Identification of Methylation-Driven, Differentially Expressed STXBP6 as a Novel Biomarker in Lung Adenocarcinoma

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DNA methylation is an essential epigenetic marker associated with the silencing of gene expression. Although various genome-wide studies revealed aberrantly methylated gene targets as molecular biomarkers for early detection, the survival rate of lung cancer patients is still poor. In order to identify methylation-driven biomarkers, genome-wide changes in DNA methylation and differential expression in 32 pairs of lung adenocarcinoma and adjacent normal lung tissue in non-smoking women were examined. This concurrent analysis identified 21 negatively correlated probes ($r \leq -0.5$), corresponding to 17 genes. Examining the endogenous expression in lung cancer cell lines, five of the genes were found to be significantly down-regulated. Furthermore, in tumor cells alone, 5-aza-2′-deoxycytidine treatment increased the expression levels of STXBP6 in a dose dependent manner and pyrosequencing showed higher percentage of methylation in STXBP6 promoter. Functional analysis revealed that overexpressed STXBP6 in A549 and H1299 cells significantly decreased cell proliferation, colony formation, and migration, and increased apoptosis. Finally, significantly lower survival rates ($P < 0.05$) were observed when expression levels of STXBP6 were low. Our results provide a basis for the genetic etiology of lung adenocarcinoma by demonstrating the possible role of hypermethylation of STXBP6 in poor clinical outcomes in lung cancer patients.

Lung carcinoma is one of the most commonly diagnosed cancer types, and it is characterized by poor survival rates. According to recent global cancer statistics, it accounts for 18% of all cancer-related deaths worldwide.1,3 Despite decades of research efforts to improve the clinical outcomes of lung cancer patients, the overall survival rates remain dismal. The mortality rates of lung cancer were the highest in Taiwan.2,3 A wide variety of risk factors, such as genetic, epigenetic, and environmental factors, may cause lung cancer. Nearly 70–90% of lung cancers in Western countries are caused due to cigarette smoking, but only 7% of female lung cancer cases are associated with smoking.4–6 Furthermore, adenocarcinoma is much more common than other subtypes of non-small cell lung carcinoma seen in non-smokers. These statistics emphasize the necessity of a better understanding of the molecular mechanisms that mediate the development of lung cancer in non-smoking female patients.

Advanced high-throughput technologies have had an important role in identifying the genetic abnormalities that drive the development and growth of various cancers.7,8 In addition to genetic changes, epigenetic changes, such as hyper- or hypomethylation, lead to the aberrant expression of tumor suppressor genes or oncogenes.9 Hypermethylation within the promoter region was responsible for the inactivation of approximately half of

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the classical tumor suppressor genes\textsuperscript{10,11}. Tumor suppressor genes undergoing aberrant hypermethylation were expressed in a non-random, tumor-specific pattern in many cancer types\textsuperscript{12}. Therefore, epigenetically disrupted gene expression was able to alter various cancer-related processes, such as cell cycle checkpoints, cell proliferation, apoptosis, signal transduction, regulation of transcription factors, cell adhesion, and angiogenesis\textsuperscript{13,14}. Also, various molecular genetics investigations revealed the impact of methylation on either resistance or sensitivity to chemotherapy or radiation\textsuperscript{15–19}. The possible role of DNA methylation in lung cancer was identified based on analysis of sputum\textsuperscript{20} and in prognosis of early-stage lung cancer\textsuperscript{21,22}. Unlike other genetic alterations, methylation-based epigenetic modification is an inherently reversible change, due to which it has gained much attention as an active target of drug development. Therefore, over the past few decades, several research groups have been focused on finding the epigenetic markers (e.g., APC\textsuperscript{23} and SHOX\textsuperscript{24,25}) or a group of gene set, such as (APC, RASSF1A, CDH13, KLK10 and DLEC1)\textsuperscript{26} and (AGTR1, GALR1, SLC5A8, ZMYND10 and NTSR1)\textsuperscript{27}, for detection or diagnosis of lung cancer. However, the role of methylation in the tumorigenesis of lung adenocarcinoma and association with prognosis in Taiwan remains largely unknown. For this reason, we performed an integrated analysis of gene expression and DNA methylation status to find novel epigenetic markers of lung cancer. With this approach, we identified STXBP6, whose expression was significantly repressed by methylation, affected cellular function in cancer cell lines, and was associated with overall survival.

Syntaxin binding protein 6, encoded by STXBP6, was initially identified in regulating the formation of the SNARE complex\textsuperscript{28} and cytogenesis\textsuperscript{29}. The regulatory role of STXBP6 in exocytosis and fusion pore stability was performed by both syntaxin-dependent and syntaxin-independent mechanisms\textsuperscript{30}. It has been reported to be associated with many diseases, such as diabetes\textsuperscript{31}, autism\textsuperscript{32,33}, and systemic lupus erythematosus\textsuperscript{34}. However, there are no studies revealing its biological role in association with lung cancer and its epigenetic regulation.

Therefore, in this study we explored the epigenetic inactivation of STXBP6 expression using lung adenocarcinoma patients. Pyrosequencing analysis using in vitro cellular models revealed the specific CpG sites that are responsible for the hypermethylation of STXBP6. Functional analysis revealed the tumor-suppressive role of STXBP6 in in vitro lung cancer cellular models. Finally, poor survival rates were observed in patients with low expression levels of STXBP6. Thus, methylation-driven, differentially expressed STXBP6 may be used as a novel biomarker to predict clinical outcomes of lung adenocarcinoma patients.

Results

Differential expression and methylation profiling in lung adenocarcinoma. In this genome-wide study, we sought to identify the genes whose expression was differentially regulated by DNA methylation in lung cancer cells. Genome-wide expression (41,789 probes) and DNA methylation profiling (27,578 probes) in 32 pairs of tumor and adjacent normal tissues were analyzed in non-smoking women with lung adenocarcinoma (Table S2). The average age of patients was 62 years old and 78% of them were in Stage I or II. To visualize the distribution of tumor and normal samples based on expression or methylation levels, principal component analyses was executed using differentially expressed probes of gene expression (Fig. 1A) and DNA methylation (Fig. 1B). Black dots denote tumor tissues, gray dots denote normal tissues, and each line indicates the paired samples from the same individual. Each dot represents the expression (Fig. 1A) or methylation values (Fig. 1B) of the same paired sample. The possible role of DNA methylation in lung cancer was identified based on analysis of sputum\textsuperscript{20} and in prognosis of early-stage lung cancer\textsuperscript{21,22}. Unlike other genetic alterations, methylation-based epigenetic modification is an inherently reversible change, due to which it has gained much attention as an active target of drug development. Therefore, over the past few decades, several research groups have been focused on finding the epigenetic markers (e.g., APC\textsuperscript{23} and SHOX\textsuperscript{24,25}) or a group of gene set, such as (APC, RASSF1A, CDH13, KLK10 and DLEC1)\textsuperscript{26} and (AGTR1, GALR1, SLC5A8, ZMYND10 and NTSR1)\textsuperscript{27}, for detection or diagnosis of lung cancer. However, the role of methylation in the tumorigenesis of lung adenocarcinoma and association with prognosis in Taiwan remains largely unknown. For this reason, we performed an integrated analysis of gene expression and DNA methylation status to find novel epigenetic markers of lung cancer. With this approach, we identified STXBP6, whose expression was significantly repressed by methylation, affected cellular function in cancer cell lines, and was associated with overall survival.

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Identification of methylation-driven down-regulated genes in lung cancer cell lines. To validate differentially expressed genes driven by methylation in non-smoking women with lung adenocarcinoma and select candidate genes for functional analysis, endogenous expression levels of the 17 genes were examined in lung cancer cell lines (A549 and H1299). Five genes, including IL11RA, GSTMS5, STXB6, RHOJ, and PECAM1, were significantly down-regulated (P ≤ 0.0001) in A549 and H1299 cells as compared to normal BEAS-2B cells (Figs 2A and S1A–D).

To validate the role of methylation in the regulation of the expression of these 5 genes, we treated A549, H1299, and BEAS-2B cell lines with 5-aza. Interestingly, only STXBP6, which expression was significantly up-regulated in a dose-dependent manner, was found when the A549 and H1299 cell lines were treated with 5-aza.
Figure 1. Identification of differentially expressed genes driven by methylation in non-smoking women with lung adenocarcinoma. (A) Principal component analysis of probes with differential gene expression in tumor and normal tissues. Differentially expressed probes were identified by paired t tests ($P \leq 10^{-6}$) and fold change ($\log_2 \geq 1$ or $\leq -1$) in tumor/normal lung tissues. Connecting lines indicate paired samples from the same individual. (B) Principal component analysis of probes with differential methylation status in tumor and normal tissues. The intensities of methylated probes versus unmethylated probes were converted to M values and examined by paired t tests ($P \leq 10^{-6}$). (C) Volcano plot of differentially expressed genes. Green dots indicate down-regulated probes ($n = 901$) and red dots denote up-regulated probes ($n = 307$). (D) Volcano plot of probes with differential DNA methylation status. Yellow dots denote hypomethylated probes ($n = 863$) and blue dots indicate hypermethylated probes ($n = 894$). (E) Heat map of the probes ($n = 273$) showing negative correlation ($r < 0$) between differential expression and methylation status. Hierarchical cluster analysis was performed using Pearson correlation distance. (F) Starburst plot of probes showing negative correlation between gene expression (y-axis) and DNA methylation (x-axis).
Table 1. Genes with negative correlation between gene expression and methylation. aProbes used for the gene expression profiling. bProbes used for the methylation analysis. cCorrelation between gene expression and methylation status. dDifferential median values of gene in tumor/normal tissue samples (log2 ratio). eStatistical significance of methylation examined by paired-t test. fStatistical significance of methylation examined by paired-t test.

Table: Genes with negative correlation between gene expression and methylation.

| Gene   | Exp. Probe ID | Methylated Probe ID | CpG Target No. | Corr. | Exp. Diff. (log2) | Beta Diff. (T vs N) | Tumor Beta value | Normal Beta value | Methyla-tion Diff. (M value) | P-value for Methyla-tion |
|--------|---------------|---------------------|----------------|-------|------------------|-------------------|------------------|---------------------|--------------------------|------------------------|
| AQPI   | 209047_at     | 4600                | cg04551925     | −0.63 | −1.83            | 2.10E-08          | 0.16             | 0.73                | 0.57                     | 1.03                    | 1.52E-07               |
| HABP2  | 206010_at     | 26621               | cg26656452     | −0.61 | 1.89             | 3.91E-07          | −0.23            | 0.58                | 0.81                     | −1.76                   | 1.33E-08               |
| SPDEF  | 220192_s_at   | 17272               | cg17240454     | −0.59 | 0.81             | 1.88E-08          | −0.19            | 0.55                | 0.74                     | −1.12                   | 2.64E-08               |
| EHF    | 232361_s_at   | 18421               | cg18414381     | −0.59 | 1.29             | 7.14E-07          | −0.17            | 0.28                | 0.45                     | −0.93                   | 1.52E-09               |
| HMGN1  | 200944_s_at   | 18826               | cg18829411     | −0.58 | 0.58             | 1.60E-08          | −0.16            | 0.31                | 0.47                     | −1.01                   | 4.89E-09               |
| SLC22A1| 204981_at     | 16913               | cg16873863     | −0.57 | 1.07             | 1.79E-07          | −0.23            | 0.40                | 0.63                     | −1.35                   | 1.90E-09               |
| ARHGEF19| 226857_at    | 18670               | cg18669385     | −0.56 | 1.20             | 7.74E-09          | −0.11            | 0.29                | 0.40                     | −0.69                   | 2.32E-07               |
| IL1RA  | 204773_at     | 3676                | cg03662459     | −0.56 | −0.74            | 8.98E-08          | 0.09             | 0.68                | 0.59                     | 0.59                    | 2.00E-07               |
| GSTM5  | 205752_s_at   | 5031                | cg14987894     | −0.55 | −0.69            | 1.57E-07          | 0.21             | 0.52                | 0.31                     | 1.04                    | 3.51E-11               |
| NIPSNAPI| 201709_s_at   | 13745               | cg13797031     | −0.50 | 0.70             | 2.14E-08          | −0.26            | 0.49                | 0.75                     | −1.79                   | 3.35E-09               |
| MESPI  | 224476_s_at   | 1105                | cg01091565     | −0.54 | 1.24             | 9.77E-07          | −0.18            | 0.62                | 0.80                     | −1.28                   | 1.72E-08               |
| EHF    | 232361_s_at   | 13011               | cg13084525     | −0.54 | 1.29             | 7.14E-07          | −0.08            | 0.20                | 0.28                     | −0.71                   | 1.36E-08               |
| CP     | 228143_at     | 17465               | cg17439694     | −0.53 | 2.57             | 2.65E-07          | −0.13            | 0.54                | 0.67                     | −0.91                   | 2.26E-07               |
| SPDEF  | 220192_s_at   | 7679                | cg07705908     | −0.53 | 1.88E-08         | 0.21             | 0.36             | 0.57                | −1.13                   | 1.90E-08               |
| HDHD3  | 221256_s_at   | 23967               | cg24012708     | −0.53 | 0.59             | 3.26E-07          | −0.15            | 0.44                | 0.59                     | −0.95                   | 1.53E-07               |
| DCC    | 238914_at     | 4292                | cg4272086      | −0.52 | −0.98            | 2.90E-09          | 0.13             | 0.20                | 0.07                     | 1.48                    | 1.21E-10               |
| STXB6  | 200994_s_at   | 6937                | cg09694294     | −0.52 | −2.07            | 8.79E-12          | 0.11             | 0.15                | 0.04                     | 1.62                    | 1.68E-08               |
| COX7A1 | 204570_at     | 42277               | cg24335895     | −0.52 | −1.27            | 7.66E-10          | 0.22             | 0.52                | 0.30                     | 1.44                    | 2.71E-11               |
| SPDEF  | 213441_s_at   | 17272               | cg17240454     | −0.52 | 0.53             | 5.98E-08          | −0.19            | 0.55                | 0.74                     | −1.12                   | 2.64E-08               |
| RHOG   | 243481_at     | 18774               | cg18771300     | −0.52 | −1.25            | 1.91E-10          | 0.21             | 0.52                | 0.31                     | 1.05                    | 1.20E-07               |
| CP     | 155803_s_at   | 17465               | cg17439694     | −0.51 | 2.52             | 3.28E-09          | −0.13            | 0.54                | 0.67                     | −0.91                   | 2.26E-07               |
| PECAM1 | 208981_at     | 3928                | cg03386110     | −0.50 | −1.49            | 1.19E-10          | 0.20             | 0.72                | 0.52                     | 1.07                    | 2.39E-11               |

Functional investigation of STXB6 in lung cancer cell lines. Since STXB6 was epigenetically down-regulated in lung cancer cells, we investigated the functional roles of STXB6 by transiently transfecting a STXB6 expression plasmid into A549 and H1299 cells. As shown in Fig. 3A, the mRNA levels of STXB6 in A549 and H1299 cells after transfection were significantly increased (P < 0.0001). Western blot analysis also validated the increased protein amounts of STXB6 in both A549 and H1299 cells upon transfection of STXB6 plasmid (Fig. 3B).

After successfully overexpressing STXB6 in lung cancer cells, we first examined the effect of STXB6 on cell growth by MTT assays. The results showed a significant decrement in proliferation in both A549 and H1299 cells overexpressing STXB6 (P < 0.05) (Fig. 3C,D). Furthermore, STXB6 overexpression markedly reduced colony formation in both A549 and H1299 cells (Fig. 3E,F). Next, the role of STXB6 in migration of lung cancer cell lines was investigated by transwell migration assays. The results revealed that STXB6 significantly suppressed the migration abilities of both A549 and H1299 cells (P < 0.001) (Fig. 4A–D).

To evaluate the possible significance of STXB6 expression in the modulation of apoptosis, annexin V-FITC and PI staining were carried out. The results showed a noticeable increment in apoptotic percentage in both A549 and H1299 cells overexpressing STXB6 (P < 0.05) (Fig. 5A–D). Furthermore, cell cycle analysis was performed on day 3 of transfection with the STXB6 plasmid. The percentage of apoptotic cells in G1 phase was significantly increased in both A549 and H1299 cells overexpressing STXB6 (P < 0.001) (Fig. 5E–H). These results demonstrated the suppressive effects of STXB6 on lung carcinogenesis.
Expression levels of STXBP6 in tumor tissues correlate with patients’ overall survival. Lastly, Kaplan–Meier analysis was used to examine overall survival in relation to the expression values of STXBP6 in adenocarcinoma patients from Shedden’s study35 (Fig. 6A) and Tomida’s study36 (Fig. 6B). Patients were divided into “high expression” or “low expression” groups based on the median value of all samples. The results showed
that patients with lower expression of \textit{STXBP6} had poorer survival than those with high expression (Fig. 6A,B). These findings indicate that epigenetic changes in \textit{STXBP6} may be useful for predicting the prognosis of patients with lung adenocarcinoma.

**Discussion**

In this study, we sought to uncover epigenetic-based molecular targets by analyzing the association between genome-wide DNA methylation and gene expression patterns in tumor and adjacent normal tissues from non-smoking Taiwanese female lung adenocarcinoma patients. First, differential expression levels and methylation status between tumor and normal tissues were identified using both gene expression and methylation microarrays. Second, selected genes with negative correlations between expression levels and methylation status were then validated by examining their endogenous levels in lung cancer cell lines. Third, pyrosequencing and 5-aza-2'-deoxycytidine treatment showed the regulatory role of methylation of \textit{STXBP6} in tumor cells. Fourth, functional analysis revealed that \textit{STXBP6} suppressed tumor growth in lung cancer cell lines. Finally, lower expression of \textit{STXBP6} was found to be associated with poor clinical outcomes in lung cancer patients.

Because of the development of targeted therapy resistance or the absence of targetable mutations in lung cancer patients, developing alternative therapeutic strategies for lung cancer in early diagnosis, prognosis prediction,
and treatment are urgent and important. Epigenetics approaches, including DNA methylation, histone modification, and miRNA regulation, may solve these problems by affecting multiple pathways that regulate major properties of the cancer cells. Among them, DNA methylation at CpG sites is the most characterized epigenetic modification described in lung cancer. Therefore, targeting DNA methylation of tumor suppressor genes or onco-genes may hold promise in lung cancer therapy.

For the last several years, more and more evidence has accumulated to emphasize the hypermethylation status of CpG islands located in the promoter regions of tumor suppressor genes. Several groups have performed epigenetic analyses of methylation in types of cancer other than lung adenocarcinoma. A recent genome-wide analysis of DNA methylation and gene expression changes in lung squamous cell carcinoma identified several methylation-driven genes, including CCDC37, CYTL1, CDO1, SLIT2, LMO3 and SERPINB5. Another genomic analysis of idiopathic pulmonary fibrosis identified methylation–gene expression relationships within genes that were either involved in fibroproliferation or were feasible candidates in this process. Suzuki et al. performed an integrative multi-omics analysis to understand how cancers harbor various types of aberrations at the genomic, epigenomic, and transcriptional levels. Additional investigations using larger numbers of samples with varied clinical features can help to reveal novel gene targets associated with methylation changes in tumorigenesis. Further research direction may also include the timing of methylation and the difference in methylation levels between epithelial and stromal tissues. In addition, more studies should focus on finding markers for epigenetic priming agents that render lung cancer more susceptible to cytotoxic chemotherapy and immunotherapy.

Worsening lung cancer statistics (e.g., increasing global incidence), particularly in women, invoke researchers to develop accurate and highly sensitive markers for the early detection of disease. Several biomarkers for the diagnosis of lung cancer have been identified; however, sensitivities of these biomarkers differ for each subtype of lung cancer. Thus, it is also highly challenging to find specific biomarkers for each subtype, as the various lung cancers are known to have diverse pathological features. Hence, we set out to find suitable biomarker genes for adenocarcinoma, to distinguish it from normal samples. To meet this expectation, we processed our data using a stringent cutoff for the negative correlation between gene expression and DNA methylation in non-smoking women with lung adenocarcinoma. From the genome-wide analysis, 167 methylation-driven, differentially expressed genes (273 probes) were identified in lung adenocarcinoma. In spite of differences in clinical features of tumor samples and selection criteria, previous genome-wide methylation studies showed results similar to our study. For instance, our hierarchical clustering analysis also resulted in a large cluster for most of the probes, which are hypermethylated and down-regulated. The current findings along with previous findings indicate that a larger number of genes may undergo hypermethylation in the case of lung carcinogenesis.

Interestingly, differential expression of some of the candidate genes in our study was in agreement with previous studies. For example, CDKN3 was found to be overexpressed in hepatocellular carcinoma and to promote cell proliferation by affecting cell cycle progression, and was also found to be overexpressed in this study. To the best of our knowledge, this is the first study reporting the regulatory role of methylation in the control of STXBP6 in lung cancer with different validation approaches, including microarrays and 5-aza and pyrosequencing analyses. Moreover, STXBP6 was significantly down-regulated or hypermethylated in many public data set (Table 2). Furthermore, negative correlation between gene expression and methylation status for STXBP6 was observed in other three publicly available datasets (Table 2), indicating the possibility of epigenetic inactivation of STXBP6 even in different races. The possible role of methylation in the control of STXBP6 expression was also suggested.
These results indicate that epigenetic silencing of STXBP6 could occur in different cancer types. Administration of methylation inhibitors, such as 5-aza, is one of the most commonly used strategies to uncover the role of aberrant methylation changes in gene inactivation. When cells were treated with 5-aza, up-regulation of STXBP6 was observed only in cancerous cell lines. Pyrosequencing results further identified the methylated CpG sites modulating the expression of STXBP6 in these cell lines.

To investigate the functional roles of STXBP6, it was overexpressed in A549 and H1299 cancer cells. Ectopic expression of STXBP6 resulted in slower cell proliferation, less colony formation, slower migration ability, and a greater percentage of apoptosis in lung cancer cells. These results suggested that STXBP6 could function as a tumor suppressor, although further experiments are warranted using in vivo studies.

Lastly, survival analysis using two publicly available datasets indicated that the survival probability of lung cancer patients increased with higher expression of STXBP6 in tumor tissues. This result suggested an avenue for developing a novel therapeutic regimen for treating lung cancer.

Figure 5. STXBP6 increases apoptosis in lung cancer cells. (A) A representative diagram of annexin V-FITC and propidium iodide (PI) staining assays in A549 cells overexpressing STXBP6. (B) Quantification of annexin V results in (A). The percentage of apoptotic cells was derived from the sum of the percentages of late and early apoptotic cells. (C) A representative diagram of annexin V-FITC and PI staining assays in H1299 cells overexpressing STXBP6. (D) Quantification of annexin V-FITC results in (C). (E) Flow cytometry analysis for apoptosis in A549 cells overexpressing STXBP6. (F) Quantification of apoptotic cells in (E). The percentage of cells in sub-G1 phase was used to identify the apoptotic cells by PI staining. Bars represent the means ± SDs of 3 independent experiments. (G) Flow cytometry analysis for apoptosis in H1299 cells overexpressing STXBP6. (H) Quantification of apoptotic cells in (G). *P < 0.05, **P < 0.001.
In conclusion, our results indicate that the pathogenesis of lung adenocarcinoma may result from epigenetically regulated expression levels of \( \text{STXBP6} \). Before this biomarker can be translated into clinical utility, further studies using larger sample sizes will help to reveal the importance of \( \text{STXBP6} \) as a novel potential biomarker for the prognosis of lung adenocarcinoma. Multicenter studies are also needed to validate the tests and analyze the reproducibility of promising results derived from limited samples.

**Methods**

**Clinical tissue samples.** Thirty-two pairs of lung adenocarcinoma and adjacent normal lung tissue samples were acquired from non-smoking female patients admitted to National Taiwan University Hospital or Taichung Veterans General Hospital. Written informed consent was obtained from all subjects and/or guardians for the use of their tissue samples. Acquisition and subsequent use of all the clinical samples were in accordance with the Declaration of Helsinki, and were approved by the Institutional Review Board of National Taiwan University Hospital (IRB approval Number: 200610015 R) and the Institutional Review Board of Taichung Veterans General Hospital (IRB approval Number: C09204). Lung tissue samples were quickly immersed in RNAlater® solution (Life Technologies, Gaithersburg, MD, USA), snap-frozen in liquid nitrogen, and stored at \(-80^\circ\text{C}\). Before the extraction of RNA and DNA, frozen tumor tissue blocks were sectioned.

**RNA extraction and cDNA synthesis.** Total RNA from sectioned tissue samples was isolated using TRIzol reagent (Life Technologies, Gaithersburg, MD, USA) and purified with the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. RNA integrity was confirmed by agarose gel electrophoresis and the Agilent 2100 Bioanalyzer RNA 6000 LabChip kit (Agilent Technologies, Santa Clara, CA, USA). The purified total RNAs were then used as templates to synthesize the labeled double-stranded cDNA and cRNA according to the Affymetrix standard synthesis protocols.

**Genomic DNA isolation, bisulfite treatment, and methylation profiling.** Genomic DNA from tumor and adjacent normal tissue samples was extracted using TRIzol reagent (Life Technologies, Gaithersburg, MD, USA). The DNA was then subjected to bisulfite conversion using an EZ DNA methylation kit (Zymo Research, Orange, CA, USA). In the bisulfite reaction, the samples were cycled 16 times for 30 sec at 95°C and 1 h at 50°C. Then, bisulfite-converted DNA was used for methylation microarrays.

**Gene expression and methylation profiling.** mRNA expression profiling was performed by Human Genome U133 plus 2.0 arrays (Affymetrix, Inc., Santa Clara, CA, USA) based on reverse transcription and probe hybridization. This platform contains 41,789 probes. Gene expression levels were detected by relative fluorescence intensity. The expression array data of this study have been submitted to the Gene Expression Omnibus database (accession number GSE19804).

To identify the DNA methylation status of 27,578 CpG sites, the Illumina Infinium Human Methylation27 beadchip (Illumina, San Diego, CA, USA) was used. The accession number for the methylation array data set in the Gene Expression Omnibus database is GSE49996. The methylation levels (beta values) of a given gene were determined by the ratio of the methylated probe intensity to the overall probe intensity of that gene. Methylation beta values were then converted to an M-value through a logistic transformation and expressed as the log$_2$ ratio of the intensities of methylated probe versus unmethylated probe. The M-value for the $i$th interrogated CpG site is defined as:

$$M_i = \log_2 \left( \frac{Beta_i}{1 - Beta_i} \right)$$
Furthermore, two-dimensional principal component analyses were used for a visual representation of differential expression patterns between tumor and normal samples. Next, hierarchical cluster analysis using Pearson correlation distances was executed to group the probes with similar expression and methylation profiles. Then, differentially expressed and methylated probes were analyzed by paired t-tests ($P < 10^{-5}$). Genes with differential expression between tumor and adjacent normal tissues were further filtered by at least 2-fold changes. A negative correlation coefficient ($r < 0$) was used to identify a converse relationship between gene expression and methylation status. A stringent correlation coefficient, defined as $r \leq -0.5$, was used for selecting genes for further validation in in vitro cell models.

Cell culture. Cancerous lung cells (A549 and H1299) and normal lung cells (BEAS-2B) were cultured in RPMI medium 1640 (GIBCO, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; Biological Industries, Beit-Haemek, Israel) and 1% antibiotics, including puromycin and streptomycin (Biological Industries, Beit-Haemek, Israel), at 37 °C in a humidified atmosphere with 5% CO$_2$. To validate the role of methylation in regulating the expression of target genes, A549, H1299, and BEAS-2B cells were seeded on a 6-well plate. After 24 h, the cell lines were treated with 5 and 10$\mu$M of 5-aza-2'-deoxycytidine (5-aza) (Sigma Chemical Company, St. Louis, MO, USA). Expression values of target genes were analyzed 3 days after treatment.

Quantitative reverse transcription PCR. The quality and quantity of the RNA were measured by NanoDrop™ 2000 (Thermo Scientific™, USA). One µg of total RNA from each cell line was reverse transcribed by the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Gaithersburg, MD, USA). The final cDNA products were used as the templates for subsequent real time PCR (RT-PCR). RT-PCR was performed with SYBR Green (Roche, Germany) on an ABI 7900 system (Life Technologies, Gaithersburg, MD, USA) according to standard protocols. The primers used in this study are listed in Table S1. All individual experiments were carried out in triplicate, and data were normalized using GAPDH as the loading control. The statistical significance of gene expression in different samples was identified by the t-test calculator in GraphPad Prism 5 (GraphPad Software, Inc., CA, USA).

Pyrosequencing assay. To quantify the methylation levels of multiple CpG sites in the 5' untranslated region of STXB6, a predesigned PyroMark CpG Assay was used (Hs_STXB6_01_PM PyroMark CpG assay, PM00057414, Qiagen, Venlo, Netherlands). Specific CpG sites including Chr14: 25518720, 25518735, 25518737, 25518743, and 25518748 were examined.

Overexpression of STXB6 in lung cancer cells. STXB6 was overexpressed in A549 and H1299 cells to evaluate its functional significance. Full-length STXB6 cDNA with a C-terminal Myc-DDK tag was inserted into the pCMV6-Entry mammalian vector (OriGene Technologies, Rockville, MD, USA). The pCMV6-Entry-Myc-STXB6 vector and an empty vector were transiently transfected into A549 and H1299 cell lines using TransIT-2020 transfection reagent (MirusBio, Madison, WI, USA) according to the manufacturer’s instruction. All sequences, including STXB6 in the vector, were verified by Sanger sequencing (the first core laboratory, College of Medicine, National Taiwan University). mRNA levels were quantified by quantitative RT-PCR using STXB6-specific primers (F-5'-GTCCTATCTTACTGGCCAGCG-3' and R-5'-GTTAAATGCCTTGTGAGCCCTC'-3'), and protein levels were examined by western blotting.

Western blot. Total cell lysates were prepared and proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins in the gel were then electrotransferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% milk and were incubated with monoclonal anti-FLAG antibody (Sigma-Aldrich, St. Louis, MO, USA) or anti-GAPDH antibody (Sigma-Aldrich, St. Louis, MO, USA) overnight. After washing, the bound primary antibodies on the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG or rabbit anti-mouse IgG (GeneTex, Irvine, CA, USA). Finally, the blots were developed with a chemiluminescent western blotting system (Millipore, Billerica, MA, USA).

Cell proliferation assay. A549 and H1299 cells were seeded into 96-well plates in triplicate and incubated for 12 h at 37 °C in a CO$_2$ incubator. Next, all cells in 96-well plates were divided into groups and transfected with STXB6 plasmid or mock vectors. At different time points (24, 48, and 72 h) of transfection, proliferative activities were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (EMD Biosciences, La Jolla, CA, USA) assay using a microtitrator plate reader (BioTek, Winooski, VT, USA) at 570 nm. The absorbance of A549 and H1299 cells was measured.

Colony formation assay. Cells were seeded in 6-well plates and incubated overnight. The adherent cells were transfected with STXB6 plasmid or mock vector. After two weeks of incubation, cells were fixed using 3:1 methanol-acetic acid and stained using 0.1% crystal violet. Finally, the dried plates were used for image acquisition with a digital camera.

Cell migration assay. Migration assays were carried out using 24-well transwell units (Corning, NY, USA). The upper chamber of each transwell unit was loaded with 4 × 10$^5$ cells/well in 0.2 mL serum-free RPMI medium and the lower chambers contained 0.6 mL of RPMI with 10% FBS as chemoattractant. Cells were then incubated for 24 h at 37 °C. Then a methanol-acetic acid (3:1) mixture was added into the lower chambers to fix the cells for 20 min at room temperature, followed by staining with 0.1% crystal violet for another 20 min. Cells on the upper side of the membrane surface were removed by scraping with a cotton swab, and the cells that passed through
the filter were destained using 10% acetic acid. The absorbance was measured at 570 nm with an ELISA reader (BioTek, Winooski, VT, USA). Images of the bottom surface of the transwell migration chambers were captured at 10X magnification before destaining.

**Apoptosis assay.** In order to perform the annexin V-FITC and propidium iodide (PI) double staining assay, cells were trypsinized, washed with phosphate-buffered saline (PBS), and resuspended in 500 μL of 1X binding buffer (Becton Dickinson, NJ, USA). Thereafter, cells were stained using 10 μL of Annexin V (5 μL) and PI (5 μL) mix (Becton Dickinson, NJ, USA) for 15 min. The suspension was passed through a nylon mesh filter and analyzed using a Beckman Coulter FC500 (Beckman, Brea, CA, USA) and CXP analysis software.

**Cell cycle analysis.** Initially, cells were trypsinized, washed with PBS, and fixed with cold 100% ethanol at −20 °C overnight. Thereafter, cells were washed twice and resuspended in PBS containing 20 μg/mL PI (Life Technologies, NY, USA), 0.1% triton-X-100 (Sigma, St. Louis, MO, United States), and 100 μg/mL RNase A (Sigma, St. Louis, MO, United States) for 30 min. The suspension was passed through a nylon mesh filter and analyzed using a Beckman Coulter FC500 (Beckman, Brea, CA, USA) and CXP analysis software.

**Survival analysis.** The gene expression signatures from GSE684655 and GSE132136 were used to elucidate the prognostic roles of STXBP6 in lung adenocarcinoma patients. Patients were categorized as “STXBP6 High” if their RNA expression levels of STXBP6 were higher than the median expression in all samples, and as “STXBP6 Low” if their RNA expression levels of STXBP6 were lower than the median expression in all samples. The association between gene expression and overall survival (up to 100 months) of lung adenocarcinoma patients was examined using Kaplan-Meier survival analysis. The statistical significance of the relationship between gene expression and survival was examined by a log-rank test.

**Statistical analysis.** Data were expressed as the means ± SDs from at least three independent experiments. The statistical significance of gene expression in different samples was identified by a t-test calculator in GraphPad Prism 5 (GraphPad Software, Inc., CA, USA). P-values less than 0.05 were considered significant.

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**Author Contributions**

G.L., M.-H.T., L.-C.L. and E.Y.C. conceived and designed the experiments. G.L. and H.-C.L. performed the experiments. G.L., J.-H.H., Y.-C.L. and T.-P.L. analyzed the data. M.-H.T., E.Y.C., J.-M.L., C.-P.H. and L.-C.L.
contributed reagents, materials and/or analysis tools. G.L., H.-C.L. and L.-C.L. wrote the paper. All authors reviewed the manuscript.

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