Hypermethylation of the PTTG1IP promoter leads to low expression in early-stage non-small cell lung cancer

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Abstract. Despite the clinical requirement for early diagnosis, the early events in lung cancer and their mechanisms are not fully understood. Pituitary tumor transforming gene 1 binding factor (PTTG1IP) is a tumor-associated gene; however, to the best of our knowledge, its association with lung cancer has not been reported. The present study analyzed PTTG1IP expression in early-stage non-small cell lung cancer (NSCLC) samples and investigated its epigenetic regulatory mechanisms. The results revealed that the mRNA level of PTTG1IP in NSCLC tissues was significantly downregulated by 43% compared with that in adjacent tissues. In addition, overexpression of this gene significantly inhibited cell proliferation. According to data from The Cancer Genome Atlas, a significant negative correlation was identified between the PTTG1IP gene methylation level and expression level in lung adenocarcinoma and lung squamous cell carcinoma cases. Reduced representation bisulfite sequencing (RRBS) analysis of six paired early-stage NSCLC tissue samples indicated that the CpG island shore of the PTTG1IP promoter is hypermethylated in lung cancer tissues, which was further validated in 12 paired early-stage NSCLC samples via bisulfite amplicon sequencing. Following treatment with 5-aza-2'-deoxycytidine to reduce DNA methylation in the promoter region, the PTTG1IP mRNA level increased, indicating that the PTTG1IP promoter DNA methylation level negatively regulates PTTG1IP transcription. In conclusion, in early-stage NSCLC, the PTTG1IP gene is regulated by DNA methylation in its promoter region, which may participate in the development and progression of lung cancer.

Introduction

Lung cancer, a complex disease involving both epigenetic and genetic changes, is the leading cause of cancer-associated mortality worldwide (1,2). Lung cancer has had a high incidence rate and a poor 5-year survival rate of <19% in the United States between 2006 and 2012 (2). One cause of the high mortality rate is the lack of specific early detection methods and the majority of patients are diagnosed with middle- or late-stage disease (3). Therefore, early detection and treatment strategies for lung cancer are urgently required.

Several imaging and cytology-based strategies have been utilized for early lung cancer detection. However, none have been demonstrated to completely reduce lung cancer mortality (3-5). Previous studies have reported that aberrant epigenetic changes are one of the most frequent cancer-associated events and are regarded as important mechanisms in carcinogenesis (6). Investigation of the associated molecular mechanisms can be exploited to diagnose early-stage lung cancer (3-5). Furthermore, methylation profiles may be potential biomarkers for early cancer diagnosis and they have been demonstrated to exhibit good prognostic value (4,7-9). Previously, accumulating evidence has confirmed that tumor tissues can be characterized by hypermethylation at promoter-associated CpG islands (CGIs) or global hypomethylation of the genome compared with normal tissues (9-11). Furthermore, certain studies have suggested that methylation of DNA CpG sites is an epigenetic regulator of gene expression that usually results in gene silencing (12,13). Hao et al (12) reported that methylation patterns can predict prognosis and survival, and identified an association between differential methylation of CpG sites and the expression of cancer-associated genes. Their findings demonstrate the utility...
of methylation biomarkers for cancer molecular characterization, diagnosis and prognosis determination. Therefore, a number of specific tumor targets can be developed for use as DNA methylation-based biomarkers (4,7,9).

At present, numerous useful cancer biomarkers have been identified. Pituitary tumor transforming gene 1 binding factor (PTTG1IP; also termed PBF) is a ubiquitously expressed proto-oncogene. PTTG1IP was first identified through its ability to bind to human securin, also termed pituitary tumor transforming gene (PTTG) (14,15). Thus far, PTTG1IP has been reported to be highly expressed in thyroid, breast, colorectal, and liver cancer (16-19). However, to the best of our knowledge, its expression levels in lung cancer have not been reported. The present study investigated PTTG1IP expression in early non-small cell lung cancer (NSCLC) and examined the correlation between the PTTG1IP promoter region methylation level and the gene expression level.

Materials and methods

Tissue samples. In total, 18 pairs of early-stage (stage I or II) NSCLC tissues and adjacent tissues were obtained from the South Hospital of Renji Hospital Shanghai Jiao Tong University School of Medicine (Shanghai, China) between January 2014 and March 2015 (Table I). A total of 12 male and 6 female patients aged between 45 and 75 years were included in the present study. During excision surgery, 50 mg fresh cancer tissue and 50 mg adjacent normal tissue (<2 cm from cancer margin) was obtained from each patient. Tissue samples were immediately frozen in liquid nitrogen following resection and stored at -80°C until use. All included samples were histologically confirmed primary NSCLC and pathological stage I or II according to the Tumor-Node-Metastasis staging system (20). Written informed consent was obtained from all patients and the study was approved by the Ethics Committee of South Hospital of Renji Hospital Shanghai Jiao Tong University School of Medicine.

Cell culture and treatments. A549 cells of the human lung adenocarcinoma cell line were cultured in Roswell Park Memorial Institute-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin/streptomycin. MRC5 cells of the human embryonic lung fibroblast cell line were cultured in minimum essential medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% FBS and 1% penicillin/streptomycin. Cell cultures were incubated at 37°C in a humidified atmosphere with 5% CO2. Following A549 cell culture to ~90% confluence, 1 µM DMSO (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), the corresponding sequences were subcloned into a pcDNA3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) that was subsequently termed pcDNA3.1/3Xmyc-PTTG1IP. Cells were plated the day prior to transfection and cultured to 70% confluence. Transfection was performed with Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 1 µg plasmid/well, according to the manufacturer’s protocol. Cells transfected with empty pcDNA3.1 vector was used as a control. The medium was replaced with new culture medium 6 h post-transfection. Cells were harvested 48 h following transfection and then prepared for subsequent assays.

Cell proliferation assay. Cell proliferation was assessed by Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) assays. Cells were seeded at 1,000 cells/well into 96-well plates with 100 µl culture medium. Subsequently, 10 µl CCK-8 solution was added to the cells at every 24 h for 5 days and the cells were incubated for 2 h at 37°C. The reaction product was quantified according to the manufacturer’s protocol by measuring the absorbance at 450 nm.

DNA and RNA extraction. Total RNA was extracted from cultured cells and tissue samples using TRIzol® reagent (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), according to the manufacturer’s protocol. Genomic DNA was extracted from cultured cells and tissue samples using a High Pure PCR Template Preparation kit (Roche Diagnostics, Basel, Switzerland), according to the manufacturer’s protocol.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT was performed with a mix of oligo dT primer and random primers for mRNA using a PrimeScript RT Reagent kit (Takara Biotechnology Co., Ltd., Dalian, China). qPCR was performed using a CFX96 Real-Time PCR detector (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and SYBR Premix Ex Taq™ (Takara Biotechnology Co., Ltd., Dalian, China). The thermocycling conditions were: 95°C for 2 min; 40 cycles of 95°C for 20 sec, 60°C for 20 sec and 72°C for 20 sec. The comparative 2−ΔΔCq method was used to calculate fold changes (21). GAPDH was used as an endogenous reference. The primers used for qPCR were as follows: PTTG1IP forward, 5′-GTCTTGAGTCCCTACCAAGC-3′ and reverse, 5′-CGCTTCAAGGGATCCCA-3′; GAPDH forward, 5′-GGAGTCCACTGGGCTTTC-3′ and reverse, 5′-GCTGTGATGTCTTGGAGGTGTTG-3′. The experiment was performed in triplicate.

Reduced representation bisulfite sequencing (RRBS). Genomic DNA was used to perform RRBS. RRBS library construction was performed as described previously (22). The library was sequenced on a next-generation sequencing (NGS) HiSeq platform (Illumina, Inc., San Diego, CA, USA). The sequencing data were aligned to a reference genome (UCSC
Table I. Basic information of the paired lung cancer tissue and adjacent tissue samples.

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pair 1^c</td>
<td>Female</td>
<td>Lung adenocarcinoma</td>
<td>II</td>
</tr>
<tr>
<td>Pair 2^e</td>
<td>Male</td>
<td>Lung squamous cell carcinoma</td>
<td>II</td>
</tr>
<tr>
<td>Pair 3^e</td>
<td>Female</td>
<td>Lung adenocarcinoma</td>
<td>II</td>
</tr>
<tr>
<td>Pair 4^e</td>
<td>Male</td>
<td>Lung squamous cell carcinoma</td>
<td>II</td>
</tr>
<tr>
<td>Pair 5^e</td>
<td>Male</td>
<td>Lung squamous cell carcinoma</td>
<td>II</td>
</tr>
<tr>
<td>Pair 6^e</td>
<td>Female</td>
<td>Lung adenocarcinoma</td>
<td>I</td>
</tr>
<tr>
<td>Pair 7^e</td>
<td>Male</td>
<td>Lung adenocarcinoma</td>
<td>II</td>
</tr>
<tr>
<td>Pair 8^e</td>
<td>Female</td>
<td>Lung adenocarcinoma</td>
<td>II</td>
</tr>
<tr>
<td>Pair 9^e</td>
<td>Male</td>
<td>Lung adenocarcinoma</td>
<td>II</td>
</tr>
<tr>
<td>Pair 10^e</td>
<td>Male</td>
<td>Lung adenocarcinoma</td>
<td>II</td>
</tr>
<tr>
<td>Pair 11^e</td>
<td>Male</td>
<td>Lung adenocarcinoma</td>
<td>II</td>
</tr>
<tr>
<td>Pair 12^e</td>
<td>Male</td>
<td>Lung adenocarcinoma</td>
<td>I</td>
</tr>
<tr>
<td>Pair 13^b</td>
<td>Male</td>
<td>Lung adenocarcinoma</td>
<td>I</td>
</tr>
<tr>
<td>Pair 14^b</td>
<td>Male</td>
<td>Lung adenocarcinoma</td>
<td>II</td>
</tr>
<tr>
<td>Pair 15^b</td>
<td>Male</td>
<td>Lung squamous cell carcinoma</td>
<td>II</td>
</tr>
<tr>
<td>Pair 16^b</td>
<td>Female</td>
<td>Lung adenocarcinoma</td>
<td>I</td>
</tr>
<tr>
<td>Pair 17^b</td>
<td>Male</td>
<td>Lung adenocarcinoma</td>
<td>I</td>
</tr>
<tr>
<td>Pair 18^b</td>
<td>Female</td>
<td>Lung adenocarcinoma</td>
<td>II</td>
</tr>
</tbody>
</table>

^a Analyzed by reverse transcription-quantitative polymerase chain reaction. ^b Analyzed by reduced representation bisulfite sequencing. ^c Analyzed by bisulfite amplicon sequencing. ^d According to the Tumor-Node-Metastasis staging system (20).

Results

Decreased PTTG1IP expression in early-stage non-small cell lung cancer. Although PTTG1IP has been reported to be abnormally expressed in a variety of tumor types (17-19,25), to the best of our knowledge, its association with lung cancer remains to be reported. The present study analyzed PTTG1IP expression in 10 paired early-stage NSCLC tissue samples (Table I). The RT-qPCR results revealed that PTTG1IP expression was decreased in all the cancer tissues except sample pair 10 (Fig. 1A). The mean mRNA level in the lung cancer tissues was significantly lower compared with that in the adjacent tissues (Fig. 1B). PTTG1IP expression was reduced by 80.1% in the lung cancer cell line A549 compared with the normal lung cell line MRC5 (Fig. 1C). To evaluate whether the expression level of PTTG1IP is associated with the proliferation capacity of lung cancer cells, PTTG1IP was overexpressed in A549 cells. The expression level of PTTG1IP was ~11 times higher in cells transfected with pcDNA3.1/3Xmyc-PTTG1IP compared with those transfected with empty pcDNA3.1 vector 2 days after transfection (Fig. 1D). A cell proliferation assay revealed that
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The proliferation of pcDNA3.1/3Xmyc-PTTG1IP-transfected cells was significantly inhibited compared with the control cells. By day five, the number of transfected cells was <50% of that in the control group (Fig. 1E).

DNA methylation analysis of the PTTG1IP promoter. To investigate the regulatory mechanism driving the decreased expression of PTTG1IP in lung cancer, the present study first downloaded RNAseq data and DNA methylation chip 450k data of lung adenocarcinoma and lung squamous cell carcinoma from The Cancer Genome Atlas (TCGA) database on the cBioportal website (www.cbioportal.org/). Correlation analysis revealed a significant negative correlation between the PTTG1IP gene methylation level and mRNA level in both lung adenocarcinoma and lung squamous cell carcinoma, with Spearman correlation coefficients of -0.415 and -0.457, respectively (Fig. 2).

To further determine the association between PTTG1IP promoter methylation and gene expression, an RRBS study was conducted with six pairs of early-stage NSCLC tissue samples (Table I). As presented in Fig. 3, a plurality of CGIs were distributed among the 2,000 bp upstream and the
2,000 bp downstream of the *PTTG1IP* transcription start site (TSS). However, regional DNA methylation analysis demonstrated that the region from 2,000 bp upstream to 1,000 bp downstream of the TSS was hypomethylated both in tumor tissues and adjacent tissues, although CpG loci were very concentrated in this region. However, a difference was identified in the CGI shore region of 1,000‑2,000 bp downstream of the TSS between lung cancer tissues and adjacent tissues, with a mean DNA methylation difference of 50%. In the region 5,000‑6,000 bp downstream of the TSS, DNA was hypermethylated and the methylation level in cancer tissues was higher compared with that in adjacent tissues. Therefore, hypermethylation of the CGI shore region within the *PTTG1IP* gene promoter might be associated with its low expression.

**DNA methylation level of the CGI shore region within the PTTG1IP gene promoter is associated with PTTG1IP expression.** Subsequently, the methylation level of a fragment composed of four CG sites in the CGI shore region within the *PTTG1IP* promoter was measured in 12 pairs of early-stage NSCLC samples using bisulfite amplicon sequencing (Fig. 4A). Hypermethylation was identified in >50% of the cancer tissues in the sample pairs. As presented in Fig. 4B, the mean methylation level of the four CG loci in tumor tissues was higher compared with that in adjacent tissues.
cancerous tissues. The mean methylation level of the four CG loci was 22.6 and 18.0%, respectively, and the difference was significant. The trend of these results was consistent with that observed in the RRBS analysis. To verify the association between DNA methylation and gene expression in this region, A549 cells were treated with 5-aza-2’-deoxycytidine (1 µM) to reduce DNA methylation levels. Following 48 h of treatment, a significant decrease in methylation of the three CG sites (except for site 46291794) was observed (Fig. 4C). The mean methylation level of the fragment was reduced from 21 to 15%. Furthermore, RT-qPCR revealed that PTTG1IP gene expression was significantly increased following treatment with 5-aza-2’-deoxycytidine (Fig. 4D). These results suggest that hypermethylation in the CGI shore within the PTTG1IP promoter is essential for silencing of PTTG1IP.

Discussion

The present study reported a negative correlation between PTTG1IP gene expression and the methylation level of its promoter region in lung cancer. In addition, it was identified that PTTG1IP was highly methylated in the early stage of lung cancer and exhibited a low expression level. Cytological experiments indicated that PTTG1IP overexpression may inhibit lung cancer cell proliferation. The present study provides a possible new mechanism for lung cancer development and a potential novel marker for early diagnosis of lung cancer.

The National Lung Screening Trial demonstrated a 20% reduction in lung cancer mortality using low-dose computed tomography (CT) screening (19). This survival benefit comes at the cost of testing numerous indeterminate pulmonary nodules, with an overall false-positive rate of 96.4% (26,27). One possible way to improve CT screening specificity is to use cancer-specific biomarkers from sputum and plasma. Previous studies have examined DNA methylation as a biomarker of cancer risk; however, the current low sensitivity and/or specificity of lung cancer screening is not sufficient (28-31). Epigenetic biomarkers, particularly DNA methylation, have become one of the most promising options for improving cancer diagnosis and have several advantages compared with other markers, including gene expression or genetic markers (32).
One surprising finding in cancer biology that has emerged from TCGA sequencing projects is the wide diversity of mutations that promote cancer development (33). DNA methylation changes are covalent modifications that are very stable and usually occur early in carcinogenesis. In addition, DNA methylation can be detected by a variety of sensitive and low-cost techniques, even in samples with low tumor cell purity (32). This epigenetic modification can also be detected in different biological fluids and is one of the most promising noninvasive cancer detection tools (32).

Previously, different epigenetic candidates have been proposed but have not yet reached clinical requirements, which is predominantly due to the fact that the majority of studies are based on a single candidate gene (34-38). For example, methylated CDKN2A, commonly referred to as p16, was an early focus in the search for diagnostic biomarkers in lung cancer plasma; however, although earlier studies identified CDKN2A promoter methylation in the plasma of patients with lung cancer (39-42), subsequent studies have described low sensitivity and specificity of this method (32,43,44). Methylated plasma CDKN2A may be used to detect lung cancer; however, it is more likely to be used as one part of a biomarker panel rather than as a single gene diagnostic marker. Other candidate genes include adenomatous polyposis coli (45,46), ras association domain family 1A gene (34,43,44,46,47), retinoid acid receptor β (43,44,46,48) and cadherin 13 (43,44,46); however, the sensitivity of these genes is generally low. The diagnostic firm Theracode identified short stature homeobox protein 2 as a potential biomarker (49); however, only 60% sensitivity (95% confidence interval, 53-67%) and 90% specificity (95% confidence interval, 84-94%) were identified (49). A multigene panel is a viable solution to the sensitivity and specificity concerns; however, more candidate genes need to be identified. Another consideration is that if early diagnosis of lung cancer requires a panel approach to assess plasma circulating tumor DNA, a panel with tumor type specificity is required, which requires a single gene methylation change in the panel or a combination of gene methylation changes indicating lung cancer. The present study demonstrated that PTTG1IP may be a new and specific gene that is aberrantly methylated in lung cancer.

PTTG1IP, also termed PBF, was originally reported to bind and promote the nuclear translocation of PTTG1 (50). PTTG1 is a marker of invasive colorectal cancer (51) and is a key signature gene associated with tumor metastasis (52). The functional interaction between PTTG1 and p53 has been demonstrated in transformed cells (53,54).

A number of studies have suggested that the subcellular localization of PTTG1IP and PTTG1 is crucial for progression of mitosis through the metaphase-anaphase transition (14,15,18). PTTG1IP promotes PTTG1 activation by promoting transfer of PTTG1 from the cytoplasm to the nucleus, thereby allowing the interaction between separase and PTTG1 (50). In addition to its role in metaphase/anaphase transition, PTTG1IP is also involved in transactivation of fibroblast growth factor 2 (50) and regulation of the human symporter in thyroid cells through its interaction with PTTG1 (55). However, to date, the full functionality of PTTG1IP has not been revealed.

PTTG1IP overexpression has been previously observed in certain types of malignancy, including thyroid (25), breast (53) and colorectal (52) cancer. However, to the best of our knowledge, PTTG1IP expression in other cancer types, including lung cancer, has not been reported. Expression data for all genes in lung adenocarcinoma, breast cancer, colorectal cancer, kidney cancer, melanoma, liver cancer and ovarian cancer (GSE1007, GSE20347, GSE32323, GSE6344, GSE3189, GSE14520 and GSE14407) were downloaded from the Gene Expression Omnibus database in NCBI. The ID_REF for PTTG1IP is 200677_at. The results of the analysis demonstrated that expression changes were not consistent among the tumor types, suggesting that PTTG1IP may perform different roles in different tumors (data not shown). Furthermore, it was revealed that the expression of PTTG1IP was regulated by the DNA methylation level. Further investigation demonstrated that DNA methylation at the shore of the CGI in the promoter region was negatively associated with PTTG1IP expression. More importantly, this region was hypermethylated in early-stage NSCLC. An appropriate gene methylation marker for early diagnosis of lung cancer may be a lung cancer-specific hypermethylated DNA site. Therefore, the unique performance of PTTG1IP in early-stage NSCLC suggests it can be used as an early biomarker for lung cancer diagnosis. Of course, prior to application in the clinic, further investigations are required to verify whether hypermethylation of the PTTG1IP promoter can be detected in body fluids, including sputum and plasma, from patients with early-stage NSCLC.

In conclusion, to the best of our knowledge, the present study investigated the expression of PTTG1IP in early-stage lung cancer for the first time. Low expression and promoter hypermethylation were identified. Furthermore, a negative correlation between PTTG1IP expression and methylation levels was revealed. These findings indicate that the methylation level of the PTTG1IP promoter region may be a candidate biomarker for early diagnosis of lung cancer.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

XT and HJ provided the samples. XT, SZ and HG performed the experiments. WH, MX and QW analyzed the data. XT and QW wrote the manuscript. XN and HJ designed and supervised the study and wrote the manuscript.
Ethics approval and consent to participate

All experimental protocols were approved by the Ethics Committee of South Hospital of Renji Hospital Shanghai Jiao Tong University School of Medicine (Shanghai, China). Written informed consent was obtained from each patient prior to participation.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


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