HBP1 promoter methylation augments the oncogenic \(\beta\)-catenin to correlate with prognosis in NSCLC

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Abstract

\(\beta\)-catenin nuclear accumulation is frequently identified in human non-small cell lung cancer (NSCLC). The HMG-box transcription factor 1 (HBP1) is a known repressor of \(\beta\)-catenin transactivation. However, the role of HBP1 in relation to \(\beta\)-catenin nuclear accumulation has not been addressed in human cancer patients. In addition, the mechanism of HBP1 gene alteration in NSCLC remains unclear, although HBP1 mutation and gene deletion of HBP1 are reported in breast and colon cancers. Here, we demonstrate that HBP1 acts as a tumour suppressor and serves as a prognostic biomarker in NSCLC clinical and cell models. The immunohistochemistry data indicated that 30.5\% (25/82) of tumours from NSCLC patients showed absence or low expression of HBP1 protein. A significant inverse correlation between mRNA/protein expression and promoter hypermethylation suggested that promoter hypermethylation is responsible for low expression of HBP1 in NSCLC patients. Reactivation of HBP1 expression by demethylation reagent or ectopic expression of HBP1 suppressed \(\beta\)-catenin transactivation. Conversely, HBP1 knockdown increased \(\beta\)-catenin transactivation. Importantly, preserved expression of HBP1 had a significantly protective effect on prognosis in patients with \(\beta\)-catenin nuclear accumulation, suggesting that low expression of HBP1 in NSCLC patients with \(\beta\)-catenin nuclear accumulation was one of the major determinants of prognosis. Our data from cellular and clinical models suggest that HBP1 is a suppressor of cancer progression, making it a potential prognostic predictor and therapeutic target to attenuate lung cancer progression.

Keywords: HBP1 • \(\beta\)-catenin • transcriptional repressor • prognosis • NSCLC

Introduction

Lung cancer is one of the most common malignancies in the world and is the leading cause of cancer deaths in industrial countries [1]. Despite the significant improvement in both diagnostic and therapeutic modalities for the treatment of cancer patients, outcome remains poor when the disease has spread to regional lymphatics [2]. Cancer cells have been characterized by multiple changes that affect tumour cells via various receptors and subsequent signalling pathways [3]. Therefore, further elucidation of the cellular signalling mechanisms in lung tumorigenesis is important to develop early diagnostic and new effective therapeutic targets.

Mounting evidence suggests that cancer may result from the aberrant activation of normally controlled developmental pathways [4]. Wnt/\(\beta\)-catenin signalling has a dual role in vertebrate development and tumorigenesis [5]. In normal and non-stimulated cells, because of the rapid turnover of \(\beta\)-catenin promoted by the destruction complex,
majority of β-catenin protein is present in the adherens junctions with very little in cytoplasmic or nuclear fractions [6]. Activated Wnt signaling inhibits phosphorylation of β-catenin, thereby causing β-catenin to dissociate from the destruction complex and preventing its degradation. The hallmark of Wnt signaling is cytoplasmic accumulation of β-catenin protein; the stabilized β-catenin enters the nucleus where it interacts with the T Cell Factor (TCF) and thereby activates the transcription of downstream target genes such as cyclin D1 and c-MYC [7, 8]. Within the cell nucleus, the activity of β-catenin/TCF complex can be inhibited by the HMGB-box transcription factor 1 (HBP1), which blocks β-catenin signaling by competing with TCF to bind with β-catenin for transcription of target genes in the Wnt/β-catenin pathway [9]. Over time, Wnt/β-catenin signaling has become a crucial pathway in non-small cell lung cancer (NSCLC) as previously demonstrated in many cancers [5, 10, 11]. Most studies have focused on the negative regulation of β-catenin accumulation in the suppression of tumorigenesis. However, transcriptional repression could also be an important mechanism for the inhibition of gene expression that is activated by stabilized β-catenin [9]. Therefore, HBP1 transcriptional repressor may be an effective way of blocking the expression of β-catenin target genes.

HBP1 may function as a tumour suppressor by inhibiting the Wnt/β-catenin signalling to block the oncogenic phenotype [9]. In addition, HBP1 is revealed to be essential for oncogene-mediated premature senescence that is lost during malignant transformation [12]. A previous study demonstrated that over half of the tested breast cancer samples (15 of 22) had reduced HBP1 mRNA levels and HBP1 mutations/variants were associated with invasive breast cancer [13]. Another study reported 18 out of 22 prostate cancer tissue samples showed reduced HBP1 mRNA levels [14]. In addition, HBP1 gene lies within the chromosome 7q31.1 that is frequently deleted in many tumours, such as colon cancer, breast cancer and myeloid leukaemia [15–17]. However, the role of HBP1 in relation to β-catenin nuclear accumulation has not been addressed in human cancer patients.

Our previous study showed that low expression of AXIN2/βTrCP in the degradation complex leads to β-catenin nuclear accumulation in NSCLC patients [11]. We also noticed that among the patients with β-catenin accumulation, some showed association with better survival outcome [18, 19], suggesting that antagonists of β-catenin transactivation may exert a protective role in patient outcome. This result prompted us to hypothesize that HBP1 may play an important role in the repression of β-catenin-mediated oncogenic effects in cell nucleus. To address this issue, we performed a comprehensive molecular analysis of the effects of HBP1 alterations on DNA methylation and mRNA/protein expression in clinical and cellular models. The mechanism of HBP1 alteration and its effect on β-catenin signalling in human NSCLC were investigated.

**Materials and methods**

**Subjects**

Paired tumour and normal lung tissues were obtained from 82 NSCLC patients prospectively recruited at the Taipei Veterans General Hospital between 2002 and 2008 after appropriate institutional review board permission and informed consent from patients were obtained. Overall survival was calculated from the day of surgery to the date of death or the last follow-up. For methylation assay, genomic DNA from primary tumour tissues was extracted by using proteinase K digestion followed by phenol–chloroform extraction. For RNA expression assay, total RNA was extracted from paired tumour and normal tissues by using Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized by using SuperScriptTM reverse transcriptase (Invitrogen) according to the manufacturer’s instructions.

**Immunohistochemical analysis**

Paraffin blocks of tumours were sectioned and processed by using standard techniques. Polyclonal antibodies against β-catenin (1:1500; Transduction Laboratories, Lexington, KY, USA), HBP1 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β-actin (1:5000; Abcam, Cambridge, MA, USA) were used as the primary antibodies to detect protein expression. Staining ‘percentage’ for HBP1 was scored as 3, 2, 1, or 0 if >75%, 50–75%, 25–50%, or <25% area were stained, respectively. A score of 1 or 0 indicated the presence of little or no HBP1 protein expression. β-catenin nuclear expression was scored as 3, 2, or 1 if >70%, 36–70%, or 0–35% area were stained, respectively. A score of 3 indicated β-catenin nuclear accumulation.

**Western blot analysis**

Cells were lysed and lysates were centrifuged. SDS gel loading buffer (60 mM Tris base, 2% SDS, 10% glycerol and 5% β-mercaptoethanol) was added and samples containing 50 μg of protein were separated on an 8% SDS-PAGE then electro-blotted onto Immobilon-P membranes (Millipore, Bedford, MA, USA). Immunoblotting was performed with antibodies against HBP1 (1:800; Santa Cruz Biotechnology); β-actin (1:5000; Abcam) was used as loading control.

**Quantitative reverse-transcriptase PCR (RT-qPCR) assays**

RT-qPCR analysis of clinical samples for HBP1 and cyclin D1 mRNA expression was performed with GAPDH gene as an internal control, while RT-qPCR of cell models for HBP1, c-myc and cyclin D1 mRNA expression was performed with β-actin gene as internal control. Primers used are listed in Table S1. The tumour samples with HBP1 expression levels <50% of corresponding normal tissues were deemed to have an abnormal pattern. The mRNA level of HBP1, c-myc and cyclin D1 were calculated using 2^[−ΔΔCt (ΔΔCt = ΔCtarget gene − ΔCinternal control)]^.

**Methylation-specific PCR (MSP) assay**

The methylation status in the promoter region of the HBP1 gene was determined by chemical treatment with sodium bisulphite and subsequent MSP analysis. Positive control samples with unmethylated DNA from IMR90 normal lung cell (U reaction) and Ss1 methyltransferase-treated methylated DNA (M reaction) were included in each PCR set.
Primers used are listed in Table S1. Bisulfite-modified DNA (100 ng) was amplified by PCR (35 cycles for U reaction; 45 cycles for M reaction) with annealing temperatures of 55°C and 65°C for U and M reactions, respectively. Hypermethylated genes were defined as those which produced more amplicons of M products than of U products from the samples.

5-aza-2′-deoxycytidine (5-Aza-dC) treatment

A549 cells (1 × 10^5 per dish) were plated in 100-mm culture dish on the day before treatment. The cells were treated with 20 μmol/l 5-Aza-dC (Sigma-Aldrich, St Louis, MO, USA) for three doubling times and then harvested for MSP, RT-qPCR and Western blot.

Low HBP1 augments the prognostic effects of β-catenin nuclear accumulation in NSCLC patients

To test our hypothesis that HBP1 exerts a protective role on survival outcome of patients with β-catenin nuclear accumulation, we performed univariate (HBP1 expression, β-catenin nuclear accumulation, smoking habit, tumour type, tumour stage and lymph node status) and multivariate Cox regression analysis. Our data showed that preserved HBP1 expression and lack of β-catenin nuclear accumulation had beneficial effects on prognosis (P = 0.039; hazard ratio [HR], 0.48; 95% confidence interval [CI], 0.24–0.96 for preserved HBP1 and P = 0.043; HR, 0.38; 95% CI, 0.24–0.99 for no β-catenin nuclear accumulation), even after adjusting for smoking habit, tumour type, tumour stage and lymph node status (P = 0.042 for preserved HBP1; P = 0.041 for no β-catenin nuclear accumulation; Table 1). In stratification analyses according to the status of β-catenin, preserved HBP1 expression, but not other factors examined, had significantly better effects on prognosis in patients with β-catenin nuclear accumulation (P = 0.007; HR, 0.15; 95% CI, 0.04–0.60; Table 2).

Low expression of HBP1 and β-catenin nuclear accumulation were apparently the major determinants of prognosis in NSCLC patients. Therefore, we classified the risk of death among NSCLC patients into two categories. The patients who had either no β-catenin nuclear accumulation or with preserved expression of HBP1 (68/82) were at low risk, whereas those having both β-catenin nuclear accumulation and low HBP1 expression (14/82) were at high risk (Fig. 1C, left panel). Interestingly, Kaplan–Meier analysis showed that NSCLC patients at high risk correlated with worse prognosis (P = 0.005; Fig. 1C, right panel). Together, these data suggested that low expression of HBP1 is a clinically relevant regulator of NSCLC that eventually leads to poor prognosis.

Results

Low HBP1 protein expression correlates with poor prognosis of NSCLC patients

To examine whether HBP1 is altered and associated with the clinical characteristics of the NSCLC patients, we first examined the protein expression level of HBP1 and β-catenin in 82 tumours from NSCLC patient by immunohistochemical analysis (Fig. 1A). The data indicated that 30.5% (25/82) of tumours showed reduced or absent expression of HBP1 while β-catenin nuclear accumulation was found in 40.2% (33/82) of NSCLC, respectively (Table S2). Low HBP1 protein expression correlated with squamous cell carcinoma patients (P = 0.046; Table S2). Next, we evaluated the prognostic effects of low HBP1 protein expression by using the Kaplan–Meier method. Unadjusted analysis showed that lower level of HBP1 protein was associated with poor survival in all patients (P = 0.034) and patients in early stage (P = 0.013) or in lymph node status (N0; P = 0.010; Fig. 1B).

Promoter hypermethylation is the predominant mechanism of low HBP1 expression and enhanced β-catenin activity in NSCLC clinical and cell models

To verify the mechanism involved in altered HBP1 protein expression, we carried out mRNA expression and DNA methylation assays of
HBP1 gene in the cohort of 82 NSCLC patients. Quantitative RT–PCR (RT-qPCR) analysis demonstrated a decrease or absence of HBP1 transcripts in 31.7% (26/82) of tumour tissues compared to their corresponding normal tissues (Fig. 2A and Table S3). Methylation-specific PCR assay showed that 53.7% (44/82) of tumours exhibited promoter hypermethylation of HBP1 gene (Fig. 2B and Table S3). Notably, promoter hypermethylation was significantly associated with low mRNA expression \( (P = 0.018; \text{Fig. 2C}) \) and low protein expression \( (P = 0.009; \text{Table S3}) \), suggesting that promoter hypermethylation is responsible for low expression of HBP1 gene. Next, we evaluated whether epigenetic silencing of HBP1 led to increased expression of β-catenin-targeted genes, such as cyclin D1, in NSCLC patients. We detected the cyclin D1 mRNA by RT-qPCR. The correlation analysis indicated that high expression of cyclin D1 in patients were frequently accompanied by low expression of HBP1 \( (P = 0.042; \text{Fig. S1}) \).

To confirm whether promoter hypermethylation of HBP1 resulted in decreased HBP1 gene expression and increased β-catenin activity, A549 cells were treated with the demethylating agent 5-Aza-dC and interrogated for the expression of HBP1 and β-catenin transactivation targets c-MYC and cyclin D1. As shown in Figure 3A–D, treatment with 5-Aza-dC successfully restored mRNA and protein expressions...
and demethylation of the HBP1 promoter which was originally methylated in A549 cells. In addition, the immunohistochemical analysis showed that accumulation of nuclear β-catenin was reduced in A549 cells treated with 5′-Aza-dC (Fig. 3D, right panel). Importantly, mRNA expression of c-MYC and cyclin D1 was decreased after restoration of HBP1 expression (Fig. 3E). Our data suggested that promoter hypermethylation of HBP1 gene could be responsible for low expression and loss of transcriptional repressive activity of HBP1 in NSCLC.

Ectopically expressed or knocked down HBP1 influences transactivation of β-catenin in NSCLC cell line

To verify the role of HBP1 in transcriptional regulation of β-catenin/TCF in lung cancer, we examined the effect of ectopically expressed HBP1 in A549 cells. By using RT-qPCR and Western blot assays we confirmed that HBP1 mRNA and protein were increased in cells ectopically expressing HBP1 compared with vector control cells (Fig. 4A and B). We further investigated whether HBP1 expression had effect on the transcriptional activity of β-catenin/TCF by using a TCF reporter/LEF reporter assay (TOPFLASH). The FOPFLASH reporter with mutated LEF/TCF site was used as a negative control. The β-catenin activity was significantly inhibited by ectopically expressed HBP1 ($P = 0.002$; Fig. 4C), whereas there was no change in the negative control FOPFLASH reporter (data not shown). Indeed, the expression of c-MYC and cyclin D1 were decreased in ectopically expressed HBP1 cells ($P = 0.007$ and $P = 0.039$, respectively; Fig. 4D).

To further confirm the reciprocal relationship between HBP1 expression and β-catenin activity in lung cancer, we examined whether HBP1 knockdown would affect β-catenin/TCF activity and increase c-MYC and cyclin D1 expression. A549 cells transfected with siRNA-HBP1 showed low HBP1 mRNA and protein expression compared with control siRNA (Fig. 5A and B). β-catenin activity was significantly increased in HBP1 knockdown A549 cells ($P = 0.032$; Table 1).

| Characteristics                  | Univariate analysis | P-value* | Multivariate analysis | P-value* |
|----------------------------------|--------------------|----------|-----------------------|----------|
|                                  | HR (95% CI)        |          | HR (95% CI)          |          |
| HBP1 IHC                         |                    |          |                       |          |
| Absent/decreased                 | 1.00               |          | 1.00                  |          |
| Preserved                        | 0.48 (0.24–0.96)   | 0.039    | 0.38 (0.15–0.96)      | 0.042    |
| β-catenin nuclear accumulation   |                    |          |                       |          |
| Yes                              | 1.00               |          | 1.00                  |          |
| No                               | 0.49 (0.24–0.99)   | 0.047    | 0.38 (0.15–0.96)      | 0.041    |
| Smoking habit                    |                    |          |                       |          |
| Non-smoker                       | 1.00               |          | 1.00                  |          |
| Smoker                           | 0.66 (0.28–1.57)   | 0.348    | 0.58 (0.20–1.72)      | 0.326    |
| Tumour type                      |                    |          |                       |          |
| ADC                              | 1.00               |          | 1.00                  |          |
| SCC                              | 1.01 (0.73–2.17)   | 0.973    | 1.17 (0.38–3.67)      | 0.784    |
| Tumour stage                     |                    |          |                       |          |
| Early (I/II)                     | 1.00               |          | 1.00                  |          |
| Late (III/IV)                    | 1.58 (0.77–3.26)   | 0.213    | 0.68 (0.21–2.26)      | 0.532    |
| Lymph node status                |                    |          |                       |          |
| N0                               | 1.00               |          | 1.00                  |          |
| ≥N1                              | 2.01 (0.99–4.10)   | 0.055    | 3.34 (1.02–10.92)     | 0.046    |

*Bold values indicate statistical significance ($P < 0.05$).
CI: confidence interval; HR: hazard ratio; IHC: immunohistochemistry; ADC: adenocarcinoma; SCC: squamous cell carcinoma.
Fig. 5C). In addition, the expression of c-MYC and cyclin D1 were induced in HBP1 knockdown A549 cells \((P = 0.045 \text{ and } P < 0.001, \text{ respectively}; \text{Fig. 5D})\). Altogether, our data suggested that HBP1 expression plays an important role in suppressing β-catenin transactivation in lung cancer.

**Discussion**

In an effort to better understand the mechanism of HBP1 alteration in NSCLC patients, we carried out a comprehensive molecular analysis including mRNA/protein expression and promoter methylation of HBP1 gene in 82 NSCLC patients. Our study unravels a new mechanism involved in HBP1 gene silencing by promoting hypermethylation and low activity in suppressing β-catenin transactivation. We also evaluated the prognostic effect of HBP1 alteration and β-catenin nuclear accumulation in NSCLC patients. Low expression of HBP1 may be the major determinant of prognosis in NSCLC patients with β-catenin nuclear accumulation (Fig. 6).

The tumour suppressor genes are known to be inactivated by genetic and epigenetic alterations [20]. HBP1 has been previously reported to be inactivated by mutations or gene deletion [13, 15–17]. However, our previous data showed that deletion at 7q is not frequently found in NSCLC patients [21], suggesting that other mechanisms may be involved in HBP1 inactivation in NSCLC. In the present study, clinical data suggested that promoter hypermethylation is involved in the deregulation of HBP1 gene. We further identified that reactivation of HBP1 by DNA demethylation indeed reduced β-catenin nuclear transactivation as evident by the low mRNA expression of several target genes of β-catenin. Our study provides a link for the transactivation of nuclear β-catenin through epigenetic inactivation of HBP1 in lung cancer.

Fig. 6). In addition, the expression of c-MYC and cyclin D1 were induced in HBP1 knockdown A549 cells \((P = 0.045 \text{ and } P < 0.001, \text{ respectively}; \text{Fig. 5D})\). Altogether, our data suggested that HBP1 expression plays an important role in suppressing β-catenin transactivation in lung cancer.

**Table 2** Hazard ratio for overall survival in the β-catenin group

| Characteristics                | HR (95% CI) | \(P\)-value* |
|--------------------------------|-------------|--------------|
| β-catenin nuclear accumulation |             |              |
| HBP1 IHC                        |             |              |
| Negative                        | 1.00        |              |
| Positive                        | 0.15 (0.04–0.60) | 0.007       |
| Tumour type                     |             |              |
| AD                             | 1.00        |              |
| SCC                            | 0.98 (0.30–3.20) | 0.970       |
| Tumour stage                    |             |              |
| Early (I/II)                    | 1.00        |              |
| Late (III/IV)                   | 1.09 (0.14–8.65) | 0.937       |
| Lymph node status               |             |              |
| N0                             | 1.00        |              |
| \(\geq N1\)                     | 2.20 (0.24–20.11) | 0.484       |
| β-catenin no nuclear accumulation |           |              |
| HBP1 IHC                        |             |              |
| Negative                        | 1.00        |              |
| Positive                        | 0.48 (0.14–1.59) | 0.228       |

*Bold values indicate statistical significance \((P < 0.05)\). CI: confidence interval; HR: hazard ratio; IHC: immunohistochemistry; ADC: adenocarcinoma; SCC: squamous cell carcinoma.

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These results suggested that HBP1 may participate in regulating DNA methylation in NSCLC cancer patients. Notably, our data showed that the accumulation of nuclear β-catenin was reduced in cells with 5'-Aza-dC treatment (Fig. 3D). The 5'-Aza-dC is a strong inducer of DNA hypomethylation that is well-known to resuscitate epigenetically silenced genes [24]. In our previous study, A549 cells showed promoter hypermethylation and low expression of AXIN2 gene, which encodes the component of β-catenin degradation complex. 5'-Aza-dC treatment successfully restored AXIN2 mRNA and protein expressions [11]. The reactivation of AXIN2 degradation complex may contribute in part to the substantial decrease in β-catenin protein expression that we observed in cells treated with 5-Aza-dC.

We found that low expression of HBP1 protein was significantly associated with poor survival in all NSCLC patients. We further performed the prognosis analyses of HBP1 mRNA expression in publicly available microarray data of NSCLC by the PrognoScan database (http://www.abren.net/PrognoScan/). As shown in Figure S2, the results support our conclusion that low HBP1 expression was associated with poor survival. These clinical data indicate the relevance of our finding which can be also seen in other cohorts of patients as part of publicly available microarray expression data [25, 26]. Notably, our Cox regression analysis revealed that low HBP1 expression was the major determinant of prognosis in NSCLC patients with β-catenin nuclear accumulation. These data suggested that low HBP1 expression in patients with β-catenin nuclear accumulation could be a useful prognostic factor in NSCLC.

Our clinical and cell model findings provide evidence that HBP1 expression can down-regulate β-catenin transactivation. Recently, Kim et al. found that epigallocatechin 3-gallate, the major phytochemical in green tea, blocks Wnt signalling by inducing the HBP1 transcriptional repressor and inhibits breast cancer tumorigenesis [27]. In addition, treatment with N-acetylcysteine, an anti-cancer
Fig. 4 Effect of ectopic expression of HBP1 on β-catenin transactivation in A549 cells. (A) RT-qPCR analysis and (B) Western blot analysis of HBP1 re-expression by ectopic expression of HBP1 in A549 cells. (C) Decreased β-catenin transactivation in A549 cells ectopically expressing HBP1. Cells expressing empty pCMV vector or HBP1 were transfected with the TOPFLASH reporter. (D) RT-qPCR analysis of the c-MYC and cyclin D1 mRNA level in cells ectopically expressing HBP1. *P*-value for each analysis is as indicated. Data represent mean ± SD from three independent experiments.

Fig. 5 Inverse correlation of HBP1 expression with β-catenin transactivation by using HBP1 knockdown in A549 cells. (A) RT-qPCR analysis and (B) Western blot analysis of siRNA-HBP1-induced gene knockdown in A549 cells. (C) Increased β-catenin transactivation in A549 cells after HBP1 knockdown. Control siRNA and siRNA-HBP1 cells were transfected with the TOPFLASH reporter. (D) RT-qPCR analysis of the c-MYC and cyclin D1 mRNA level in cells after HBP1 knockdown. *P*-value for each analysis is as indicated. Data represent mean ± SD from three independent experiments.
compound, suppresses cell growth by increasing the expression of HBP1, but HBP1 knockdown attenuated growth arrest and apoptosis in oral cancer [28]. Therefore, strategies to increase HBP1 expression may be useful for cancer prevention or treatment. The search for antagonists or agonists of the HBP1 may also lead to the discovery of compounds that can potentially be used for lung cancer treatment.

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Conflicts of interest

The authors declare that they have no competing interests.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 (a) Bar graph shows the percentage of expression of different proteins in NSCLC patients.

Figure S2 Low HBP1 mRNA expression correlates with poor survival of NSCLC patients in the publicly available microarray datasets in (a) jacob-00182-CANDF project [25] and (b) GSE31210 project [26]. Overall survival curve of Kaplan-Meier method was performed in patients with preserved (red lines) and low (blue lines) HBP1 mRNA expression. P values were determined using log-rank test.

Table S1 List of primer sequences used in the present study.

Table S2 Correlation between HBP1 or β-catenin expression and clinicopathological parameters of lung cancer.

Table S3 Correlation between HBP1 mRNA expression or promoter methylation and clinicopathological parameters of lung cancer.

Table S4 Correlation between HBP1 expression and promoter methylation of AXIN2, BTRCP and HIC1 genes in lung cancer patients.

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