Clinical significance of expression of *Hint1* and potential epigenetic mechanism in gastric cancer

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Received December 22, 2010; Accepted February 24, 2011

DOI: 10.3892/ijo.2011.994

Abstract. HINT1, a member of the evolutionary highly conserved HIT protein superfamily, is ubiquitously expressed in diverse species including mammalian tissues. Accumulating evidence shows that HINT1 is a haploinsufficient tumor suppressor. In the present study, we explored possible correlations between the expression level of HINT1 and clinicopathological features in tissues from gastric cancer (GC) patients. Decreased expression of HINT1 detected by qPCR and Western blotting in tumor tissues was found in 58.82 and 39.2% of the patients, respectively. Significantly reduced expression of HINT1 was found in poorly differentiated tumor tissues (p=0.027). Environmental interference (either *H. pylori* or EBV infection) (p=0.005) was associated with the expression of HINT1. Moreover, compared with the GC tissues, the level of *Hint1* detected by qPCR was significantly higher in the adjacent non-cancerous tissues (p=0.03). We treated AGS cells, a GC cell line with low expression level of HINT1, with 5 and 10 μmol/l 5-Aza-dC for 72 h and found that HINT1 could be induced by 5-Aza-dC in a dose-dependent manner. These results suggested that *Hint1* expression was lower in GC tissues and some etiological factors, such as *H. pylori*/EB infection or promoter hypermethylation may play a role in gastric tumorigenesis.

Introduction

Gastric cancer (GC), as the fourth most common cancer and the second cause of death worldwide, causes nearly 1000,000 new cases and over 850,000 deaths every year (1). The epidemiology of GC has obvious geographic and demographic variations, occurring mainly in Africa and Asia. The infection of *H. pylori* is a common event and is shown to induce aberrant methylation in gastric cancer (2). Epstein-Barr (EB) virus infection is considered as another important carcinogenic factor in gastric carcinogenesis (3). The development of GC is a complicated and multistep process involving multiple genetic and epigenetic aberrations, which cause cell proliferation, differentiation and genetic instability.

The HINT1 protein, as a member of the evolutionary highly conserved HIT protein super family, is ubiquitously expressed in diverse species including mammalian tissues. The HIT protein superfamily can be classified into the Hint branch, the Fhit branch and the GalT branch (4). In previous studies, *Hint1* knockout mice had a marked increase in susceptibility to chemical carcinogen-induced gastric tumors (5), mammary tumors (6) and ovarian tumors (6). These studies in mice provided evidence that HINT1 may be a haploinsufficient tumor suppressor. HINT1 protein can interact with several proteins and has corresponding effects, which include pro-apoptosis by up-regulation of p53 and Bax and down-regulation of Bcl-2 in human colon cancer cell line SW480 and breast cancer cell line MCF7 (7); cell cycle regulation by up-regulation of cellular levels of p27 (8); inhibition of several transcription factors including β-catenin (9), MITF (10), USF2 (11) and activator protein-1 (AP-1) (12); cellular responses to ionizing radiation via interaction with the product of ATDC gene to repress fos transcription (13); modulating the μ-opioid receptor signaling pathway through interaction with the μ-opioid receptor and/or RGSZ1 (14,15). It had been reported that *Hint1* is transcriptionally silenced in SW480 and increased expression of *Hint1* inhibits growth of the cell (12). Similar effects have been seen in non-small cell lung cancer (NSCLC) cell lines H522 and H538 (9,17).

A growing number of genetic and epigenetic alterations in tumor suppressor and tumor-related genes, such as *APC*, CHFR, COX2, *DAP-kinase, DCC, E-cadherin, GSTP1,* HRK, LOX, *hMLH1, MGMT, p14, p15, p16, PTEN, RASSF1A, RUNX3, 14-3-3 sigma, THBS1, TIMP-3* and *TSLC1* (18-43), have been found to be involved in gastric carcinogenesis. In addition, more and more genes have been identified with aberrant promoter hypermethylation, suggesting that promoter hypermethylation is an important molecular mechanism for gastric carcinogenesis. In a previous study, promoter hypermethylation of *Hint1* was found in hepatocellular carcinoma (HCC) tissues and normal liver tissues (16), and cell lines such

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Key words: Hint1, GC, *H. pylori*, EB virus, promoter methylation

as SW480 (12), Hep3B and HepG2 (44). However so far there is no report suggesting the relationship of *Hint1* and GC tissues or GC cell lines.

Based on the above findings in the present study, we investigated *Hint1* protein and mRNA expression in GC and paired adjacent non-tumor tissues and its clinical significance.

Materials and methods

Tissue samples and cell lines. The human gastric cancer (GC) cell lines AGS and BGC-823 were obtained from cell bank of Chinese Academy of Sciences. Both the cell lines were maintained in medium containing RPMI-1640 (Invitrogen) supplemented with 10% fetal bovine serum. Cells were incubated in a 100% moist incubator with 5% CO_2 at 37˚C. GC and adjacent non-cancerous tissue specimens diagnosed by pathologists were obtained from the Nanjing First Hospital affiliated Nanjing Medical University. Tumor differentiation, depth of invasion, and lymph node metastasis was judged by routine pathological diagnosis. The clinicopathological features are shown in Table I. The age range was 38-91 years. Appropriate consent was obtained from each patient.

Reverse-transcription (RT)-PCR. Total RNA was isolated by using TRIzol Reagent (Invitrogen, USA) and first-strand cDNA was prepared from total RNA with Oligo(dT) 18 primer and AMV reverse transcriptase (BioFlux, Japan) according to the manufacturer's instructions. Primers used in subsequent PCR: *Hint1*: 5'-GAGATGGCAGATGAGATT-3' (forward) and 5'-TTAACCAGGAGGCCAATG-3' (reverse). PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide, and visualized under UV illumination. The intensities of the specific bands were analyzed and quantified.

Detection of H. pylori and EB virus in GC tissues. The presence of *H. pylori* and EB virus infection was determined by PCR for *H. pylori* 16S rDNA and the EBNA-1 region, respectively. The primers for *H. pylori* were as follows: 5'-GCCAATGGTAAATTAGTT-3' (forward) and 5'-CTCCTTAATTGTTTTTAC-3' (reverse). The primers for EB virus were as follows: 5'-GTCATCATCATCCGGGTCTC-3' (forward) and 5'-TTCGGGTTGGAACCTCCTTG-3' (reverse). The DNA templates obtained from the GC patients were extracted by using the Biospin Tissue Genomic DNA Extraction Kit (BioFlux, Japan). *H. pylori* infection was also determined by Giemsa-stained histological staining. Cases that showed positivity in any assay were regarded as *H. pylori*-positive or EB virus-positive.

Real-time RT-PCR. To test HINT1 expression in normal GC tissues, Western blotting and real-time RT-PCR was carried out on the tissues. Primers used in the RT-PCR: *Hint1*: 5'-TTCTTCCGAGCCTCTCCTCT-3' (foward) and 5'-GACGATACCCACCTCAGCAG-3' (reverse); β-actin: 5'-AAAGACCTGTACGCCAACAC-3' (foward) and 5'-GTCATACTCCTGCTTGCTGAT-3' (reverse). Examination of the mRNA expression of *Hint1* was carried out by using a SYBR-Green PCR kit (Takara, Japan) under the following cycling conditions: PCR reactions were performed in 20 μl of total volume with 2 U polymerase (Takara) and cDNA samples equivalent to 0.5 ng of RNA In 7300HT Fast Real-Time PCR System (Applied Biosystems, USA). As an endogenous standard, β-actin was quantified synchronously in order to normalize the cDNA input among samples. The relative level of expression of *Hint1* among the different tissues was then calculated referring to the amount of β-actin (ABI PRISM 7300 Detection System, USA). Each reactions was performed in duplicate and the mean of the two experiments was used as the relative quantification value. At the end of 42 amplification cycles, the reaction products were collected and electrophoresed on 3% agarose gel, to confirm that non-specific products were not obtained during the process of amplification.

Table I. Correlation of HINT1 expression level with clinicopathological features in 51 GC cases.

Parameters	Cases	T<N	T≥N	P-value
Age				0.839
≤60	17	7	10	
>60	34	13	21	
Gender				0.839
Male	34	13	21	
Female	17	7	10	
Differentiation				0.027[a]
Poor	25	14	11	
Moderate/well	24	6	18	
Invasive degree				0.665
Early stage	6	3	3	
Progression	43	16	27	
Lymph node metastasis				0.903
Yes	33	13	20	
No	17	7	10	
EB infection				0.101
Yes	22	11	11	
No	29	8	21	
HP infection				0.304
Yes	21	10	11	
No	30	10	20	

[a]Significant difference.

5-Azadc treatment of GC cell. 5-Azadeoxycytidine (5-Aza-dC) was purchased from Sigma (USA). The cell line AGS was cultured at $2x10^5$ in dishes on the first day. After incubated for 24 h the cells were treated with solvent or with 5-Azadc (5 or 10 μM) for 72 h, and then their medium was changed to fresh for the last 24 h. At last, the cells were harvested for RNA or protein extraction. All the assays were done in triplicate.

Protein extraction and Western blot assays. Cells were cultured and treated with the various conditions as described above.

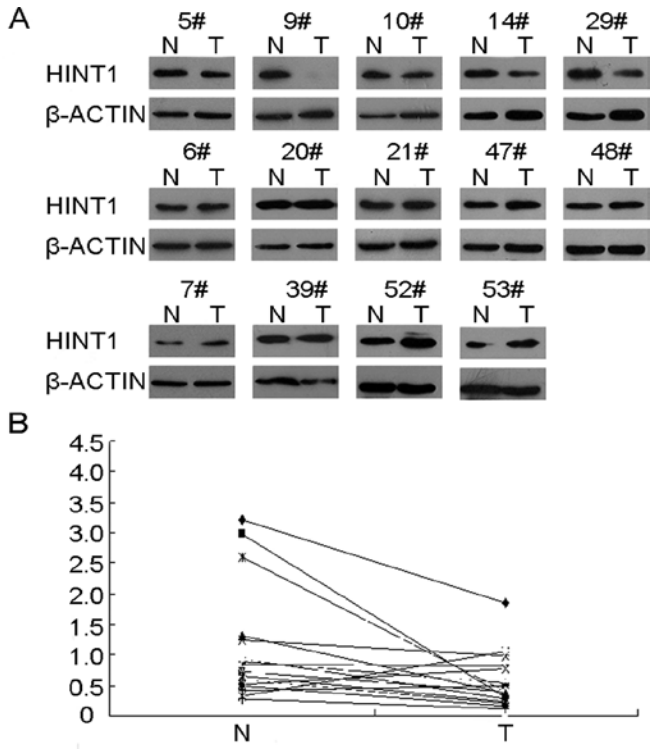

Figure 1. Expression of the Hint1 in GC and adjacent non-cancerous tissue samples. (A) Protein expression level was detected by Western blotting, 20 cases display T<N and 27 cases display T=N in the 51 pairs of GC and adjacent non-cancerous tissue samples. Representative examples are shown above. (B) In the RNA expression level detected by real-time RT-PCR, 58.82% of the cases display T<N and 29.41% display T=N.

Protein extracts of tissues were then prepared as described. The proteins were separated by SDS-PAGE with 15% polyacrylamide gels and then blotted with the indicated antibody. The primary antibodies included anti-HINT1 antibody (ProteinTech Group, USA), anti-β-actin antibody (Sigma, USA). Anti-rabbit and anti-mouse IgG antibodies were used as the secondary antibodies, respectively. The intensities of specific protein bands were quantified with Gel Pro 3.2 (UVP, CLL, USA), corrected for the intensity of the respective β-actin band.

Immunohistochemistry (IHC) detection of HINT1 protein in paraffin-embedded sections. Tissue sections were obtained from Pathology Department of the Nanjing First Hospital affiliated Nanjing Medical University. IHC was used to detect the location and expression of HINT1. After deparaffinization in dimethylbenzene and rehydration in the gradient ethanol, the sections were heated in 10 mM citric acid (pH 6.0) in a microwave oven for antigen retrieval. Staining was carried out according to the manufacturer's instruction: sections were incubated with the primary antibody (1:100 dilution) overnight at 4˚C, and then incubated with the secondary antibody (1:50 dilution). Color development was carried by using ABC reagent and DAB. Slides were counterstained with hematoxylin. The following categories were used for scoring: percentage of positive staining, <5% (0), 5-25% (1), 25-50% (2), > 50% (3) of cells, and intensity of staining, none (0), mild (1), moderate (2), strong (3). Combining percentage and intensity staining resulted in the following score 0-1; negative (-); 2-6 positive (+).

Table II. Correlation of *Hint1 mRNA* expression level with clinicopathological features in 34 GC cases.

Parameters	Cases	T<N	T≥N	P-value
Age				0.29
≤60	11	5	6	
>60	21	15	6	
Gender				0.226
Male	23	16	7	
Female	10	4	6	
Differentiation				0.912
Poor	16	9	7	
Moderate/well	12	7	5	
Invasive degree				0.492
Early stage	2	2	0	
Progression	24	13	11	
Lymph node metastasis				0.683
Yes	18	11	7	
No	8	4	4	
EB infection				0.868
Yes	14	8	6	
No	20	12	8	
HP infection				0.226
Yes	17	8	9	
No	17	12	5	

Statistical analysis. SPSS 13.0 software for Windows (Microsoft, USA) was used for statistical analysis. Correlation of the expression of *Hint1* between GC and adjacent non-cancerous tissues were analyzed through χ^2 test, differences were analyzed by the Fisher's exact test. Results were considered statistically significant at $p<0.05$.

Results

Expression of Hint1 in GCs and the corresponding normal gastric mucosae. The mRNA expression levels of the *Hint1* were detected by qPCR in 34 pairs of matched tumor and non-tumor tissues and decreased expression of *Hint1* was 58.82% in tumor tissues, while the protein expression of HINT1 was detected by Western blotting in 51 cases and decreased expression was 39.2% in tumor tissues comparing to the non-tumor tissues (Fig. 1). In addition, the mRNA expression level of *Hint1* in GC was significantly lower than that in non-tumor tissues (p=0.030) (Fig. 2).

Although we did not find any significant correlations between *Hint1* mRNA expression levels and the clinico-pathologic data (Table II), significantly decreased protein expression of HINT1 was observed in poorly differentiated tissues (p=0.027, Table I). There was no obvious statistical

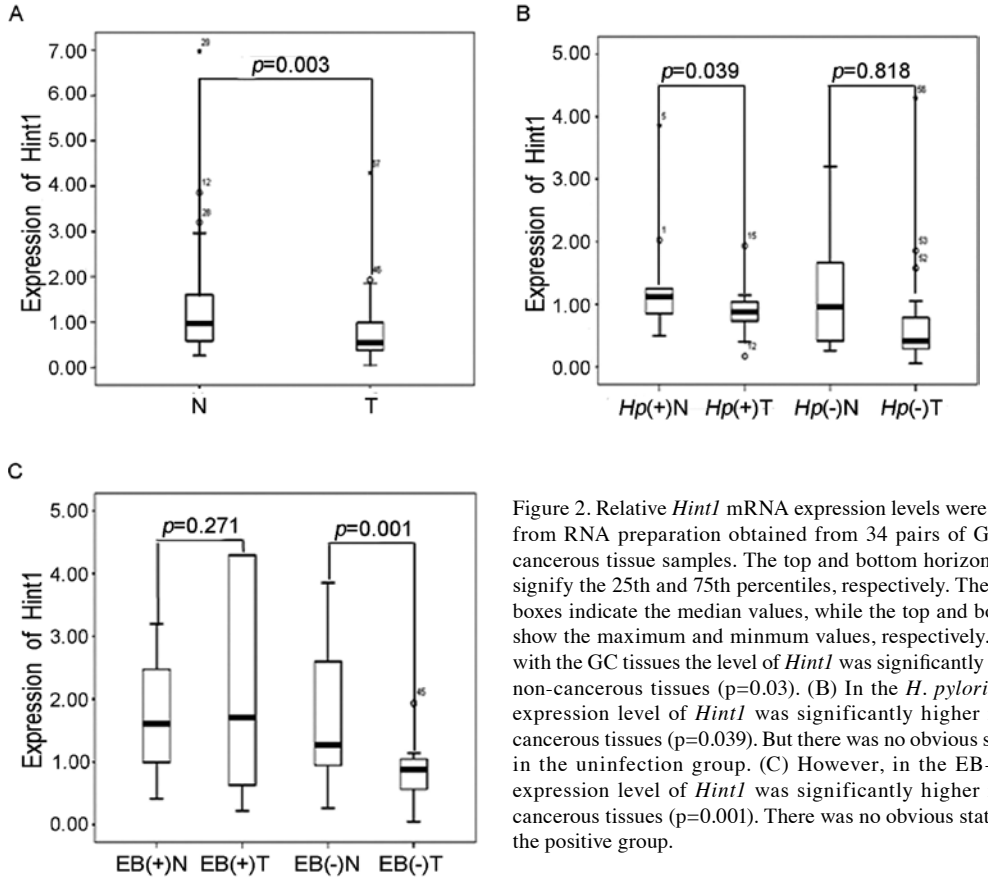

Figure 2. Relative *Hint1* mRNA expression levels were determined by qPCR from RNA preparation obtained from 34 pairs of GC and adjacent non-cancerous tissue samples. The top and bottom horizontal lines of the boxes signify the 25th and 75th percentiles, respectively. The bold lines within the boxes indicate the median values, while the top and bottom horizontal bars show the maximum and minmum values, respectively. (A) When compared with the GC tissues the level of *Hint1* was significantly higher in the adjacent non-cancerous tissues (p=0.03). (B) In the *H. pylori* infection group, the expression level of *Hint1* was significantly higher in the adjacent non-cancerous tissues (p=0.039). But there was no obvious statistical significance in the uninfection group. (C) However, in the EB-negative group, the expression level of *Hint1* was significantly higher in the adjacent non-cancerous tissues (p=0.001). There was no obvious statistical significance in the positive group.

Table III. The correlation of *H. pylori* or EB infection and tumor invasive degree detected by Western blot analysis.

	Cases	T<N	T≥N	P-value
EB infection				
Yes	22	11	11	1
Early stage	2	1	1	
Progression	18	9	9	
No	29	8	21	0.568
Early stage	4	2	2	
Progression	25	7	18	
HP infection				
Yes	21	10	11	1
Early stage	3	2	1	
Progression	17	8	9	
No	30	11	20	1
Early stage	3	1	2	
Progression	26	8	18	

Table IV. The condition of *H. pylori* and EB infection detected by Western blot analysis.

	Cases	T<N	T≥N	P-value
Hp and EB infection				0.007[a]
Both	9	3	6	
Either	25	15	10	
Neither	17	2	15	
Hp and EB infection				0.005[a]
(+)	34	18	16	
(-)	17	2	15	

[a]Significant difference.

significance when we analyzed the relevance between *H. pylori* or EB infection and the result of Western blotting. We found obvious statistical significance when we combined the *H. pylori* and EB infection with the result of Western blotting (Table IV). Therefore, we could affirm that the environmental interference (either *H. pylori* or EB infection) was interrelated to lower expression of HINT1 in GC tissues.

IHC was used to show the immunostaining of HINT1 in gastric mucosal cells. Representative examples of HINT1 expression in adjacent non-cancerous tissues showed expression of the HINT1 in both tumor cell nuclei and cytoplasm (Fig. 3B and D). GC tissue with moderate differentiation displayed expression of the HINT1 in both tumor cell nuclei and cytoplasm (Fig. 3A) while GC tissue with poor differentiation showed lack of expression in tumor cells (Fig. 3C). PBS substituted for the primary antibody of HINT1 and BGC-823 were used as blank and positive controls, respectively (Fig. 3E and F).

Expression of Hint1 in GC cells. There is increasing evidence that *Hint1* is a novel tumor suppressor gene. In view of the

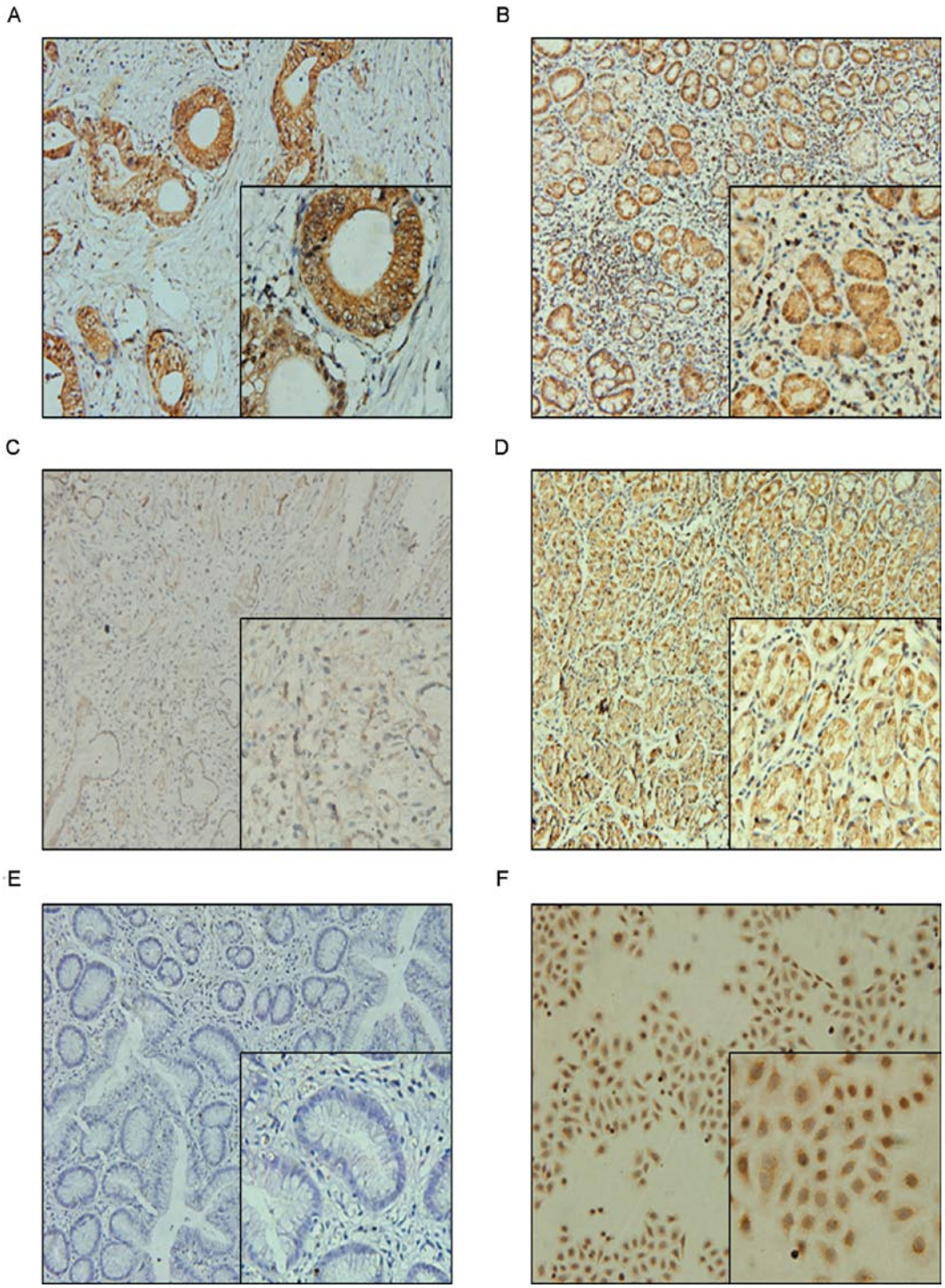

Figure 3. Immunohistochemical detection of HINT1 expression in GC cases. (A) GC tissue with moderate differentiation shows expression of the HINT1 protein in both tumor cell nuclei and cytoplasm, x200 and x400 (lower right corner). (B) Paired adjacent non-cancerous tissue of (A) shown expression of the HINT1 protein in both tumor cell nuclei and cytoplasm, x200 and x400 (lower right corner). (C) GC tissue with poor differentiation lacks expression in tumor cells, x200 and x400 (lower right corner). (D) Paired adjacent non-cancerous tissue of (C) shows expression of the HINT1 protein in both tumor cell nuclei and cytoplasm, x200 and x400 (lower right corner). (E) PBS substituted for the primary antibody of HINT1 and used as a blank control, x200 and x400 (lower right corner). (F) BGC-823 was used as a positive control, x200 and x400 (lower right corner).

low expression of *Hint1* in GC cases, it was of interest to examine the level of *Hint1* in GC cell lines. Among the two GC cell lines, AGS displays lower level of *Hint1* than BGC-823. That is, the expression of *Hint1* was more significantly inhibited in GC cell line AGS. Similar results were obtained in repeat studies.

The level of Hint1 ascends after AGS treatment by 5-Azadc. The expression of specific tumor suppressor genes is often inhibited in cancer cells via methylation of specific cytidine residues in the corresponding promoter regions of these genes and also via other modifications that alter chromatin structure. There was a previous study which suggested that the relatively

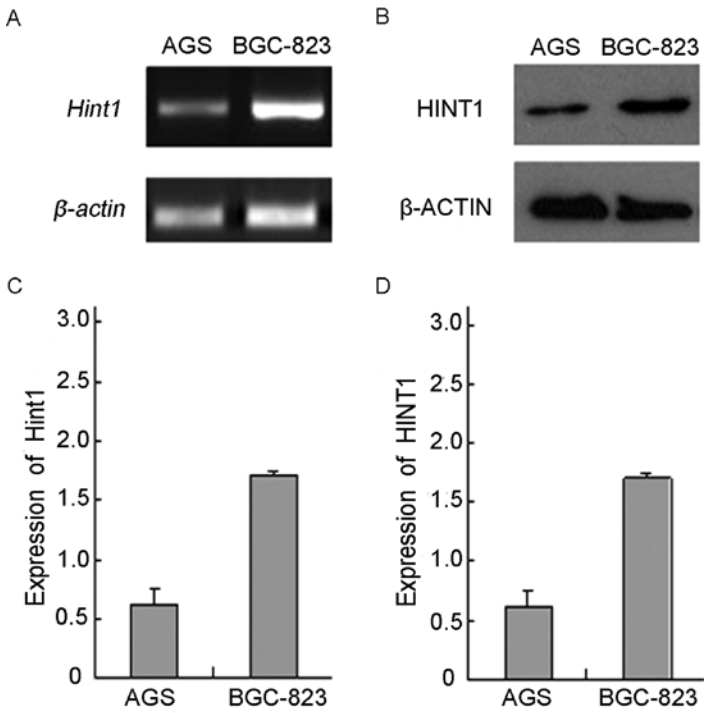

Figure 4. (A and C) The cells were cultured and then treated as described previously in Materials and methods. When it was detected by RT-PCR, the expression of *Hint1* in AGS was lower than that in BGC-823. (B and D) Similar result was confirmed by Western blotting. Expression ratios for HINT1 were calculated after normalization for *β-actin*. Assays were done in duplicate and the same results were obtained.

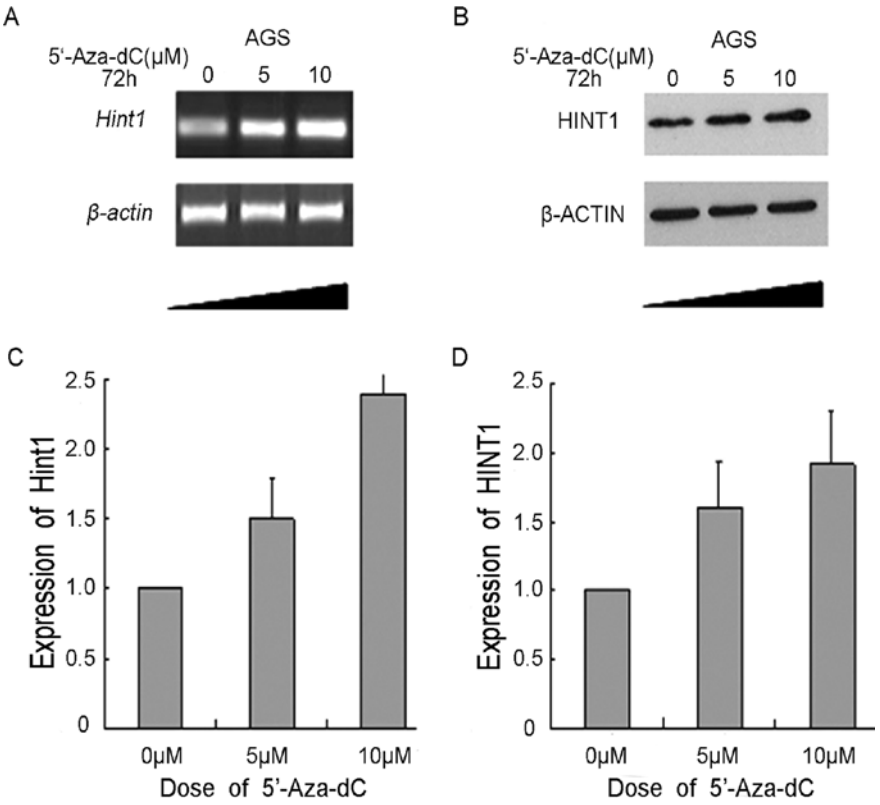

Figure 5. (A and C) The GC cell AGS was treated with 0, 5 and 10 μmol/l 5-Azadc for 72 h. Total RNA was isolated and semiquantitative RT-PCR was done with specific *Hint1* and *β-actin* (internal loading control) primers. PCR products were analyzed on 2% agarose gels and visualized under UV illumination. (B and D) Cells were treated with 5-Aza-dC as described above, and the same results were gained by Western blot analysis.

low levels of HINT1 in human HCC cell lines HepG2 and Hep3B (10), NSCLC cell line H522 (17) and colon cancer cell line SW480 (12) may be due, at lease in part, to promoter methylation. Therefore, we treated AGS with 5 and 10 μmol/l

5-Aza-dC for 72 h and then examined by RT-PCR and Western blotting the levels of expression of HINT1 mRNA and protein, respectively. We found that the level of HINT1 could be induced by treatment with 5-Aza-dC and the elevation was dose-dependent. The same results were obtained in triplicate studies.

Discussion

Previous investigations with genetically engineered mice evidenced that *Hint1* is a novel haploinsufficient tumor suppressor gene (6). Its potential mechanism of tumor progression was characterized in human hepatocellular carcinoma (HCC) (16). However, the mechanism and clinical significance of HINT1 is unclear in other types of human cancer. In the present study, we examined HINT1 protein expression levels by Western blotting in tissues from GC patients and we found 20 cases showing T<N, 70% (14 cases) displayed poor differentiation, but 29 cases showed T≥N, only 37.93% (11 cases) displayed poor differentiation (p=0.027). This result suggested that comparing with moderately/well differentiated cases, the expression of HINT1 was decreased more in poorly differentiated GC tissues. Infection of *H. pylori* and EB are considered as important carcinogenic factors in gastric carcinogenesis (2,3). In the present study, we found at least in protein level either *H. pylori* or EB infection was associated with the reduced expression of HINT1 in GC tissues (p=0.005). Moreover, in the *H. pylori* infection group, *Hint1* mRNA expression was significantly lower in the GC tissues than in the non-tumor tissues (p=0.039), while in the uninfection group, there was no significantly statistical difference. However, in the EB-negative group, the mRNA expression level of *Hint1* was significantly higher in the adjacent non-cancerous tissues (p=0.001), whereas, there was no obvious statistical significance in the positive group. Therefore, we found that the protein level the expression of HINT1 was more likely repressed in GC tissues with poorly differentiated, *H. pylori* or EB infection. Furthermore, the mRNA expression of *Hint1* was suppressed more in *H. pylori*-positive and EB-negative GC tissues.

Previous studies indicate that HINT1 is recruited to ionizing radiation-induced foci (IRIF) and associates with γ-H2AX and ATM in response to ionizing radiation (IR)-induced or radiomimetic drug bleomycin-induced DNA damage responses, and plays an important role in modulating the appropriate responses to DNA damage in mammalian cells (45). In addition, HINT1-deficient cells exhibit resistance to IR-induced apoptosis and several types of chromosomal abnormalities (5,45). Overexpression of HINT1 in the LM217 cell line increases the sensitivity of these cells to ionizing radiation (46). These findings clarify that HINT1 may play a role in radiation sensitivity at least by either ionizing radiation or radiomimetic drug such as bleomycin. As stated previously, we can suggest that patients with low or deficient expression of HINT1 may clinically appear resistant to radiotherapy or chemotherapy.

In the present study, we found HINT1 expression in AGS was lower than that in the other human GC cell line BGC-823. Previous investigations found decreased expression of *Hint1* in human HCC cell lines HepG2 and Hep3B is due to methylation of the promoter region of the Hint1 gene (44). Similar evidence was obtained in human NSCLC cell lines (17) and colon cancer cell SW480 (12) based on studies using 5-Aza-dC. When we treated AGS with 5-Aza-dC we found that the level of HINT1 could also be induced and the elevation was dose-dependent. Thus, we suggest that decreased expression of *Hint1* in human GC cell line AGS is, at least in part, due to methylation of the promoter region of the *Hint1* gene.

Based on the above findings, we investigated promoter hypermethylation of *Hint1* in several DNA samples from GC patients. In four cases with HINT1 underexpression, methylation bands were determined in GC tissues and unmethylation bands were determined in paired adjacent non-tumor tissues (data not shown). The result suggested that promoter methylation status may inversely correlate with the HINT1 expression in GC tissues.

Immunohistochemical (IHC) data showed that HINT1 was localized in both nuclei and cytoplasm in the majority of moderately differentiated GC tissues while it was undetectable in poorly differentiated GC tissues. Besides, it was widely expressed in the paired adjacent non-cancerous tissues. These results were in line with the result from Western blotting. However, there were still 2 of 10 moderately diffe-rentiated and poorly differentiated GC tissues that showed inverse results (data not shown). Previous IHC study suggested that 83% of unmethylated HCC samples demonstrated positive nuclear and cytoplasmic staining, and 64% of methylated HCCs showed loss of expression of HINT1, and in adjacent non-tumor samples 87% of unmethylated samples demonstrated positive nuclear and cytoplasmic staining and 53% of methylated samples showed loss of expression of HINT1 (16). Therefore, we can infer that in our study the 2 of 10 moderately differentiated GC tissues with lack of HINT1 expression detected by IHC may due to promoter methylation, and likewise the poorly differentiated GC tissues with positive HINT1 expression detected by IHC may due to unmethylation of the promoter.

In conclusion, this was the first study on detection of HINT1 expression in GC tissues. HINT1 was underexpressed in GC tissue in protein and mRNA levels. Its downregulation was associated with poorer tumor cell differentiation and viral infection such as by *H. pylori*/EB virus, suggesting patients with underexpressed HINT1 may have biologically aggressive tumor and poor prognosis. Underexpression of HINT1 in GC may also be due to promoter methylation and plays a role in gastric tumorigenesis.

Acknowledgements

This work was supported by Nanjing Medical Key Scientific Foundation, (no.: ZKX08012) and the Open Project Program of Key Laboratory of Developmental Genes and Human Diseases, Ministry of Education, Southeast University (no.: 200903). We are most grateful for the experimental platform provided by the Key Laboratory.

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