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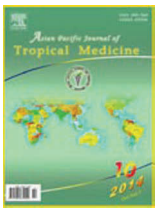

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HTRA1 gene expression in gastric epithelial cells

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ABSTRACT

Objective: To explore *HtrA1* gene expression and its regulation in human gastric cancers. **Methods:** The HtrA1 mRNA levels were examined by QPCR analysis and confirmed its expression with Northern blot analysis. The HtrA1 protein levels in all six gastric epithelial cell lines were investigated by Western blot analysis. Gene copy number was accessed and then sequenced the coding region from each mRNA in all six cell lines. The HtrA1 promoter region DNA methylation status was detected by using bisulfite sequencing analysis. Effect of decitabine and TSA on HTRA1 expression in gastric cancer cell line was determined by RTPCR. **Results:** HIC analysis indicated that HtrA1 was highly expressed in normal epithelium, but dramatically down-regulated in gastric carcinoma tissues and variably expressed in tumor-adjacent tissues. *HtrA1* gene expression was dramatically decreased in gastric carcinoma cells compared to non-tumorigenic counterparts. The *HtrA1* gene loss in any of the 4 breast cancer cell lines was not detected. Total 14 CpGs in this region were all methylated in gastric cancer cells, whereas two normal cells, GES-1 and HFI-145, were having several unmethylated cytosines in this region. HtrA1 showed as ~Mr 44,000, Expression of HtrA1 protein was not observed in any of the four gastric cancer cell lines, BGC-823, MKN-45, SGC-7901and MKN-28. HtrA1 expression was observed in the HFI-145and GES-1 cell lines. **Conclusions:** The epigenetic silencing for *HtrA1* gene expression could provide a possible strategy for re-activating *HtrA1* gene expression in gastric cancer cells, thus facilitating further investigation of HtrA1's role in chemotherapy.

1. Introduction

Serine protease 11 (PRSS11, HTRA1) belongs to the tumor inhibitory proteas. HTRA1 expressed in all kinds of human solid tumors. Studies have shown that HTRA1 mRNA expression is decreased or missing in ovarian cancer and melanoma, which may play a role in tumor suppression[1-4]. Mullany etc detected HTRA1 expression using Western Blot and found completely missied expression in seven kinds of uterine papillary serous carcinoma. Immunohistochemistry also showed its low expression in endometrial carcinoma[5]. Other studies have suggested HTRA1 expression in liver cancer tissue significantly lower than the corresponding cancer-surrounding tissue[6]. However, in the process of malignant cell transformation, specific mechanism of HTRA1 expression is still unclear. In clinical research, Chien found that HTRA1 can modulate the tumor suppression effect of cisplatin and paclitaxel. Further research showed that the gastric cancer patients with higher expression of HTRA1 is more sensitive to chemotherapy than those with lower expression of HTRA1[8]. Folgueira team found that the combined detection of HTRA1, MTSS1, CLPTM1 can identify the chemotherapy sensitive of breast cancer patients to doxlubincin, and the accuracy rate reach 5%[9]. But the role of HTRA1 in gastric cancer is unclear. It is well known that the lack of certain gene expression in the process of cancer development may be caused by many genetic and epigenetic changes[10-12]. From the perspective of the genetics, the silence of gene expression may be caused by point mutation, lost of heterozygosity (LOH)

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and homozygous deletion. And from the perspectives of epigenetics, promoter DNA methylation, histone acetylation, and chromatin remodeling are interrelated mechanism that cause the silence of gene expression[13,14]. In tumor, abnormal cytosine methylation is closely related to the gene expression[15,16]. CpG islands of promoter regions are usually non-methylation in normal tissue.

But in the process of aging or pathology, CpG islands of promoter regions may be methylated. In this research, for the purpose of exploring the mechanism of *HTRA1* gene silencing, we adopted the bisulfite sequencing method to detect DNA methylation in the promoter region of HTRA1 during malignant cell transformation. The epigenetic mechanism of gene silencing is a genetic process of mitosis, expecially DNA methylation. This reversible process relies on methyltransferase activity and protein deacetylase[17,18]. Decitabine is DNA methyltransferase inhibitors, trichostatin A is a inhibitor of histone deacetylase, which has been widely used to study the regulation of gene expression, DNA methylation and histone deacetylation. Therefore, in this research, we adopted decitabine and TSA alone or combination to treat gastric cancer cell lines, and then explore the relation between decreased HTRA1 expression and epigenetic modifications in gastric cancer cell line.

2. Materials and methods

2.1. Specimen collection

114 cases of samples were collected from patients with gastric cancer from January 2010 to December 2010. The patients included 73 males and 41 females aged from 31 years to 84 years old (average 63.2 years). All the patients did not receive preoperative radiotherapy or chemotherapy. All patients were confirmed by pathology of gastric cancer, and had complete clinical data and follow-up records. In each case, gastric cancer tissue, nearer-tumor region and further-tumor region were collected. Nearer-tumor region refers to edge within five cm away from cancer lesions; further-tumor region refers to tissue beyond nearer-tumor region. Tissue specimens were fixed with 10% methyl alcohol.

2.2. Cell culture

GES HFI - 145-1, BGC-823, MKN-45, SGC-7901 and MKN-28 cells were stocked in our lab. All cell lines were cultured in high glucose DMEM culture medium (containing 10% fetal bovine serum, dual antibioses). Cells were cultured in 37℃, with 5% CO_2 and 95% humidity. When the color of medium was changed, it was changed with fresh medium. When the alignment was approximately 80%, the cells were digested to extend.

2.3. HTRA1 expression detection in gastric cancer tissue using IHC

Specimens were fixed by 10% formaldehyde, and were dehydrated with alcohol, transparent with xylene, embedded in paraffin. Then serial 4 μm sections were made. Following deparaffinization, sections were heated in a microwave oven (30 minutes at 90℃) in citrate buffer for antigen retrieval. Then, they were washed in phosphate-buffered saline for 10 minutes and incubated overnight at 4℃ in the presence of mAb HTRA1 in the form of 1:200 diluted hybridoma supernatant or control reagents. Bound antibodies were visualized by using the avidin-biotin complex method according to the ecommendations of the supplier (Vectastatin Elite ABC Kit; Vector Laboratories Inc., Burlingame, CA). Diaminobenzidine was used as chromogen. HTRA1 staining was classified as follows: "positive" indicating positive cell percentages more than 20% of cells; "negative" indicating positive staining of no more than 20% of cells.

2.4. HTRA1 mRNA expression detection in gastric cancer cell lines by real-time PCR

Total RNA was extracted with Trizol method. Reverse transcription was in according to kit reference (Taqman, American ABI). Real-time PCR were performed on ABI9700 type PCR (the United States ABI company). HTRA1 primer sequences for F: 5′-TTGTTTCGCAAGCTTCCGTT-3′, R: 5′-ACGTGGGCATTTGTCACGAT-3′. Beta actin primers sequences for F: 5′- CACGGCACTGATTTTCAGTTCT-3′, R: 5′-TTCTTGCTGCCAGTCTGGACT-3′. Real time PCR reaction conditions: 95℃×5 min, 1 cycle; 58℃ 95℃ 25 s, ×20 s, 72℃×30 s, 45 cycle. Threshold was set by machine. Results calculation was as follow: relative amounts c (t) = $2^{-\triangle\triangle C(t)}$ = C(t) gene a internal gene C(t).

2.5. Northern hybridization analysis

30 μg of total RNA were from six kinds of gastric cancer cells. cDNA of corresponding gene sequence were designed according to synthesis of primers, mRNA as a template, the synthesis probe hybridization by RT-PCR, using beta actin as reference.

2.6. DNA PCR of gastric epithelial cell lines

Genomic DNA was extracted from six gastric epithelial cell lines in logarithmic phase. 20 ng genomic DNA from each samples were taken as a template. A 500 bp fragments of *HTRA1* gene were amplified, the first exon can obtain form this fragment by PCR reaction. primers for *HTRA1* gene: 5′-TTGTTTCGCAAGTAAAGAGA-3′ (forward), 5′-ACGTGGGCATTTGTCACGAT-3′ (reverse). As internal reference, clotting factor Ⅸ was amplified by PCR. 24, 26, 28 and 30 cycles amplification were performed to avoid the amplification saturation phenomenon.

2.7. HTRA1 coding sequence in gastric epithelial cell lines

Total RNA extraction and reverse transcription were performed. 1.5 kB *HTRA1* gene coding sequence were amplified through the PCR reaction. Primers sequence: 5′-CAGAGTCGCCATGCAGATCC-3′ (forward), 5′-GAAGTCCAG CTC ATG CCTS CTG-3′ (reverse). PCR products were cloned to the TOPO carrier. Correctly cloned were screened by *EcoR* Ⅰ endonuclease. In each cell lines, correctly connected clones were sequenced and compared using Blast software (HTRA1 Genebank access number is: NM_002775).

2.8. DNA methylation detection of HTRA1 promoter regions

Human *HTRA1* gene promoter sequences were obtained From the UCSC genome bioinformatics website (WWW. Genome.ucsc.edu). the possible transcription factor binding sites were tested within 2 000 bp sequence among promoter ahead of the transcription start site through TRANSFAC database (www.gene-regulation.com/databases.html).

For Methylation analysis, DNA bisulphite conversion and purification recovery were performed using QIAGEN Company EpiTech Bisulfite kit. The operation steps as follows: 1 μg genome DNA was incubated in sodium bisulfite buffer at 37℃ water bath circulation for 5 hours. After the incubation, DNA was purified and recovery. Purified DNA was used for PCR and sequencing. Primer sequence cover -561 to -266 bp of promoter region: the forward primer 5′-TTTATTATTTTATTGTGGGTTTGGG, reverse primer 5′-AATAAAACTTTACAAAAAAACCCTAC.

2.9. Effect of HTRA1 on gastric cancer cells expression

One day before drug intervention, four types of gastric cancer cells were seeded in 6 wells plate in the density of 5×10^5/mL. the cells were treat with in DMSO, decitabine (5 microns), the TSA (300 nm) separately or combination of decitabine and TSA for 72 hours. Then total RNA were collected. Real time PCR was performed to analyze the expression of HTRA1.

2.10. HTRA1 protein expression in gastric cancer cells by Western blot

Gastric cancer cells in logarithmic phase were harvested and washed with PBS. Cells pellets were extracted in cell lysis buffer. An additional step was applied using ultrasonic dismembrator (240 times for each sample with frequency ultrasonic for 2 seconds, interval for 2 seconds, power 300 W). extracts were clarified by centrifugation with 10 000 r/min at 4℃ for 5 minutes, and protein concentrations were measured by Bradford assay. 10 μg of total protein for each sample was resolved by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The protein was transferred to polyvinylidene nitrocellulose membrane, and the blot was blocked with 5% nonfat dry milk in 20 mM TBST for 1 h at room temperature. The blot was incubated with HTRA1 primary antibody (1:500 dilution) over night at 4℃, and the bound antibody was detected using horseadish peroxidase-conjugated secondary antibody and chemiluminescence. After striping, the blot was reblocked with β-actin antibody as internal. The films were scanned and its gray zone was determined with Bio-Rad Ouantity one software, and ratio of grey value of targeting protein was calculated and compared with beta actin.

2.11. Statistical analysis

Data was expressed as percentage. SPSS 18.0 statistical analysis software was applied. Comparison among groups was performed by *Chi*-square test. Correlation of Hierarchical data was evaluated using nonparametric Spearman rank correlation test. $P<0.05$ was considered as significant different.

3. Results

3.1. HTRA1 expression in gastric epithelial tissue

Immunohistochemistry results showed that the HTRA1 expression increased in further-tumor region, but decreased in gastric cancer tissue, and was differently expressed in nearer-tumor region (Figure 1).

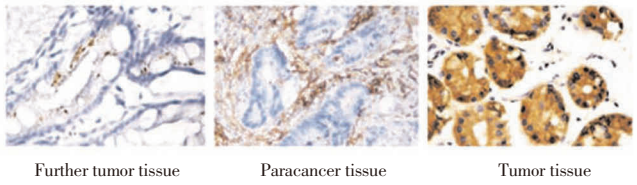

Figure 1. HTRA1 expression in gastric epithelial tissue.

3.2. Real time PCR and Northern blot

In this research, we detected HTRA1 mRNA level using real-time PCR in four gastric cancer cell lines BGC-823, MKN-45, SGC-7901 and MKN-28, and two normal gastric mucosa epithelial cell line HFI-145, GES-1. Results showed that the HTRA1 mRNA were significantly higher in normal gastric mucosa epithelial cell lines than in gastric cancer cell lines (Figure 2 a). Northern blot further confirmed the differential expression of HTRA1 mRNA in normal gastric mucosa epithelial cell lines and gastric cancer cell lines (Figure 2b).

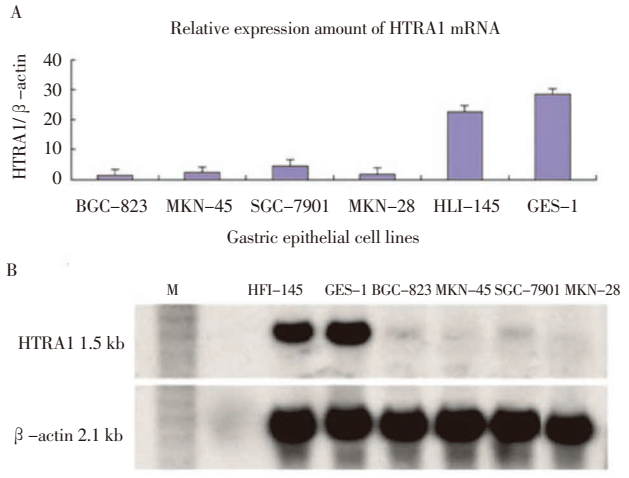

Figure 2. HTRA1 mRNA level in gastric epithelial cell lines
A: Real-time PCR; B: Northern blot.

3.3. Genome PCR and sequence of coding area

Decreased HTRA1 express may be caused by chromosome aberration. Firstly, we inspect the relationship between the decreased HTRA1 expression and the chromosome aberration through PCR on HTRA1 genomic DNA. The PCR product was 500 bp, containing exon 1 area of *HTRA1* gene. The results do not show *HTRA1* gene loss in four types of gastric cancer cell line (Figure 3). To rule out the possible effect of point mutations on *HTRA1* gene silencing in cancer cells, we match the RT-PCR products of HTRA1 mRNA in gene bank Blast. The results showed no point mutations or deletions in all cell lines.

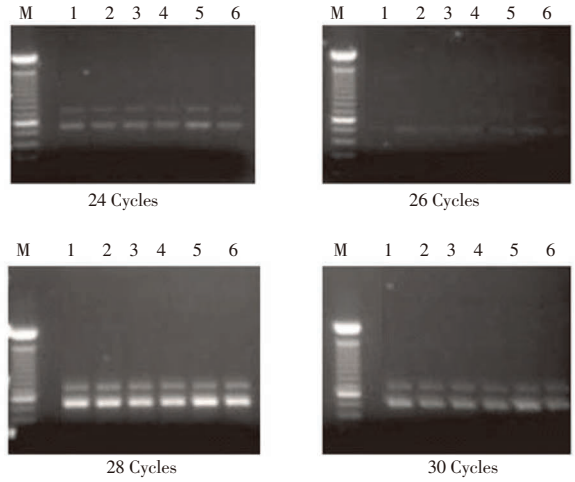

Figure 3. mRNA of gDNA HTRA1 in gastric epithelial cells.

3.4. Results of bisulfite sequencing

Decreased *HTRA1* gene expression in gastric cancer cells may be caused by epigenetic modifications, but not the HTRA1 sequence changing. Because the area 800 bp upstream the *HTRA1* gene transcription start site were rich in GC (including two CpG island), therefore epigenetics DNA methylation was likely related to the decreased *HTRA1* gene expression.

The results of sodium bisulfite sequencing showed a negative correlation between DNA methylation status of -561 bp to 266 bp HTRA1 promoter regions and mRNA expression level. There are 35 CpG island in this area. In cancer cell lines HFI-145 and GES-1, CpG island methylation rate were 39.9% and 37.4%, respectively. In MKN-45, SGC-7901 and MKN-28 and BGC-823, at the same area, average methylation levels were 71.4%, 92.6%, 93.7% and 95.1% (Figure 4).

By comparing real-time PCR results of HTRA1 mRNA and Northern blot results, we know HTRA1 regulation site mainly located in the promoter -511 to -423 bp area (Figure 4b). This area contained 14 CpG island, and all the CpG islands were methylation in four types of gastric cancer cell lines, but there was a lot of unmethylated CpG island and in normal gastric epithelial cell lines GES-1 and HFI-145.

GDNA extracted from gastric epithelial cell lines were performed sodium bisulfite sequencing. The results showed that transcription start site located in the -511 bp to 423 bp region containing 35 CpG islands. A: blue column indicated unmethylated cytosine, red column indicated starting sites. B: expression regulatory regions of *HTRA1* gene located

between −511 bp to 423 bp. In this area, forty CpG islands of four types of gastric cancer cells are all methylate. But there were a lot of unmethylated CpG islands in normal gastric epithelial cell lines HFI-145 and GES-1.

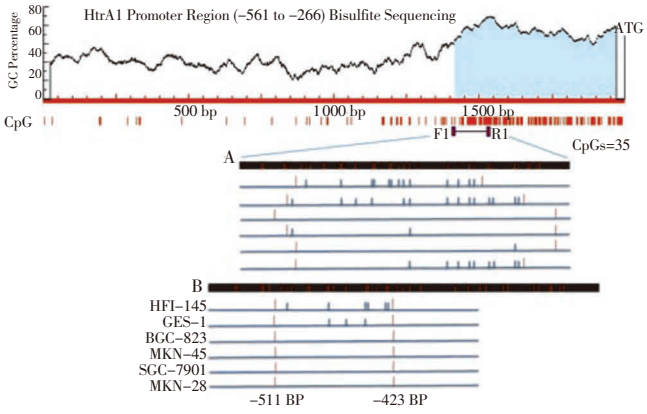

Figure 4. Methylation on *HTRA1* gene promoter.

3.5. Effect of decitabine, TSA alone or combined treatment on HTRA1 expression

To explore the effect of epigenetic modifications on regulating *HTRA1* gene expression in gastric cancer cells, Decitabine (DNA methyltransferase inhibitor), TSA (histone acetylation enzyme inhibitor) alone or combined treatment were used to treat gastric cancer cells. GES-1 cell was used as positive control when HTRA1 mRNA expression was assayed by real time PCR. In BGC-823, MKN -45 cell lines, HTRA1 expression was inhibited by histone acetylation, but was not affected by DNA methylation. In SGC-7901 and MKN-28 cell lines, HTRA1 expression was inhibited mainly through DNA methylation (Figure 5). In SGC-7901 cell lines, DNA methylation and histone acetylation can synergistically controlled the *HTRA1* gene expression. In MKN-45, however, in SGC-7901 and MKN-28 cell lines, *HTRA1* gene expression level was still far less than in gastric epithelial cell line GES-1 even after its expression was restored. There were two possibilities for this results, one was incomplete treatment of inhibitors on DNA methylation and histone acetylation. Other mechanisms except for DNA methylation and histone acetylation regulated the *HTRA1* gene expression. bisulfite sequencing were also used in this study to further sequence *HTRA1* gene promoter region in SGC-7901 cells after treatment with Decitabine. All CpG island within −511 and −423 bp area are demethylated, but all CpG island within this area of SGC-7901 cells are methylated (Figure 4b). These results demonstrated that DNA methyltransferase were completely suppressed in SGC-7901 cell line. Therefore, *HTRA1* gene expression level did not return to normal levels even after treated with DNA demethylase and histone deacetylase. The possible reason for this phenomenon is not completely inhibiting histone deacetylase treatment, or in addition to the uncompleted inhibition on histone deacetylase or other mechanisms involved in the regulation of *HTRA1* gene expression, such as chromatin remodeling.

The methylation status of promoter region in SGC-7901 cells after treated with decitabine.

3.6. HTRA1 expression in gastric epithelial cell line by Western Blotting

Western Blotting was used to examine HTRA1 expression in four gastric cancer cells and two kinds of normal gastric epithelial cells (Figure 6). HTRA1 protein was about 44 kDa. HTRA1 was not expressed in cell lines including BGC-823, MKN-45, SGC-7901 and MKN-28 but expressed in HFI-145 and GES 1. GES-1 was used to create HTRA1 overexpression cell line or gene knockout cells.

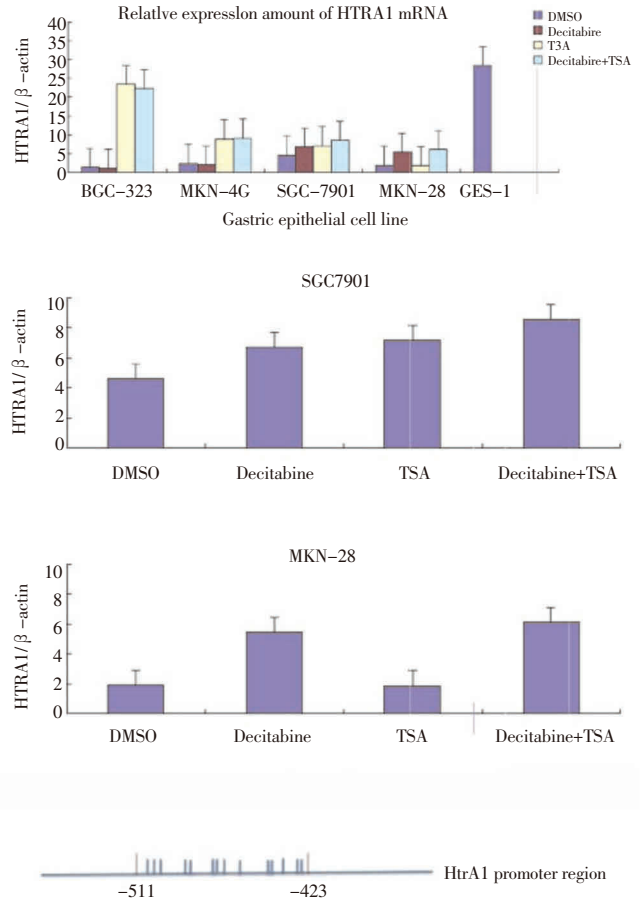

Figure 5. *HTRA1* gene expression in gastric cancer cell lines after decitabine treatment.

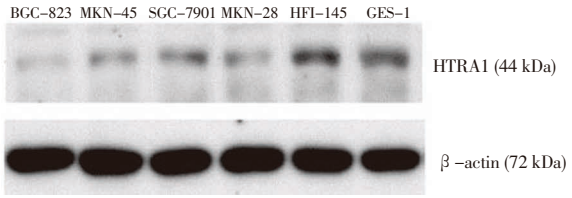

Figure 6. HTRA1 expression in gastric epithelial cells.

4. Discussion

Results showed HTRA1 expression in gastric cancer cell line is significantly lower than in normal gastric epithelial cell lines. Semi-quantitative PCR results showed no change of gene copy number in gastric cancer cell lines. No gene mutation is observed in normal gastric epithelial cell line by cDNA sequencing. The decrease of HTRA1 expression in gastric cancer cells is associated with epigenetic silencing, including DNA methylation, histone acetylation or both. A growing number of studies confirmed that epigenetic changes play an important role in tumorigenesis and progression. Epigenetic changes are heritable mitosis, so they led to the occurrence of the disease through combination of genetic alterations. DNA methylation, histone modification and chromatin remodeling are three interrelated mechanisms that cause the cancer genome gene silencing[19-21].

In addition to the DNA methylation confirmed in our research, histone acetylation is another important reason for the decrease of *HTRA1* gene expression in gastric cancer cell lines. Zupkovitz etc provides an important basis for this theory. Their study found that rats *HTRA1* gene is one of the genes being negatively regulated by acetylation enzyme protein 1 (HDAC1)[22]. Through chromatin precipitation of two regions in HTRA1 gene, Zupkovitz *etc* found the acetylation level of histone H3 (AcH3) and H4 (AcH4) of mouse is higher in HDAC1 mutation ES cells than in wild-type cells. Their study also found the inhibition function trimethylation lost in HDAC1 mutation cells causing trimethylation on H3K9 and H3K27. Studies have confirmed the close relation between class Ⅰ acetylation enzyme (including HDAC1) and methyl protein and methyl transferase[23,24]. Therefore, our study shows the close relation of DNA methylation and histone acetylation.

Chromatin contains DNA and the histone surrounded by tDNA, and its basic structure is nucleosome[25,26]. Nucleosome, link histone and the non-histone component are closely integrated into advanced structure. In this structure, DNA are unable to access to the transcription initiator, therefore they could not enter transcription. As a result, the chromatin structure must be dynamically regulated to perform gene expression[27,28].

Chromatin remodeling is performed by multi-subunit complexes, whice consists of two kinds of basic molecular functional groups. Multi-subunit complexes can covalently modify histone, DNA and ATPase dependent remodeling complex[29]. The first set of molecular functional groups consists of factors that can modify histone through acetylation, methylation, phosphorylation, ubiquitination, and they also participate in *HTRA1* gene expression regulation[30,31].

TESS database (http://www.cbil.upenn.edu) provides a potential transcription start site of multiple genes and DNA binding sites. In this research,for the purpose of understanding the key transcription factors regulating *HTRA1* gene expression, we analysis the *HTRA1* gene proximal promoter regions by using TESS database. Results showed, in HTRA1 promoter sequence super methylation area (511-511 bp), there are a variety of binding sites for transcription factor, including c-Myc, c-Myb, T-Ag, NF-1, AP-1, Sp1, ER, E2F and HOXA5. *HTRA1* gene chromatin structure, and the interaction between DNA and histone modification may inhibit the expression by affecting the transcription factors binding to the promoter regions.

In conclusion, epigenetic silencing of the *HTRA1* gene expression could provide a viable strategy to reactivate *HTRA1* gene expression in gastric cancer cells, thus promoting further research to explore the role of *HTRA1* in chemotherapy.

Conflict of interest statement

We declare that we have no conflict of interest.

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