

## Expression Profile of Histone Deacetylase 1 in Gastric Cancer Tissues

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Although histone deacetylases (HDACs) appear to play a crucial role in carcinogenesis, the expression status of HDACs in primary human cancer tissues has not yet been reported. In this study, we investigated the expression level of HDAC1 in 25 paired primary human gastric cancer (GC) tissues and corresponding normal tissues through semi-quantitative RT-PCR and immunoblot analysis. The HDAC1 expression pattern was also topologically examined through immunohistochemistry. Overexpression of HDAC1 mRNA was detected in 68% of GC tissues (17 of 25), and the relative density of HDAC1 mRNA in GC tissue was increased 1.8-fold versus the normal counterpart ( $P < 0.01$ ). Elevated expression of HDAC1 protein was also detected in 61% of GC samples (11 of 18), which also showed an increased mRNA level of HDAC. Immunohistochemically, overexpression of HDAC1 was predominantly localized in the nuclei of most neoplastic cells, including embolic tumor cells, whereas normal glandular epithelial cells revealed only weak HDAC1 expression that was focal in distribution. Thus, the present study clearly demonstrates that HDAC1 is overexpressed in GC and probably plays a significant role in gastric carcinogenesis.

Key words: HDAC1 — Gastric cancer — RT-PCR — Immunoblotting — Immunohistochemistry

Recent extensive studies have shown that histone modification through reversible acetylation is a crucial event in gene expression. The status of histone acetylation is controlled by histone acetyltransferase (HAT) and histone deacetylase (HDAC).<sup>1,2</sup> HAT acetylates four lysine residues of core histones, resulting in activation of gene expression, whereas HDAC deacetylates the acetylated lysine residues, leading to an inactive chromatin structure.<sup>3</sup> Notably, deletion or inactivation of the *HAT* gene causes a genetic disease known as Rubinstein-Taybi syndrome that shows high incidence of malignant tumor.<sup>4</sup> Furthermore, several tumors are also known to have higher HDAC activities than the normal cells.<sup>5</sup> These data imply that loss or decrease of histone acetylation could be implicated in tumorigenesis.<sup>2</sup>

Thus, HDAC is of interest as a novel anti-tumor therapeutic target.<sup>6</sup> Eight HDAC isotypes have been cloned from human so far. Moreover, HDAC has been cloned from *Drosophila*, yeast, and maize. These findings thus suggest a conserved function of HDAC in eukaryotes.<sup>7–9</sup> Several studies have demonstrated that the inhibition of HDAC by a selective inhibitor, such as trichostatin A or trapoxin, induces the silencing of tumor suppressor genes in various human cancer cell lines and leads to growth arrest.<sup>6, 10–12</sup> Recently, a synthetic HDAC inhibitor, MS-

27-275, was also revealed to inhibit the growth of several tumor lines implanted into nude mice.<sup>13</sup> Thus, HDACs appear to play a crucial role in human cancer. However, the expression status of HDACs in human fresh cancer tissues has not yet been reported.

In this study, we investigated the expression level of HDAC1 in primary human gastric cancer (GC) tissues through semi-quantitative RT-PCR and immunoblot analysis. The expression level of HDAC1 was compared in paired normal and GC tissues. The expression pattern was also topologically examined through an immunohistochemical method.

### MATERIALS AND METHODS

**Patient samples** A total of 25 samples were collected from gastric cancer patients during partial or total gastrectomy. Immediately after removal, portions of about 0.5 to 1 g of tumor tissues and normal gastric mucosa distant from the cancerous area were quickly frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  until analysis. The remaining portions were fixed in 10% phosphate-buffered neutral formalin, routinely processed, and stained with hematoxylin and eosin (H&E) for pathologic diagnosis. This study was performed in accordance with the Declaration of Helsinki.

**RNA isolation and semi-quantitative RT-PCR** Total RNA was isolated from normal and GC tissues using “Trizol reagent” (GIBCO BRL, Life Technologies, Inc.,

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Gaithersburg, PA). Purity and the amount of RNA were measured using a spectrophotometer. RNA (0.3  $\mu\text{g}$ ) was used for reverse transcription, and each PCR reaction was carried out according to the manufacturer's protocol using the GeneAmp RNA PCR kit (Roche Molecular Systems, Inc., Branchburg, NJ). The PCR primer pairs of HDAC1 used were as follows: sense, 5'-CCACATCAGTCCTTCAATA-3', and antisense, 5'-TTCTCCTCCTTGGTTTCTC-3'. After the PCR reaction, the products were analyzed on 1.5% agarose gel and visualized through ethidium bromide staining.

**Immunoblot analysis** The frozen tissues were homogenized in lysis buffer containing 50 mM Tris HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 100  $\mu\text{g}/\text{ml}$  phenylmethanesulfonyl fluoride (PMSF), 1  $\mu\text{g}/\text{ml}$  aprotinin, and 1% Triton X-100 using a glass-on-glass homogenizer on ice. After sonification for 5 min, the homogenate was centrifuged at 12 000 rpm for 15 min, and the supernatant was collected. Protein concentration was determined using a spectrophotometer set at 280 nm. Thirty micrograms of total protein was separated on 6% polyacrylamide gel containing 10% lauryl sulfate, 7.5 mM Tris-HCl, 0.035% TEMED, and 7 mg of ammonium persulfate. They were transferred onto a nitrocellulose membrane at 36 mA in a transfer buffer containing 39 mM glycine, 48 mM Tris base, 0.037% lauryl sulfate, and 20% methanol. Non-specific binding was prevented by treatment with a blocking buffer containing 5% non-fat dry milk and 0.1% Triton X-100 in PBS (pH 7.4). The membrane was subsequently incubated for 2 h with polyclonal rabbit anti-human HDAC1 antibody (Upstate Biotechnology, Lake Placid, NY) at 1:2000 dilution. Horseradish peroxidase-conjugated anti-rabbit immunoglobulin (Amersham Pharmacia Biotech, Buckinghamshire, UK) was used as the secondary antibody at 1:1500. HDAC1 was then detected using the ECL detection reagent (Amersham Pharmacia Biotech).

**Immunohistochemistry** Immunohistochemical staining was performed to detect the expression of HDAC1 in tissues. Sections of paraffin-embedded tissues (3  $\mu\text{m}$  thick) from patients with GC and matching normal tissues were placed on Probe-On slides (Fisher Scientific, Pittsburgh, PA), deparaffinized, and rehydrated. After inhibition of the endogenous peroxidase activity with methanol containing 3%  $\text{H}_2\text{O}_2$ , tissue sections were heated in 0.01 M sodium citrate buffer (pH 6.0) in a pressure cooker for 6 min to retrieve the antigen. After blocking of non-specific binding by treating the slides with 10% normal goat serum at 37°C for 60 min, the slides were incubated at 4°C overnight with rabbit anti-human HDAC1 (Upstate Biotechnology) at 1:100 dilution. The sections were washed and incubated with biotinylated goat anti-rabbit IgG (Vector Lab, Burlingame, CA) for 90 min at 1:200 dilution. They were then washed again and incubated with Streptavidin (DAKO, Copenhagen, Denmark) for 60 min at 1:200 dilu-

tion. 3,3-Diaminobenzidine was used as a chromogen to reveal the antigen, and the sections were counterstained with Harris hematoxylin. Negative control tissues were prepared in the same manner as described above, except for the omission of primary antibodies and the substitution of an isotype-matched but irrelevant antibody.

**Densitometry and statistical analysis** Densitometric measurements of the bands in RT-PCR analysis and western blotting were performed using the Scion image computer program (Scion Corp., Frederick, MD). The results were analyzed by using Student's *t* test. A value of  $P < 0.05$  was considered to be statistically significant.

## RESULTS

To investigate the mRNA level of HDAC in GC tissues, we used 25 patient-matched normal and cancer tissues, in order to minimize the influence of individual variation. Overexpression of HDAC1 was detected in 68% of GC tissues (17 of 25) (Fig. 1A). As shown in Fig. 1B, the relative density of HDAC1 mRNA in 25 GC tissues was  $1.34 \pm 0.54$ , an 1.8-fold increase, compared to  $0.82 \pm 0.36$  in

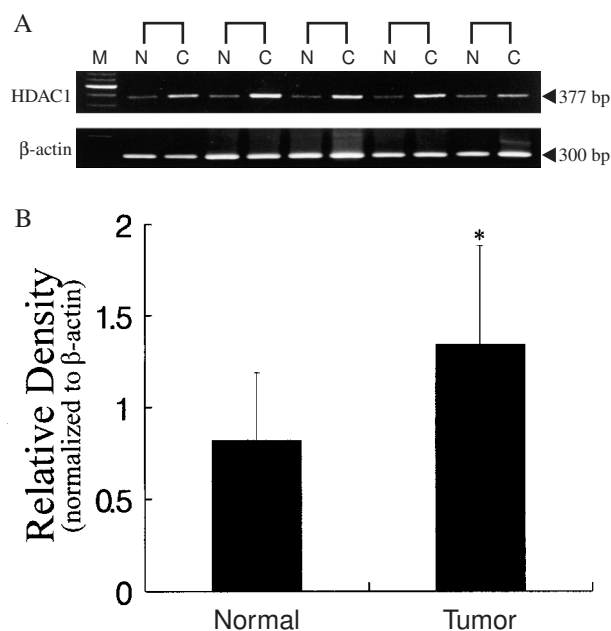


Fig. 1. Increased expression of HDAC1 mRNA in human gastric cancer. (A) A representative RT-PCR analysis of HDAC1 mRNA expression in pair-matched normal and gastric cancer tissues. Lanes: N, normal tissue; C, cancer tissue; M, molecular marker. (B) Mean relative density of HDAC1 PCR product of normal and cancerous tissues. A 1.8-fold increase in the amount of HDAC1 mRNA was detected in gastric cancer compared to the normal tissue ( $P < 0.01$ ).

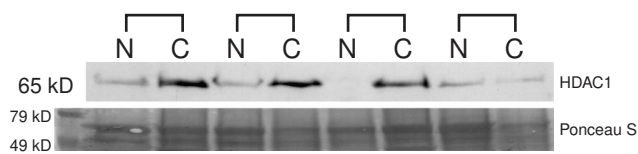


Fig. 2. A representative immunoblot analysis of HDAC1 in pair-matched normal (N) and gastric cancer tissues (C) obtained from the same patient, upper panel. Lower panel, Ponceau S staining. The first 3 pairs: overexpression of HDAC1 was noted in C compared to N. The last paired sample showed similar levels of HDAC1 expression in C and N.

the paired normal tissue ( $P < 0.01$ ). We also examined the HDAC1 protein levels of 18 available samples through immunoblotting. Compared to the normal gastric mucosal tissues, a  $6.3 \pm 2.1$ -fold increase in the expression of HDAC1 protein was detected in 61% of GC samples (11 of 18) (Fig. 2). All 11 samples with HDAC1 protein overexpression also concomitantly had increased HDAC1 mRNA levels.

Immunohistochemical staining was considered positive only if a strong nuclear staining was observed, since HDAC1 is known to be present in nuclei.<sup>14)</sup> The expression of HDAC1 was predominantly localized in the nuclei of normal glandular epithelial cells, whereas non-neoplastic elements such as stromal, inflammatory, and smooth muscle cells showed negligible staining. In normal pyloric glandular epithelium, the distribution of HDAC1 expression appeared random (Fig. 3A). However, in gastric cancer tissues, some degree of heterogeneity of HDAC1 expression was detected even within some morphologically similar areas of the tumors, though most tumor tissues showed diffuse expression (Fig. 3B). Clusters of embolic tumor cells in the vessels were also strongly positive for HDAC1 (Fig. 3C).

Based on the results of semi-quantitative RT-PCR, western blotting and immunohistochemistry, overexpression of HDAC1 may be a common event in gastric cancer and may play a significant role in the tumorigenesis. The immunohistochemical method may be useful for detecting the status of HDAC expression in gastric cancer tissues.

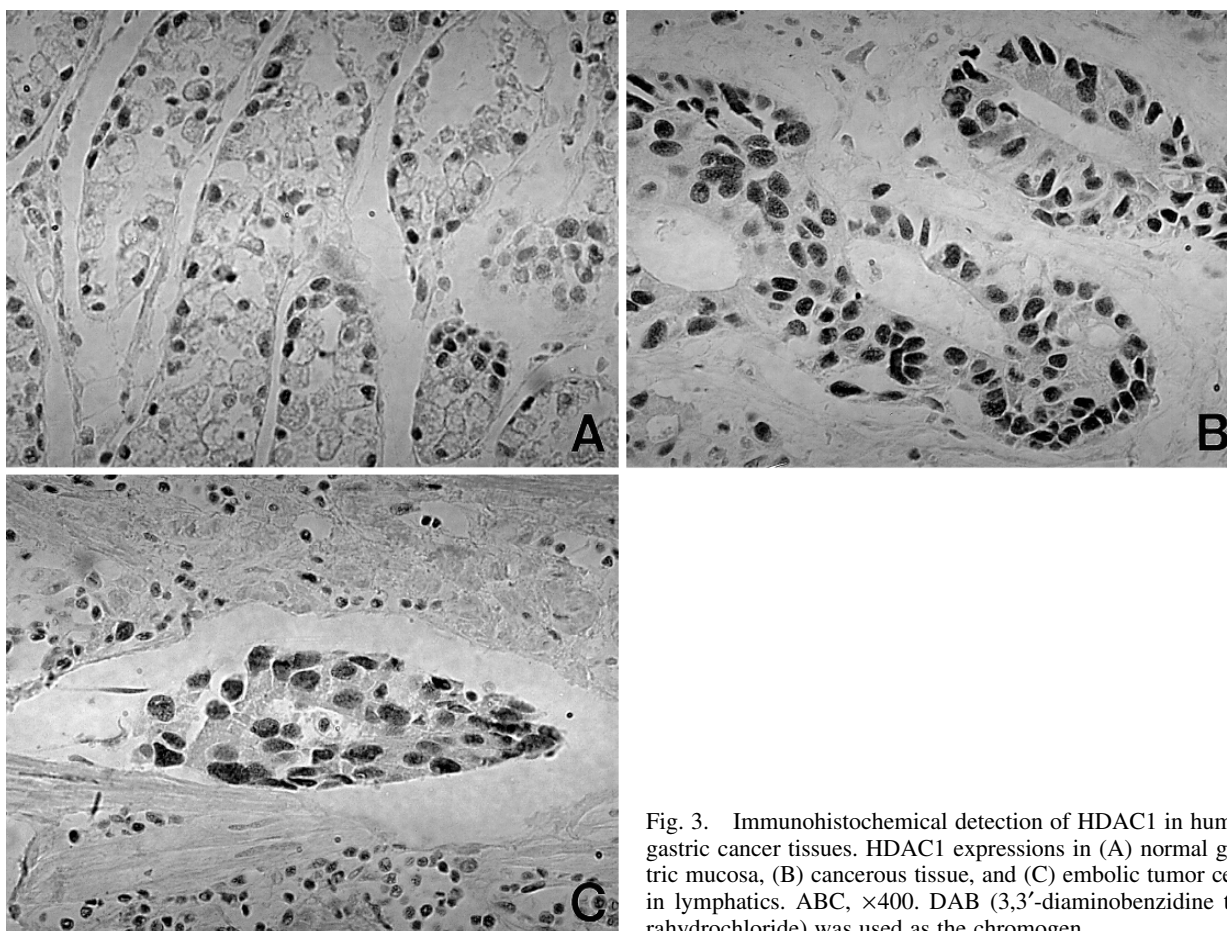


Fig. 3. Immunohistochemical detection of HDAC1 in human gastric cancer tissues. HDAC1 expressions in (A) normal gastric mucosa, (B) cancerous tissue, and (C) embolic tumor cells in lymphatics. ABC,  $\times 400$ . DAB (3,3'-diaminobenzidine tetrahydrochloride) was used as the chromogen.

## DISCUSSION

Despite extensive investigation of the biological functions of HDACs, little is known about the expression status of HDACs in primary neoplastic tissues. Our results clearly show that HDAC1 is upregulated in GC tissues at both transcriptional and translational levels. The overexpression of HDAC1 causes downregulation of tumor suppressor genes, such as *p53* and von Hippel-Lindau.<sup>5)</sup> These data imply that elevated HDAC1 expression may cause histone hypoacetylation leading to silencing of several tumor suppressor genes in GC. Supporting this idea, the physiological consequence of hypoacetylation due to deletion or inactivation of HAT in Rubinstein-Taybi syndrome is a high incidence of malignant tumors.<sup>4)</sup>

Gray and Ekstrom reported that TSA treatment of Hep 3B cell line causes the upregulation of HDAC1.<sup>15)</sup> The effect of TSA on the expression of HDAC1 decreased to the normal level as the cells reached 70% and 100% confluence, with a reduced growth rate. In peripheral blood mononuclear cells, TSA and butyrate are also known to elevate the expressions of HDAC1, 2, and 3.<sup>16)</sup> These results suggest that the expression of HDAC may be upregulated via an auto-regulatory loop, leading to elevated HDAC expression in rapidly growing cells, in agreement with the results of our immunohistochemical analysis. Thus, in human GC, a regulatory factor related to the auto-regulatory loop may be altered, causing the overexpression of HDAC. Details of the mechanism responsible for the HDAC auto-regulatory loop remain to be determined.

Although several genes have been shown to be upregulated with the increasing level of acetylated histone, not all appeared to be upregulated.<sup>17)</sup> A specific inhibitor of HDAC provides an important approach for studying the

function of chromatin acetylation. Only 2% of the genes were upregulated following treatment with HDAC inhibitor.<sup>6)</sup> These genes include regulators of the cell cycle and apoptosis, such as *p21<sup>waf1</sup>*, *c-myc*, plasminogen activator, gelsolin, and *p53*.<sup>6, 18)</sup> Thus, the inhibition of HDAC leads to the arrest of the cell cycle at the G1 and G2 phases and to differentiation or apoptosis of the tumor cells. Recently, it was reported that TSA inhibited the growth of GC and induced apoptosis of the cells through modulation of the expression of cell cycle regulators and the apoptosis-regulating protein.<sup>19)</sup> Shin *et al.* also showed that the inhibition of HDAC by *n*-butyric acid or TSA leads to the activation of *p21* and *p57* genes without activating *p27* in gastric cancer cells.<sup>20)</sup> Thus, the formation of deacetylated histone by HDAC appears to play a significant role in the neoplastic transformation of gastric glandular epithelial cells in part through the inactivation of the cell cycle regulatory genes.

In conclusion, the present study clearly demonstrated that HDAC1 is overexpressed in GC and probably plays a significant role in gastric carcinogenesis. Our results also indicate that a new HDAC inhibitor with less cellular toxicity could have potential as a cancer therapeutic agent. Further studies using larger numbers of samples and covering other HDACs are needed to identify the cell cycle regulators affected by HDACs and to assess the feasibility of using HDACs as prognostic factors in gastric cancer.

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