Hypermethylation of the PTTGIIP 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 (PTTGIIP) 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 PTTGIIP 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 PTTGIIP 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 PTTGIIP 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 PTTGIIP 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 PTTGIIP mRNA level increased, indicating that the PTTGIIP promoter DNA methylation level negatively regulates PTTGIIP transcription. In conclusion, in early-stage NSCLC, the PTTGIIP 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 survivin, also termed putative 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 gene expression level and methylation 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 normal tissue 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 5-aza-2'-deoxycytidine (5-aza-dC, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used for RNA extraction. The corresponding sequences were subcloned into a pcDNA3.1 vector (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 downstream applications.

Plasmid construction and cell transfection. To generate a vector expressing myc-PTTG1IP, the corresponding sequences were cloned into a pcDNA3.1 vector (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.

RNA and DNA 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 CFL96 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 15 sec, 60°C for 20 sec, and 72°C for 30 sec. The comparative 2−ΔΔCt 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'-GCTCTGACTACCAGTTACAAGC-3' and reverse, 5'-CGCCTCAAAGTTCCACCCA-3'; GAPDH forward, 5'-GGAGTCTCAGGCGTCTTTC-3' and reverse, 5'-GCTGATGATCTGGAGGCTGTTG-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...
hg19) using Bismark (a flexible aligner and methylation caller for Bisulfite-Seq applications) with default parameters. The methylation level of each cytosine was calculated using the R package MethylKit (version 1.0.0; http://code.google.com/p/methylkit), which is a comprehensive R package for analysis of genome-wide DNA methylation profiles (23).

**Bisulfite amplicon sequencing.** The DNA methylation level of the **PTTG1IP** promoter was analyzed in cells or tissue samples via bisulfite amplification followed by NGS. A total of 500 ng DNA was bisulfite treated using an EZ DNA Methylation Gold-kit (Zymo Research Corp., Irvine, CA, USA). The bisulfite-converted DNA was used to amplify the candidate fragment with a Takara EX Taq Hot Start Version kit (Takara Biotechnology Co., Ltd., Dalian, China). The PCR products were loaded on a 1.5% agarose gel for analysis and recovered for library construction and NGS using a MiSeq platform (Illumina, Inc., San Diego, CA, USA). The DNA methylation level of candidate fragments was determined by analyzing the NGS data. The primers for amplification were as follows: **PTTG1IP** forward, 5'- GTA TTG TTG AAG GGT GTA GAG ATG-3' and **PTTG1IP** reverse, 5'-CCA CCC ACC AAA ACT TTAATAATTA-3'.

**Statistical analysis.** The statistical significance of mean values in a two-sample comparison was determined with Student's t-test. P<0.05 was considered to indicate a statistically significant difference. Data are presented as the mean ± standard error. The lung adenocarcinoma and lung squamous cell carcinoma data sets from The Cancer Genome Atlas (TCGA) were used to further validate the relationship between promoter methylation and gene expression of **PTTG1IP**. Gene expression data (RNASeq) and DNA methylation data (Illumina methylation beadchip HM450 K) from 456 lung adenocarcinoma samples and 370 squamous cell carcinoma samples were downloaded from TCGA database on the cbioportal website (www.cbioportal.org). Spearman's non-parametric correlation test was performed to evaluate the correlation between gene methylation and expression using R software (version 3.3.2; http://www.R-project.org) (24).

**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 via bisulfite amplification followed by NGS. A total of 500 ng DNA was bisulfite treated using an EZ DNA Methylation Gold-kit (Zymo Research Corp., Irvine, CA, USA). The bisulfite-converted DNA was used to amplify the candidate fragment with a Takara EX Taq Hot Start Version kit (Takara Biotechnology Co., Ltd., Dalian, China). The PCR products were loaded on a 1.5% agarose gel for analysis and recovered for library construction and NGS using a MiSeq platform (Illumina, Inc., San Diego, CA, USA). The DNA methylation level of candidate fragments was determined by analyzing the NGS data. The primers for amplification were as follows: **PTTG1IP** forward, 5'- GTATTTGTGCAAGGGTGTAGAG ATG-3' and **PTTG1IP** reverse, 5'-CCACCCACCCAAA ACT TAAATAATTA-3'.

**Table I. Basic information of the paired lung cancer tissue and adjacent tissue samples.**

| Sample no. | Sex | Diagnosis                      | Stage |
|------------|-----|--------------------------------|-------|
| Pair 1c    | Female | Lung adenocarcinoma          | II    |
| Pair 2ac  | Male   | Lung squamous cell carcinoma | II    |
| Pair 3ac  | Female | Lung adenocarcinoma          | II    |
| Pair 4ac  | Male   | Lung squamous cell carcinoma | II    |
| Pair 5ac  | Male   | Lung squamous cell carcinoma | II    |
| Pair 6ac  | Female | Lung adenocarcinoma          | I     |
| Pair 7ac  | Male   | Lung adenocarcinoma          | II    |
| Pair 8ac  | Female | Lung adenocarcinoma          | II    |
| Pair 9ac  | Male   | Lung adenocarcinoma          | II    |
| Pair 10ac | Male   | Lung adenocarcinoma          | II    |
| Pair 11ac | Male   | Lung adenocarcinoma          | II    |
| Pair 12c  | Male   | Lung adenocarcinoma          | I     |
| Pair 13b  | Male   | Lung adenocarcinoma          | I     |
| Pair 14b  | Male   | Lung adenocarcinoma          | II    |
| Pair 15b  | Male   | Lung squamous cell carcinoma | II    |
| Pair 16b  | Female | Lung adenocarcinoma          | I     |
| Pair 17b  | Male   | Lung adenocarcinoma          | I     |
| Pair 18b  | Female | Lung adenocarcinoma          | II    |

*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).
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

Figure 1. PTTG1IP expression is decreased in lung cancer and increased expression in a cancer cell line decreases cell proliferation. (A) The relative PTTG1IP mRNA expression levels in paired early-stage lung cancer tissue samples. (B) The mean PTTG1IP expression levels in paired tissue samples. P<0.05. (C) The relative PTTG1IP mRNA levels in normal lung cells (MRC5) and lung cancer cells (A549). *P<0.01. (D) PTTG1IP overexpression was achieved in A549 cells. (E) The proliferation of PTTG1IP-overexpressing cells and control cells was determined by Cell Counting Kit-8 assay at 24 h intervals over 5 days. Overexpression of PTTG1IP significantly decreased cell proliferation. *P<0.05, **P<0.01 vs. PTTG1IP. Data are presented as the mean ± standard error (n=3). PTTG1IP, pituitary tumor transforming gene 1 binding factor.
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.
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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 treat-
ment, 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 exper-
iments 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).

Figure 4. Validation of the hypermethylation in the PTTG1IP promoter region and its association with gene expression. (A) The methylation level of CpG sites in the PTTG1IP promoter in paired tumor samples based on bisulfite amplicon sequencing. (B) The mean methylation level of CpG sites in the PTTG1IP promoter in paired tumor samples. (C) The methylation level of CpG sites in the PTTG1IP promoter and (D) the expression level of PTTG1IP in A549 cells following treatment with 1 µm 5-Aza. Data are presented as the mean ± standard error *P<0.05. PTTG1IP, pituitary tumor transforming gene 1 binding factor; 5-Aza, 5-aza-2'-deoxycytidine.
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), retinoic 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.

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