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BAP1 is a good prognostic factor in advanced non-small cell lung cancer

Abstract

Purpose: Non-small cell lung cancer (NSCLC) is the leading worldwide source of cancer-related deaths. Although some drugs targeting epidermal growth factor receptor (EGFR) mutations have been developed, most advanced NSCLC is still incurable and new targets for anticancer drugs are in demand. BRCA1-associated protein-1 (BAP1) is a component of the ubiquitin proteasome system (UPS). UPS has emerged as a potential target for anti-cancer drugs. The aim of the present study was to investigate the expression of BAP1 protein in patients with NSCLC.

Methods: BAP1 expression was measured using Western blot analysis in 103 cases patients with advanced NSCLC.

Results: Results revealed 49 (47.5%) patients were classified with high expression of BAP1. Squamous cell carcinomas were more likely to be observed in BAP1 high expressers compared with adenocarcinomas (55.8% vs. 32.3%, $p = 0.001$). High BAP1 expression was associated with no lymph node metastasis ($p = 0.002$). There was also a significant association between BAP1 expression and histological type ($p = 0.014$), while expression of BAP1 was not correlated with other clinical or pathological characteristics. Kaplan-Meier survival analysis showed that patients with high BAP1 expression had a longer median survival compared with patients with low BAP1 expression (23.2 vs. 14.7 months, $p = 0.021$). Multivariate analysis revealed that high BAP1 expression was an independent lower risk for all 103 patients (HR = 0.61, 95% CI 0.32-0.71, $p = 0.003$).

Conclusions: BAP1 may be a useful prognostic factor of NSCLC patients and potential target for anticancer drugs.

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Non-small cell lung cancer (NSCLC), especially advanced NSCLC, is one of the leading worldwide sources of cancer-related deaths [1]. Some epidermal growth factor receptor (EGFR)-targeting drugs, such as erotinib and gefitinib, have been successfully applied in treating advanced NSCLC in past years; however, erotinib and gefitinib are only suitable for treating some patients with NSCLC. New targets for anticancer drugs are required.

BRCA1-associated protein-1 (BAP1) is a component of the ubiquitin proteasome system (UPS) and has been implicated in lung cancers [2]. Mutations in the UPS genes have emerged as potential targets for anticancer drugs [3,4].

BAP1 was originally identified as a protein that interacts with the RING finger domain of the breast cancer susceptibility gene product, BRCA1 [2]. BAP1 is a nuclear-localized DUB with an NH₂-terminal ubiquitin COOH-terminal hydrolase (UCH) domain and two predicted nuclear-localization signals (NLS). The UCH family includes UCH-L1, UCH-L3 and UCH-L5 (UCH37), all of which possess a conserved catalytic domain containing an invariant histidine, cysteine and aspartic acid catalytic triad [5]. Although UCH family members were initially associated with the maturation and turnover of ubiquitin, these enzymes possess isopeptidase activity and, thus, might selectively regulate protein stability or activity [6-8]. Remarkably, BAP1 possesses a large C-terminal domain that is not present in other UCH members and is predicted to play an important role in regulating and coordinating its deubiquinating enzyme (DUB) activity through selective association with potential substrates or regulatory components. Although its cellular function is still elusive, several lines of evidence support a role for BAP1 as a tumor suppressor [9]. The BAP1 gene locus undergoes frequent loss of heterozygosity in cancer. Moreover, large rearrangements, deletions and missense mutations in the BAP1 gene locus have been found in lung and in sporadic breast tumors and lung cancer cell lines [4], and BAP1 suppresses growth of breast cancer cells in vitro [2]. Interestingly, recent studies indicate that RNA interference (RNAi)-mediated depletion of BAP1, can also exert an inhibitory effect on cell proliferation [10-12]. In further support of this notion, homozygous-inactivating mutations in BAP1 have been found in subsets of lung carcinoma and breast cancer cell lines, suggesting that this DUB is a tumor suppressor [5,13,14]. These controversial results can be at least partially attributed to the use of different cell types, reflecting the complicated function of BAP1 in biological progression of different cancers.

Substantial evidence from clinical data is needed to support BAP1 as a potential target for anticancer drugs. The expression of BAP1 protein in patients with NSCLC has not yet

been reported. Here, we measure BAP1 expression in 103 cases of patients with advanced NSCLC to investigate, for the first time, the impact of BAP1 on survival. Western blot and immunohistochemistry (IHC) were used to measure BAP1 protein levels and classify tumors according to levels of BAP1 expression in NSCLC patients.

Methods

Patients

A total of 103 patients with advanced NSCLC (III and IV stage) were studied. Patients were admitted to the Department of Oncology of The Affiliated Sixth People's Hospital, Shanghai Jiaotong University from February 2006 to September 2010. All samples were obtained by biopsy. Clinical and radiological examination identified patients suspected with NSCLC, and then definitive pathological diagnosis of NSCLC had made carried out by the CT-guided puncture or incisional biopsy.

The 7th AJCC Staging system was used to categorize patients and two groups of patients were used in this study: stage III NSCLC and stage IV NSCLC. Among patients with Stage III NSCLC, metastatic recurrence was characterized as limited metastatic or extensive metastatic disease as described in a previous report [15]. All patients were assessed with diagnostic CT or PET/CT imaging to identify metastatic lesions. Brain metastases were assessed with MRI. In some patients bone metastases were identified with bone scan and/or MRI.

All patients were followed up after diagnosis. The patients in this study were treatment naive when tissues were collected. Western blot analysis was performed on fresh frozen samples. Immunohistochemical assays were performed on paraffin-embedded tissue.

Western blot

For Western blot, samples were homogenized in a solution of 10 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT, 0.5 mM PMSF, 10 µg/ml aprotinin and 10 µg/ml leupeptin, at pH 7.9. Protein extracts were then subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The monoclonal antibody against BAP1 was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The membrane was incubated with primary and secondary antibodies for 1 hour at room temperature then developed using an ECL Kit (Amersham Pharmacia Biotech Inc., Buckinghamshire, UK). Cell lysates of A431 cell line that expresses BAP1 protein was used as positive control (Datasheet of sc-48386, Santa Cruz Biotechnology Inc.). Blot quantitation was done with a Mo-

FIGURE 1. BAP1 expression in NSCLC tissues of all 103 patients were investigated by Western blot and IHC. **A.** Western blot: Lane 1 in upper figure indicates the BAP1 peptide as positive control. Lane 2-10 in upper figure indicates parts of 103 NSCLC tissues randomly. Lower figure indicates the expression of beta-actin. 100 μ g total protein were added per lane. **B.** IHC: A case was considered BAP1 high expression if more than 30% cells were strongly stained.

lecular Dynamics Laser Densitometer (Model PSD) and the Image Quant Version 1 software [16]. When the densitometric ratio of BAP1 expression in the tumor sample to that in the A431 cell line is ≥ 0.5 , the sample is termed BAP1 high expression.

Immunohistochemistry (IHC)

Anti-BAP1 (Santa Cruz Biotechnology Inc.) monoclonal antibody (diluted at 1:5000) was used in these experiments. Five- μ m-thick sections were cut and mounted on SuperFrost Plus slides. Sections were left at room temperature until immedi-

ately before use, at which point they were heated for 1 h at 60°C. After heating, sections were deparaffinized through three changes of xylene and rehydrated through graded alcohols into water. Heat-induced epitope retrieval and endogenous peroxidase blocking was performed. Sections were then incubated with normal goat serum at 1:50 (Vector, Burlingame, CA) for 15 min at room temperature. The goat serum was then shaken off and sections were incubated with primary antibody overnight at 4°C and then washed. After washing, sections were incubated with either Biogenex Multilink secondary antibody at a dilution of 1:20 for 20 min at room temperature. Sections were again washed and then stained using the ABC elite kit

(Vector Laboratories) with diaminobenzidine according to the manufacturer's protocol. The staining results were read by a group of senior pathologists. In each case, 2,000 tumor cells were counted from 10 randomly selected areas, ensuring that the whole section was scanned. A case was considered BAP1 high expression if more than 30% cells were strongly stained. Immunostaining was analyzed with an Olympus Corp. BX-50 microscope, and pictures were taken with an Olympus Corp. Oly 760 video camera.

Statistical analysis

The statistical significance of the correlation between expression of BAP1 and several clinicopathological parameters was assessed by Fisher's exact test, χ^2 test, or χ^2 test for trends as indicated. The probability of overall survival as a function of time was determined by the Kaplan-Meier method and the log-rank test. Multivariate survival analysis was performed using the Cox regression model. P values smaller than 0.05 were considered as significant. For the statistical evaluation, the SPSS software version 12.0 was used (SPSS, Inc., Chicago, IL).

TABLE 1. Patient demographics.

Characteristic	No. of Patients (n = 103)	Percentage (%)
Age (years)		
<62	50	48.5
≥62	53	51.5
Sex		
Male	62	60.2
Female	41	39.8
Tumor size		
< 3 cm	39	37.9
≥ 3 cm	64	62.1
Lymph node metastasis		
No	37	35.9
Yes	66	64.1
Remote metastasis		
No	44	42.7
Yes	59	57.3
Histological type		
Ad	50	48.6
SCC	48	46.5
Ad+SCC	5	4.9
Clinical stage		
III	47	45.6
IV	56	54.4
Smoking status		
Ever	61	59.1
Never	42	40.9

Ad, adenocarcinoma; SCC, squamous cell carcinoma; Ad+SCC, Adenosquamouscarcinoma.

TABLE 2. BAP1 expression levels for various clinicopathological factors in patients with NSCLC.

Characteristic	Cases	BAP1 expression (%)		P value
Age (years)		High	low	0.235
<62	50	25 (50)	25 (50)	
≥62	53	24 (45.3)	29 (54.7)	
Sex				0.102
Male	62	30 (48.4)	32 (51.2)	
Female	41	19 (46.3)	22 (53.7)	
Tumor size				0.395
< 3 cm	39	20 (51.3)	19 (48.7)	
≥ 3 cm	64	29 (45.3)	35 (54.7)	
Lymph node metastasis				0.002
No	37	24 (64.9)	13 (35.1)	
Yes	66	25 (37.8)	41 (62.2)	
Remote Metastasis				0.058
No	44	25 (56.8)	19 (43.2)	
Yes	59	24 (40.7)	35 (59.3)	
Histological type				0.014
Ad	50	19 (38)	31 (62)	
SCC	48	28 (58.3)	20 (41.7)	
Ad+SCC	5	2 (40)	3 (60)	
Clinical stage				0.063
III	47	26 (55.3)	21 (44.7)	
IV	56	23 (41.1)	33 (58.9)	
Smoking status				
Ever	61	31 (50.8)	31 (49.2)	0.091
Never	42	18 (42.9)	24 (57.1)	

Ad, adenocarcinoma; SCC, squamous cell carcinoma; Ad+SCC, Adenosquamouscarcinoma.

Results

Clinical and Pathological Characteristics of Patients with NSCLC.

The basic demographics and pathological characteristics of this group are shown in Table 1.

Correlation between high BAP1 expression and clinicopathological factors in patients with NSCLC.

The expression of BAP1 was investigated by Western blot (Fig. 1A) and IHC (Fig. 1B), and the correlation between high expression of BAP1 and clinicopathological factors of patients with NSCLC was analyzed. BAP1 expression levels determined by these two different techniques were consistent. The high expression of BAP1 was significantly associated with an absence of lymph node metastasis ($P = 0.002$) and trended towards an association with no remote metastasis ($P = 0.058$). There was also a significant association between BAP1 expression and histological type ($P = 0.014$), while expression of BAP1 was not correlated with tumor size, gender, age, remote metastasis, smoking status and clinical stage (Table 2).

High BAP1 expression is a prognostic factor for overall survival (OS) and progression-free survival (DFS) in univariate Kaplan-Meier analysis.

In Kaplan-Meier survival analysis, high expression of BAP1 was a significant prognostic indicator for overall survival ($P = 0.005$) as well as disease free survival ($P = 0.011$) (Fig. 2). In

addition, the presence of metastases and the histological grade were significant prognostic indicators for both overall survival and disease-free survival. Lymph node metastasis, remote metastasis, clinical stage and smoke status were also significant prognostic indicators for overall survival and progression-free survival.

Multivariate survival analysis.

Multivariate survival analysis demonstrated that high BAP1 expression was significantly related to longer survival. In contrast, lymph node metastasis, remote metastasis and later clinical stage were significantly related to shorter survival (Table 3).

Discussion

Here we present, for the first time, evidence that the expression levels of BAP1 have prognostic significance in patients with advanced NSCLC. Specifically, individuals whose tumors had relatively higher BAP1 expression survive longer in comparison with patients with relatively lower levels of BAP1 expression. The correlation between high expression of BAP1 and clinicopathological factors of patients with NSCLC was also investigated.

Although its cellular function is still elusive, BAP1 has previously been shown to play a role as a tumor suppressor. Deletions, loss of heterozygosity, missense mutations and large rearrangements in the BAP1 gene locus have been found in lung and sporadic breast tumors and lung cancer cell lines [3,4]. In addition, BAP1 suppresses growth of breast cancer cells in vitro [3]. RNAi-mediated depletion of BAP1, can exert

FIGURE 2. High BAP1 expression is a prognostic factor for overall survival (OS) and disease free survival in univariate Kaplan-Meier analysis. A: $P=0.005$; B: $P=0.011$.

TABLE 3. Relative risk of death (RR) of 103 patients with advanced NSCLC in this study by multivariate survival analysis.

Characteristic	RR (95%CI)	P value
BAP1 expression		
High	1.00	
Low	2.59 (0.97-4.65)	0.011
Age (years)		
< 62	1.00	
≥ 62	2.32 (1.85-6.72)	0.155
Sex		
Male	1.00	
Female	2.11 (0.76-4.83)	0.097
Tumor size		
< 3 cm	1.00	
≥ 3 cm	2.62 (0.85-6.72)	0.098
Lymph node metastasis		
No	1.00	
Yes	2.83 (0.45-4.36)	0.038
Remote metastasis		
No	1.00	
Yes	2.15 (1.01-5.19)	0.006
Histological type		
Ad	1.00	
SCC	2.29 (1.65-3.92)	0.053
Ad+SCC	1.73 (0.55-4.21)	
Clinical stage		
III	1.00	0.027
IV	1.41 (1.76-5.57)	
Smoking status		
Ever	1.00	0.328
Never	1.51 (0.67-3.41)	

RR, relative risk of death; CI, confidence interval; Ad, adenocarcinoma; SCC, squamous cell carcinoma; Ad+SCC, Adenosquamous carcinoma.

an inhibitory effect on cell proliferation [13,14]. No clinical evidence on the relationship between BAP1 gene and tumor cell proliferation in the NSCLC has yet been reported. Our data demonstrated that the high expression of BAP1 was significantly associated with lower levels of lymph node metastasis. When BAP1 expression is low, the tumors of NSCLC pa-

tients tended to more easily metastasize to the lymph node. Although no significant difference is observed in remote metastasis, the tendency toward an association between high BAP1 expression and less remote metastasis was shown by our data.

There was also a significant association between BAP1 expression and histological type. Adenocarcinomas had lower BAP1 expression than squamous tumors (38% vs. 58.3%, respectively). Although non-squamous histology is a criterion for bevacizumab [17,18,19] as well as pemetrexed treatment [17,20,21], many patients with adenocarcinoma are not sensitive to these drugs [22,23,24]. Thus, new targets for effective drugs are always needed. It is interesting to note that the prognostic power of BAP1 is somewhat stronger in patients with adenocarcinoma and in patients who never smoked. BAP1 could potentially be developed as a novel therapeutic target and contribute benefit in adenocarcinoma patients who do not respond to current treatment modalities. The prognostic power of BAP1 is higher in adenocarcinoma, and this suggests that it may be more useful as a prognostic marker in adenocarcinoma as opposed to squamous cell carcinoma. This finding also demonstrates the importance of stratifying lung cancer subtypes when making clinical decisions. A survival benefit for BAP1 expression was demonstrated in patients without histological evidence of adenocarcinoma = and this observation confirms the effect of adenocarcinoma histology for prognostic stratification of NSCLC patients. Although the mechanistic basis for this subgroup bias is not yet known, such stratification might still be relevant for future targeted therapy and/or early predictions of survival of patients with adenocarcinoma.

Better clinical stage tends to be related to low expression of BAP1, but no significant difference was observed (P values were 0.058 and 0.063, respectively). BAP1 expression was not correlated with gender, age and smoking status in this study.

BAP1 may be a potential therapeutic target; however, it can be difficult to use tumor suppressor-like genes as therapeutic targets in a clinical setting. Considering that the mutation of BAP1 may contribute to the carcinogenesis of lung cancer, targeting on the mutation of BAP1 could be a strategy for NSCLC treatment.

Given the known tumor suppressor role of the BAP1, one would expect that high BAP1 expression would correspond to less remote metastasis and a better clinical stage. One possible explanation for this unexpected finding is that the number of patients recruited for this study is too low to draw a more specific conclusion. Another potential limitation of this study is the retrospective nature of the collection of patients. A prog-

nostic trial of a single marker such as this can rarely be prospectively designed outside the setting of a clinical trial.

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