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Histone deacetylase 1 expression in gastric cancer

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Abstract. The aim of this study was to identify and evaluate novel prognostic markers for gastric cancer. Differential mRNA displays comparing paired tumor/normal stomach samples were assessed. Several differentially expressed cDNA fragments of candidate genes were identified, and one of these was further studied using quantitative reverse transcription-PCR in 140 human gastric carcinomas. To evaluate protein expression, immunohistochemical staining was performed in selected cases. One of the genes abundantly expressed in tumor tissue on the differential mRNA displays was identified as histone deacetylase 1 (HDAC). HDAC was overexpressed in the tumor tissue in 77% of the cases as determined by quantitative reverse transcription-PCR. Immunohistochemical staining revealed analogous results, showing strong expression in cancer cells. Patients were then classified into high (n=78) and low (n=62) expression groups according to the mean value of HDAC expression. High frequencies of lymph vessel and vascular vessel permeations, and advanced stage of the disease were recognized in the high expression group compared to the low expression group ($p<0.05$). Prognosis was significantly worse for the high expression group than for the low expression group ($p<0.05$), and multivariate analysis demonstrated that HDAC expression was an independent prognostic factor. Although not significantly different, lymph node metastasis was recognized more frequently in the high expression group ($p=0.07$). In conclusion, the findings show that HDAC expression is associated with aggressive behavior of primary gastric cancer, and imply that determination of the HDAC expression status is useful for predicting prognosis in patients with gastric cancer.

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Abbreviations: HDAC, histone deacetylase 1; mRNA, messenger RNA; cDNA, complementary DNA; RT-PCR, reverse transcription polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Key words: histone deacetylase 1, gastric cancer, prognostic factor, differential display

Introduction

We previously used the techniques of differential mRNA display or cDNA microarray between tumor/normal paired samples of gastrointestinal cancers to determine novel prognostic markers (1-3). After the reports, other research groups identified differentially expressed mRNAs related with tumorigenesis and lymph node metastasis in gastric cancer using similar techniques (4,5). In the present study we further identified several differentially expressed cDNA fragments between tumor/normal paired samples of the stomach. One of these cDNA fragments was cut from the gel, cloned and sequenced to reveal its identity as histone deacetylase 1 (HDAC).

It is known that major epigenetic mechanisms that modulate chromatin structure and regulate gene transcription involve DNA methylation and histone acetylation (6). Up-regulation of transcription is often noted in conjunction with hypomethylated DNA sequences associated with acetylated core histones. Conversely, down-regulation is often noted in cases with hypermethylated DNA sequences associated with deacetylated core histones. The status of histone acetylation is controlled by histone acetyltransferase and histone deacetylase (6).

HDAC expression has been reported to be up-regulated in malignant tissue compared to benign tissue in various organs such as breast (7,8), lung (9), pancreas (10) or prostate (11). With respect to the stomach, Choi *et al* reported overexpression of HDAC in malignant tissues in 68% of 25 cases (12). However, the significance of its overexpression was not studied from the clinical and pathological viewpoints. Thus, we evaluated the expression status of HDAC and extended this to include the clinical and pathological significance in 140 cases of gastric carcinoma. We herein report the isolation of HDAC from differential displays, the results of quantitative RT-PCR of expression of this gene in clinical gastric cancer cases, and a correlation between the gene expression status and clinicopathological factors. Notably, this study suggests that HDAC expression may be a new prognostic marker for patients with gastric cancer.

Materials and methods

Identification of HDAC from differential displays. The mRNA obtained from human gastric cancer tissue and that from corresponding normal tissue were analyzed by the modified protocol of fluorescent differential display (13). In brief, a total of 2.5 μg of RNA was mixed with 50 pmol of the fluorescein isothiocyanate (FITC)-labeled 3'-anchored olig-dT primer, heated at 70˚C for 10 min and chilled. Ten microliters of th 2X RT solution was added and incubated at 25˚C for 10 min, 42˚C for 50 min and 90˚C for 5 min. The reaction mixture was diluted 5-fold by addition of 80 μl of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Subsequently, 1 nmol dNTP, 1 unit of Taq DNA polymerase, 10 pmol of arbitrary primer, 5 pmol FITC labeled 3'-anchored oligo-dT primer and 2 μl of cDNA solution were added to the reaction mixture. The mixture was subjected to a secondary process, and the thermal cycling protocol was as follows: 94˚C for 3 min, 37˚C for 5 min and 72˚C for 5 min for second-strand synthesis, followed by 20-25 cycles at 95˚C for 15 sec, 55˚C for 1 min and 72˚C for 2 min for amplification. Each PCR product was mixed with the same amount of dye solution and denatured at 90˚C for 2 min. Samples was applied to 6% polyacrylamide gel, and electrophoresis was performed at 800 V for 2.5-3 h. One particular band that was markedly increased in the cancer tissue compared to normal tissue was cut out, cloned and sequenced.

Northern blot analysis. To confirm the expression of the gene isolated from the differential displays, Northern blot analysis was performed as described elsewhere (1,2). The paired samples of tumor/normal tissues obtained from 5 cases of gastric cancer were used. These 5 cases were randomly selected from the 140 patients described below. In brief, equal amounts (15 μg) of total RNA were loaded on each lane of 1.0% agarose-formaldehyde gels and electrophoresed overnight. The RNAs were transferred to nylon membranes (GeneScreenPlus; Dupount, Boston, MA). After overnight hybridization at 42˚C, followed by washing, the membranes were exposed to X-ray film (Kodak X-OMAT AR, Rochester, NY) at -70˚C. After the membranes were washed, they were used for another hybridization with actin probe as an internal control.

Immunohistochemistry. An immunohistochemical study of HDAC was performed on specimens available from 10 of 140 cases of gastric carcinoma described below using the avidin-biotin-peroxidase method (LSAB2 kit; Dako, Kyoto, Japan) on formalin-fixed, paraffin-embedded tissues (14). Tissue sections were de-paraffinized, soaked in 0.01 M sodium cytorate buffer, and boiled in an electronic oven for 15 min at 500 W to retrieve cell antigens. The tissue sections were immunohistochemically stained using the streptavidin-biotin peroxidase method (Universal Dako Cytomation LSAB® kit; Dako) with a primary antibody against rabbit anti-human HDAC1 anti-sera (Upstate Biotechnology, Waltham, MA) used at a dilution of 1:150. In brief, the sections were blocked by 3% H_2O_2 for 5 min and incubated overnight with the primary antibody at 4˚C. The samples were then washed with TBS buffer and subsequently incubated with the secondary antibody for 30 min. All sections were counterstained with hematoxylin.

Clinical samples and RNA extraction. One hundred and forty fresh surgical specimens of primary gastric cancer were used. The samples were stored at -90˚C until use, and total RNA was prepared (1,2). To avoid contamination by genomic DNA, 50 mg of total RNA was treated with one unit of DNase I (Message Clean kit, GenHunter Corp., Nashville, TN) at 37˚C for 1 h in the presence of one unit of RNase inhibitor, followed by phenol/chloroform purification and ethanol precipitation. The treated RNA was stored at -90˚C until use.

Quantitative RT-PCR analysis. cDNA was synthesized from 8 μg of total RNA (15,16). Real-time PCR for HDAC1 and GAPDH cDNA was performed using a LightCycler thermal cycler system (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. The primers used were as follows: HDAC1 sense primer, 5'-CCACATCA GTCCTTCCAAT-3' and antisense primer, 5'-TTCTCCTCC TTGGTTTTCTC-3'; GAPDH sense primer, 5'-TTGGTATC GTGGAAGGACTCA-3' and antisense primer, 5'-TGTCA TCATATTTGGCAGGTTT-3'. For PCR, 1 μl RNA was placed into a 19-μl reaction volume containing 0.67 μl primer, 1.2 μl 25 mM $MgCl_2$, and 2 μl LightCycler-FastStart DNA Master SYBR-Green I mix (Roche Diagnostics). The protocol included a denaturation step at 95˚C for 60 sec followed by 26 cycles that each included denaturation at 95˚C for 20 sec, annealing at 56˚C for 20 sec, and extension at 72˚C for 20 sec. Negative controls were run concomitantly to confirm that the samples were not cross-contaminated. A sample with 1 μl diethylpyrocarbonate-treated water instead of RNA was concomitantly examined for each of the reactions described above. After amplification, the products were subjected to a temperature gradient from 68˚C to 95˚C at 0.2˚C/sec with continuous fluorescence monitoring to produce a melting curve of the products. After proportional background adjustment, the fit point method was used to determine the cycle in which the log-linear signal was distinguished from the background, and this cycle number was used as a crossing-point value. The standard curve was produced by measuring the crossing point of each standard value (2-fold serially diluted cDNAs of AZ521) and plotting them against the logarithmic value of the concentrations. The concentrations of each sample were then calculated by setting their crossing points to the standard curve. The expression levels were normalized to GAPDH. The cases were classified into two groups according to the mean value of 1.6; a HDAC high expression group (T/N ≥1.6; n=78) and an HDAC low expression group (T/N <1.6; n=62).

Clinicopathological data. Clinical variables and follow-up data were available for all patients, as shown in Table I. The data were compared between the HDAC high and low expression groups.

Statistical analysis. The BMDP Statistical Package program (BMDP, Los Angeles, CA) for the main frame computer (4381; IBM, Armonk, NY) was used for all analyses.

Table I. Clinicopathological data and HDAC1 expression.

Variables	HDAC1 expression		P-value
	High (78)	Low (62)	
Age (years)	66.3±12	68.0±13.1	N.S.
Gender			
Male	52	42	N.S.
Female	26	21	
Histological differentiation			
Well	15	12	N.S.
Moderate	30	21	
Poor	33	29	
Depth of invasion			
T1-T2	37	34	N.S.
T3-T4	41	28	
Lymph vessel invasion			
Absent	32	37	N.S.
Present	46	25	
Venous invasion			
Absent	46	47	N.S.
Present	32	15	
Lymph node metastasis			
Absent	36	38	0.07
Present	42	24	
Clinical staging			
Ia-Ib	13	15	<0.05
II	19	23	
IIIa-IIIb	27	17	
IV	19	7	

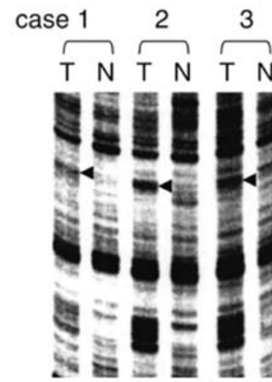

Figure 1. Differential display of cDNA subsets between gastric carcinoma (T) and adjacent non-neoplastic gastric mucosa (N). The PCR band which is strong in the carcinoma sample but weak in the non-neoplastic gastric mucosa, represents HDAC1, as shown by the arrow.

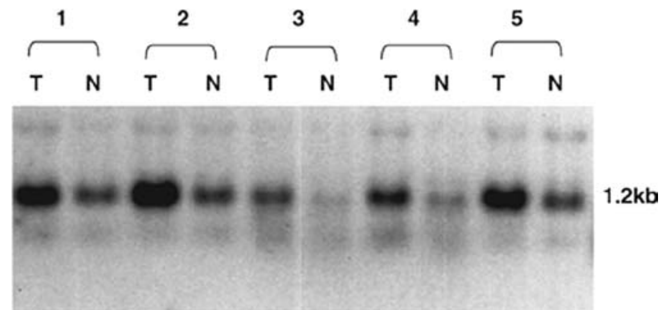

Figure 2. Northern hybridization with tumor (T) and non-tumor (N) paired RNA samples from 5 patients. The HDAC1 expression is strong in tumor tissue, but faint in non-neoplastic tissue. The filter was stripped and rehybridized to a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe to verify that the mRNA was intact and that equal amounts of RNA were loaded onto the gels.

Associations between the variables were tested by the Fisher's exact probability test. The BMDP P1L program was used for survival analysis (Kaplan-Meier method) and testing of the equality of the survival curves (Mantel-Cox method). The BMDP P2L program was used for multivariate adjustments for all covariates simultaneously, with a backward stepwise logistic regression analysis.

Results

Isolation of HDAC. One result of differential display subsets of cDNA between samples of three tumor/normal pairs of gastric tissues is shown in Fig. 1. The PCR product (arrow) was identified by differential display analysis. The expression of the product was strong in the tumor samples whereas the expression was faint in the normal samples. The strongly expressed band was cut out, and the contained product was cloned as mentioned in Materials and methods. The sequence of the product was matched with that of *HDAC*.

Northern blot analysis. The 377-base pair cDNA fragment obtained by PCR with the above-mentioned primers was used as a probe in the Northern blot analysis. All of the five tumor samples showed HDAC mRNA signals, while the normal samples showed only weak signals (Fig. 2). The results prompted us to further examine the significance of the difference between the high and low HDAC expression cases.

Immunohistochemistry. A relatively strong HDAC staining was recognized in the nucleus of the carcinoma cells (Fig. 3). In some cases, a certain extent of heterogeneity was noted within the tumor. On the other hand, only a weak staining was recognized in the normal glandular epithelium.

Real-time RT-PCR and clinicopathological data. Expression of *HDAC1* in tumor and normal tissues in 4 representative cases by Northern blotting is exhibited in Fig. 2, and the amplified RT-PCR products were measured by LightCycler 2000 as shown in Fig. 4. The patient group consisted of 93 men and 47 women. Their age ranged from 33 to 82 years with a mean of 66.9 years. The analysis between HDAC expression status and clinicopathological factors is shown in Table I.

According to the quantitative RT-PCR results 144 cases were divided into an *HDAC* high expression group and *HDAC*

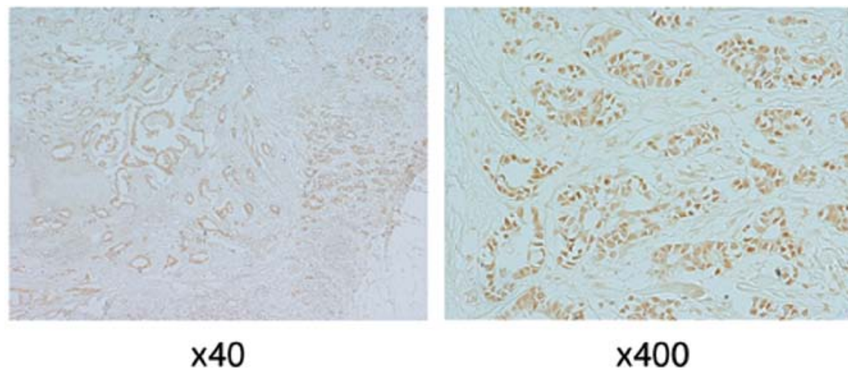

Figure 3. Immunohistochemical staining of HDAC1 in a gastric cancer case. A relatively strong HDAC staining was recognized in the nucleus of the carcinoma cells.

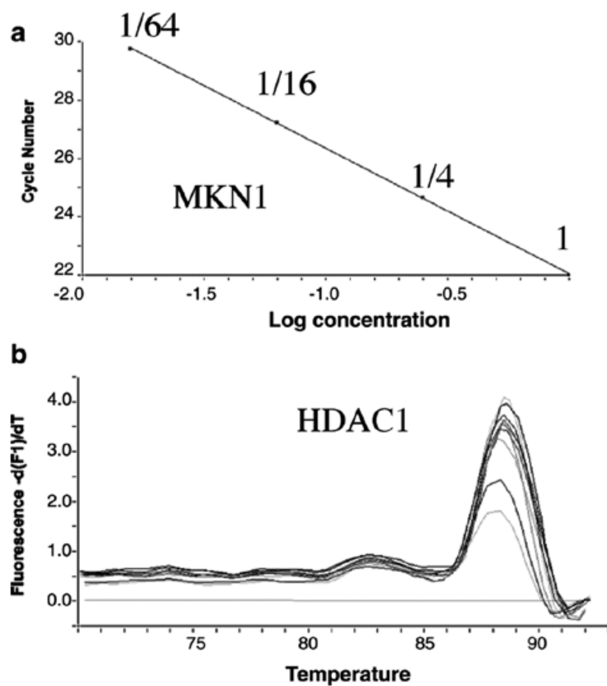

Figure 4. (a) The standard curve constructed by measuring the crossing point of each standard value (4-fold serially diluted cDNAs of MKN1). We plotted these against the logarithmic value of the concentrations. (b) Measurement of the RT-PCR product evaluated by the LightCycler 2000. The above ten samples consisting of 5 pairs of tumor and normal tissues show amplification of HDAC1 at the identical temperature.

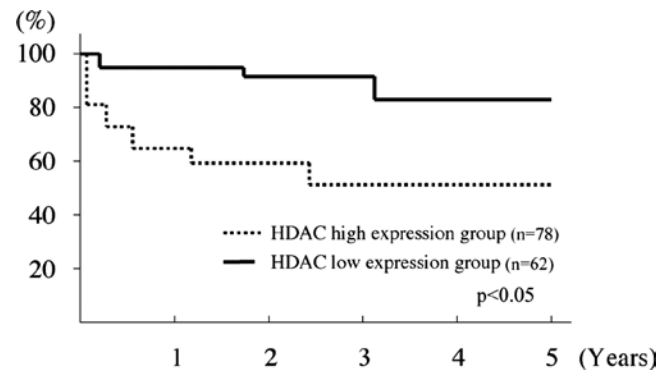

Figure 5. The survival curve of gastric cancer patients according to the expression status of HDAC. The patients with high HDAC expression demonstrated a worse survival than those with low HDAC expression ($p<0.05$).

low expression group. The 78 cases (55.7%) were classified into a high group and 63 cases (44.3%) were classified into a low expression group. Statistical analysis was performed to assess the correlation between HDAC expression and clinico-pathological factors. As shown in Table I, there were no significant differences between *HDAC* expression status and age, gender, histological differentiation, and depth of gastric wall invasion. In contrast, *HDAC* expression was significantly associated with depth of lymph vessel invasion, venous vessel invasion and stage of disease ($p<0.05$). Although not significantly different, lymph node metastasis was more frequently recognized in the high expression group than in the low expression group ($p=0.07$).

The 5-year survival was compared between the *HDAC* high expression group and the low expression group. As shown in Fig. 5 the patients with high expression showed a significantly worse survival than those with low expression ($p<0.05$).

Each of the ten variables of age, gender, histological differentiation, depth of tumor invasion, lymph vessel invasion, venous vessel invasion, lymph node metastasis and *HDAC* status were used in the Cox regression analysis. Lymph node metastasis and *HDAC* status were found to be significant prognostic factors (Table II).

Discussion

There are two protein families with HDAC activity: the SIR2 family and the classical HDAC family. The classical HDAC family consists of class I and class II members (17). The class I HDACs include HDAC1, 2, 3 and 8, and are most closely related to the yeast transcriptional regulator RPD3. The class II HDACs include HDAC4, 5, 6, 7, 9 and 10, and share domains with similarity to HDA1, another deacetylase found in yeast. The class I HDACs are expressed in most cell types, whereas the class II HDACs are expressed in a more restricted manner, suggesting that they correlate with cellular differentiation and developmental processes.

Table II. Results of the multivariate analysis.

Variables	Regression coefficient	Standard error	Odds ratio	P-value
Lymph node metastasis	2.20	0.55	9.22	<0.01
HDAC1 expression	1.84	0.63	6.15	<0.01

Recent studies have demonstrated that the loss or decrease of histone acetylation function, including that by HDAC overexpression, may be involved in tumorigenesis. Halkidou *et al* reported that HDAC was highly expressed in hormone refractory prostate cancer, and its overexpression led to an increase in proliferation and a shift towards the undifferentiated cytokeratin profile (18). Kawai *et al* reported that overexpression of HDAC in stable transfected breast cancer cell (MCF-7) clones induced loss of estrogen receptor (ER)-α and significantly increased cell proliferation and colony formation, whereas treatment of this clone with an HDAC inhibitor induced re-expression of ER-α mRNA and protein. They concluded that overexpression of HDAC modulates breast cancer progression by negative regulation of ER-α (7).

With respect to gastric carcinoma, expression of histone acetylation and deacetylation have both been studied. Ono *et al* reported that the acetylated histone H4 expression was reduced in gastric carcinomas, and its reduction was correlated with advanced stage, depth of tumor invasion, and lymph node metastasis. On the other hand, HDAC was reported to be overexpressed in 17 of 25 gastric carcinomas (19). In the present study, we investigated a larger number of cases and disclosed a similar up-regulation of HDAC. Furthermore, and notably, multivariate analysis demonstrated that the HDAC expression status as determined by quantitative RT-PCR was an independent prognostic factor for patients with gastric carcinoma.

The reasons why increased expression of the HDAC1 gene is associated with tumor progression remains uncertain. There are several postulated explanations. For example, Kim *et al* reported that overexpressed HDAC down-regulated expression of p53 and the von Hippel-Lindau tumor-suppressor genes and stimulated angiogenesis of human endothelial cells (20). The expression of HDAC is related to hypoxia, angiogenesis through suppression of hypoxia-responsive tumor-suppressor genes. In addition to p53 and von Hippel-Lindau tumor-suppressor genes, HDAC may regulate p21waf1, semaphorin III, gelsolin, and plakoglobin (β-catenin). These genes were revealed to be up-regulated following treatment with HDAC inhibitors, leading to the arrest of the cell cycle at the G1 and G2 phases and differentiation or apoptosis of tumor cells. Shim *et al* reported that HDAC inhibition leads to the activation of p21 and p57 genes without activating p27 in gastric cancer cells, suggesting an important role of HDAC in the neoplastic transformation of the stomach partly through the inactivation of cell cycle regulatory genes (21). Furthermore, the existence of small RNAs called microRNAs (miRs) that suppress vast numbers of genes (by only a thousand of miRs) are now understood to play significant roles in cancer cell proliferation, differentiation and apoptosis. HDAC is also suppressed by miR transcription, and Scott *et al* revealed that miR expression was significantly up-regulated by exposure to HDAC inhibitors in breast cancer cells (22). Considering these studies, HDAC has significant influence not only on tumor-suppressor-related genes but also on miR expression and function. The same phenomenon must be observed in gastric cancer.

HDAC inhibitors are an exciting new class of chemotherapeutic drugs (6,9,23). HDAC inhibitors function by displacing the zinc ion and thereby rendering the charge-relay system dysfunctional. TSA, with its hydroxamic acid group and its five-carbon atom linker to the phenyl group, has the optimal conformation to fit into the active site (10). HDAC inhibitors induce activation of differentiation programmers, inhibition of the cell cycle, induction of apoptosis, activation of the host immune response and inhibition of angiogenesis, thus playing varied roles as drugs for cancer treatment. Shim *et al* demonstrated that p21 is up-regulated in tumor cells treated with these agents in the absence of p53 (21). This is important for cancer therapy, as many types of cancer have no functional p53 and are therefore unable to arrest cells in a p53-dependent fashion. Recently, a variety of HDAC inhibitors are being developed, and several studies reported satisfactory results in cancer therapy (24-27). The discovery and development of specific HDAC inhibitors may be helpful for cancer treatment, and for elucidating the molecular mode of action for HDAC.

In conclusion, the status of HDAC expression may represent a novel prognostic marker for patients with gastric cancer. Further study is required to clarify the precise mechanism of HDAC gene expression in gastric cancer progression or metastasis for potential future therapeutic applications.

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