BP1-overexpressing (BP1-OS1 and BP1-OS2) and control T47D cells in SCID mice. Increased tumorigenicity was observed with cells overexpressing BP1. Tumor volumes were bidimensionally determined every 3 days for 42 days. Values are presented as mean tumor volume ± SD ($n = 6$).

with empty construct: 169 ± 34 (BP1-OS1, $n = 3$, $P < 0.001$) and 291 ± 46 (BP1-OS2, $n = 3$, $P < 0.001$) versus 43 ± 8 ($n = 3$), suggesting that a higher level of BP1 is associated with enhanced proliferation and oncogenicity in breast cancer. To confirm these findings, we next examined the tumor formation ability of cells overexpressing BP1 *in vivo*. Our experiments showed that all mice injected with these cells, as well as empty control cells, developed tumor. As displayed in Fig. 2b, *in vivo* BP1-overexpressing cells recapitulated their *in vitro* growth characteristics, growing much faster than the control line, showing that the cells with ectopically elevated BP1 levels were more tumorigenic.

Discussion

The mammary gland undergoes a variety of developmental cycles of branching morphogenesis, functional differentiation, secretion, and involution in which some of the homeobox genes play critical regulatory roles, particularly for maintaining the proper differentiated state.[27] Therefore, deregulated expression of these genes might result in loss of the differentiated state and/or adoption of an alternative cell identity, such as loss of cell cycle control, evasion of apoptosis, acquisition of invasiveness or reduced sensitivity to 'antigrowth' signals.[8,28] Several lines of evidence have shown that cells harboring these inappropriate characteristics are much easier to undergo neoplastic transformation.[7] In addition, since the processes of normal embryogenesis and neoplasia share many similar pathways, it is increasingly accepted that neoplastic transformation is an aberrant form of organogenesis.[27]

We have been able to show, in the present study, that breast tumors with a high *BP1* mRNA level are significantly associated with aggressive phenotype such as high histological grade, positive lymph node metastasis, and negative ER status. Combining our findings in the Chinese population with previous reports,[17,26] we consider that the expression of BP1 among patients from different ethnic backgrounds and different areas is relatively consistent, indicating that the *BP1* gene is functionally highly conserved and might be an important participant in breast cancer development. It is also worthy to note that BP1 is expressed in the premalignant stage and in all tumor grades, as well as in both small and large tumors,[17,20] all of which strongly support the concept that this gene is probably activated at the very early stage of neoplastic transformation and then functions through all the progress of tumor progression. BP1 protein expression in breast tumors has been poorly documented. Currently, our laboratory is generating and testing a monoclonal antibody for BP1 in order to further analyze the association of BP1 expression between the protein and transcriptional levels. In addition, using this antibody, we may conduct a large-scale tissue microarray analysis, which will be of great importance to confirm several observations in this study.

Breast cancer is a hormone-dependent disease. ER/PR status reflects the level of estrogen and progesterone *in vivo* and the acquisition of estrogen-independent phenotype even determines the metastasis potential of cancer cells, especially during tumorigenesis in estrogen-dependent organs.[29,30] It has been shown that ER-negative tumors are unresponsive to anti-estrogen therapy and most of them have a higher histological grade and a higher proliferative rate.[18,25] In our recent studies, we found that the expression of BP1 in ER-positive breast cancer cells could be cooperatively regulated by estrogen (1×10^{-9} mol/L) and different dosage of tamoxifen treatment (3×10^{-7}–3×10^{-6} mol/L; Yu *et al*., unpublished data, 2006). These preliminary results imply that the expression of *BP1* mRNA might be directly or indirectly regulated by ER-mediated transduction pathway(s) and the signal regulation of estrogen might be involved in the complex functions of BP1 in breast cancer development. Furthermore, overexpression of BP1 has potential clinical value for breast cancer, at least in a certain subset of patients. Our survival analyses revealed that BP1 is a powerful prognostic predictor for breast cancer patients with negative ER. We also obtained evidence that a higher level of BP1 expression was significantly relevant to shorter DFS rate in patients with tumor of histological grade III. Therefore, if *BP1* mRNA in those patients could be examined preoperatively, BP1 could have potential to serve as an adjuvant predicting factor for clinical oncologists to guide surgery, optimize therapeutical regimens systemically, and lower the chance of recurrence.

As well as the ER-mediated pathway, there might exist other molecular mechanisms leading ultimately to BP1 overexpression in breast tumors. Our results, together with recent data, show that breast cancer T47D cells stably overexpressing BP1 show a significantly higher proliferative activity *in vitro* or *in vivo*, and short interfering RNA-mediated inhibition of BP1 could promote apoptosis in ER-positive breast tumor cells (Yu *et al*., unpublished data, 2006). Recently, Song *et al*. also reported that there was a high percentage of cyclin D1 and BP1 co-expression in 82 cases of breast cancer specimens.[31] Thus, we consider that BP1 is probably capable of acting with cell cycle modulator(s) to aggravate tumor growth while preventing cells from undergoing

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apoptosis, regarded as a direct cause of malignancy. The expression of BP1 might also result from its interaction with other tumor-associated factors, because, as a transcription factor, BP1 could bind to DNA and/or other proteins of the transcriptional machinery, directly leading to expression, and in some cases, to repression, of key genes involved in tumorigenesis.[32] For instance, double immunostaining showed that BP1 is co-localized with HER2 in a subset of tumor cells, and the number of cells with co-localized BP1 and HER2 increases with tumor progression.[26] Moreover, BP1 is mapped at 17q21-22, a hotspot region of DNA amplification in breast cancer and spatially close to HER2.[14,33,34] Consequently, BP1 might act on HER2 and trigger its overexpression by the means of over-replication. Further identification of the downstream targeting genes for BP1 will be necessary to provide new possibilities for clarifying its specific role in breast cancer.

In conclusion, despite the limited number of patients, the significant positive correlations between *BP1* mRNA level and lymph node metastasis, histological grade, ER negativity, as well as poor outcome, indicate that the *BP1* gene might be involved in breast neoplastic transformation and/or tumor progression and suggest for the first time that *BP1* mRNA expression could have prognostic significance for breast cancer. Further studies involving assessment of a larger number of cases over a longer follow-up period, combined with determination of BP1 expression at the protein level and insights into its molecular targets, will be beneficial to elucidate the functional significance of BP1 in breast cancer.

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