ORIGINAL ARTICLE

Expression of HDAC1 and CBP/p300 in human colorectal carcinomas

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Background: The histone-modifying enzymes histone deacetylase (HDAC) and histone acetyltransferase (HAT) control gene transcriptional activation and repression in human malignancies.

Aims: To analyse the expression of HDAC/HAT-associated molecules such as HDAC1, CREB-binding protein (CBP) and p300 in human colorectal carcinomas, and investigate the relationship between their expression levels and clinicopathological parameters.

Methods: Expression levels of HDAC1, CBP, and p300 in human colorectal cancer were investigated by immunohistochemistry. In situ hybridisation (ISH) and reverse transcription (RT)-PCR analyses were also carried out to confirm mRNA expression levels of these genes. Immunoreactivity was evaluated semi-quantitatively using a staining index (SI). The relationships between the SIs and clinicopathological findings were analysed and survival curves were calculated using the Kaplan–Meier method and log-rank tests.

Results: The mean SIs for HDAC1, CBP, and p300 in this series of tumours were much higher than those in normal colonic mucosa. The presence of *HDAC1* and *CBP* mRNAs on colorectal carcinoma cells as well as normal epithelial cells was confirmed by ISH analysis. A marked increase in *p300* mRNA levels was detected in a majority of cases by RT-PCR. Among the patients with colorectal cancer, overexpression of p300 (SI > 11.9) correlated with a poor prognosis, whereas high CBP expression levels (SI > 16.6) indicated long-term survival.

Conclusion: Results showed the up-regulation of these three histone-modifying molecules in this series of colorectal cancers and suggested that monitoring of CBP and p300 may assist prediction of the prognosis in patients with colorectal adenocarcinoma.

Histones are essential for packaging of DNA in eukaryotic cells. At the centre of the chromatin structure are highly conserved histone proteins that function as building blocks for packaging DNA into repeating nucleosomal units.¹ Recently, the histone-modifying enzymes histone deacetylase (HDAC) and histone acetyltransferase (HAT) have been shown to be involved in transcriptional activation and repression.^{1–2}

Eight HDAC isotypes have been cloned and classified into three distinct families (classes I, II, and III).² As class I HDACs, which are related to the yeast transcriptional regulator RPD3, are expressed in most types of cell, they are thought to be involved in cellular differentiation and developmental processes.³ In particular, HDAC1 is believed to play a key role in the development of various human malignancies, such as acute myeloid leukaemia, breast cancer, prostatic cancer, lung cancer, and gastric cancer.^{4–10}

CREB-binding protein (CBP) and p300, which are known as HAT molecules, are considered to be important transcriptional coactivators that act with other factors to regulate gene expression.¹¹ In total, they have about 60% homology.¹² Oncoprotein E1A activates HAT activity of CBP,¹³ while it disturbs HAT activity of p300,¹⁴ suggesting that CBP and p300 play different roles in cancer cells.¹⁵ These HATs regulate p53-dependent transcription and function as pleiotropic coactivators that facilitate activation by a wide variety of sequence-specific transcription factors.¹¹ Germline mutations in the *CBP* gene have been reported in Rubinstein–Taybi disease, while somatic alterations have been detected in hepatocellular carcinoma and acute myeloid leukaemia.^{16–18} In addition, somatic mutations in the *p300* gene have been found in gastric cancer, colon cancer, glioblastoma and acute myeloid leukaemia.^{19–21} Thus, dysfunction of CBP and/or p300 is believed

to contribute to tumourigenesis in various human malignancies.

In the current study, we evaluated expression levels of HDAC1, CBP, and p300 in human colorectal cancers in order to determine their roles in the development and progression of such tumours. Immunohistochemical, in situ hybridisation (ISH), and reverse transcription (RT)-PCR analyses were performed to investigate the expression levels of these HDAC/HAT molecules and mRNAs in the colorectal carcinoma tissue. We then examined the relationships between HDAC/HAT expression levels and various clinicopathological findings.

MATERIALS AND METHODS

Tissue samples

Tissue samples were obtained from 64 patients with colorectal carcinoma who underwent surgical resection (table 1). The patients had not received any therapeutic agents or irradiation before surgery. Histological classification and clinicopathological staging were performed according to the general rules for clinical and pathological studies on cancer of colon, rectum and anus along with the International Union against Cancer Classification.^{22–23} The depth of tumour invasion was classified into four groups: none or minimal invasion within muscularis mucosa (m), invasion of the submucosa (sm), invasion of the proper muscle (mp), and beyond mp (ss, se, si, a1, a2). A total of 64 patients with colorectal carcinoma were followed-up for 6 years; 17 cases were lost.

Abbreviations: CBP, CREB-binding protein; HAT, histone acetyltransferase; HDAC, histone deacetylase; ISH, in situ hybridisation; SI, staining index

Table 1 Relationship between clinicopathological findings and staining index (SI) for HDAC1 and CBP/p300 proteins

Clinicopathological parameter	Number of cases	SI, mean (SD)		
		HDAC1	CBP	p300
Gender				
Male	40	11.2 (3.2)	15.4 (4.4)	13.8 (3.4)
Female	24	10.9 (3.4)	17.2 (5.0)	15.9 (6.4)
Age (years)				
≤ 64	38	11.0 (3.0)	16.3 (5.3)	13.2 (3.6)
> 65	26	11.3 (3.3)	16.0 (4.3)	15.4 (5.3)
Tumour size (cm)				
≤ 5.0	25	10.8 (3.9)	16.1 (3.8)	13.3 (4.3)
> 5.1	39	11.3 (2.8)	15.7 (4.7)	15.4 (5.0)
Histology†				
Normal	20	5.3 (1.7)**	8.5 (3.7)**	7.1 (2.0)**
Adenocarcinoma	64	11.1 (3.2)	16.1 (4.7)	14.6 (4.8)
Well differentiated	23	10.7 (3.3)	16.8 (5.1)	12.6 (3.2)*
Moderately differentiated	19	10.6 (2.8)	15.8 (3.5)	12.5 (3.9)
Poorly differentiated	22	13.4 (4.0)	15.8 (4.3)	18.4 (4.6)
Medullary type	13	13.9 (5.8)	16.8 (2.5)*	20.1 (5.5)*
Pleomorphic type	9	12.3 (4.6)	13.2 (4.2)	16.3 (2.7)
Lymph node metastasis†				
Negative	29	11.7 (3.5)	16.1 (5.3)	13.7 (4.2)
Positive	35	10.6 (3.0)	16.2 (4.3)	15.2 (5.2)
Depth of invasion†				
m, sm, mp	12	12.3 (3.2)	16.6 (4.9)	14.5 (4.7)
ss, se, si, a1, a2	52	10.8 (3.2)	16.0 (4.7)	14.6 (4.9)
Stage†				
0	1	11.3	11.9	10.8
I	10	12.7 (3.4)	17.9 (4.5)*	15.2 (5.0)
II	18	11.1 (3.6)	15.9 (3.9)	13.7 (3.8)
III	29	10.8 (2.9)	16.5 (5.3)	14.7 (4.8)
IV	6	9.9 (3.3)	12.3 (2.8)	16.8 (8.0)

†Histological classification and clinicopathological staging were performed according to the General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus along with the International Union Against Cancer Classification.

* $p < 0.05$, ** $p < 0.01$ by Kruskal–Wallis test or Mann–Whitney test in poorly differentiated adenocarcinoma. HDAC, histone deacetylase; CBP, CREB-binding protein.

In addition, a total of 20 normal colonic mucosae from the resection margin of colorectal carcinomas were also used as controls. We also examined 17 fresh specimens of colorectal cancer and the corresponding normal colorectal mucosa for RT-PCR analysis. These tissues were immediately frozen in liquid nitrogen and stored until use. Informed consent was obtained from all patients before surgery; this study was carried out in accordance with the ethics code for Human Experimentation, Yamagata University School of Medicine.

Immunohistochemistry

Immunohistochemistry was performed with an avidin–biotin complex immunoperoxidase technique as described previously.¹⁴ The primary antibodies against HDAC1, CBP, and p300 were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). After blocking of the endogenous peroxidase activity each 4 µm of formalin-fixed and paraffin-embedded tissue was placed in 0.1 mM EDTA (pH 8.0, Nacalai, Kyoto, Japan) and microwaved for 23 min at 90°C to retrieve the antigenicity. The immunostaining was independently

evaluated by two observers (KI and MY). The staining index (SI) was calculated as described previously.^{24–25} Briefly, at least 50 fields in each tumour and non-tumour colorectal mucosa sections were evaluated to determine the proportion of tumour cells and the staining intensity of the nuclei in entire fields of the sections. The tumour cellularity was scored from 1 to 6 based on the proportion of tumour cells and the staining pattern of the section. The staining score was firstly estimated on a five-point scale (0, ±, 1+, 2+, and 3+) and a score was obtained according to that intensity.^{24–25} Where a homogeneous pattern was obtained, a visual estimate was made of both the predominant intensity and the minority value. The SI was calculated by multiplying the cellularity and staining scores.^{24–25} Finally, the SI for a section was calculated as the mean of the SIs of all the fields examined in each section. To confirm the reproducibility of the result, all the sections were scored at least three times.

ISH analysis

Twenty of 64 colon cancers were examined by ISH for *HDAC1* and *CBP* mRNAs to confirm the presence of their messages on cancer cells. The antisense oligonucleotide probes, *HDAC1* and *CBP*, were as follows: *HDAC1*, 5'-GAG GAG AAG CCA GAA GCC AAA GGG GTC AAG GAG GAG GTC AAG TTG GC-3'; *CBP*, 5'-GAA CTG TCC GTT GGT GAT ACC ACG GGA GAC ACA CTA GAA AAG TTT GTG GAG GGT TTG-3'. The probes were hybridised to entire mRNA transcript of the *HDAC1* and *CBP* genes, respectively, including the 5' and 3' untranslated regions. Each 4 µm of formalin-fixed (within 12 h) and paraffin-embedded sections were deparaffinised and rehydrated. After digestion with 0.4% pepsin, the specimens were

Table 2 Intraobserver and interobserver variations

HDAC/HAT	No. of cases	Intraobserver variation	Interobserver variation
HDAC1	64	$p = 0.947$	$p = 0.912$
p300	64	$p = 0.972$	$p = 0.928$
CBP	64	$p = 0.933$	$p = 0.901$

HDAC, histone deacetylase; HAT, histone acetyltransferase; CBP, CREB-binding protein.

Table 3 Results of univariate analysis

Predictive variables	HR	CI	p-Value
HDAC1 SI: >10.7 (baseline ≤ 10.6)	1.725	0.219 to 4.426	0.2236
p300 SI: >11.9 (baseline ≤ 11.8)	3.560	1.021 to 12.410	0.0464
CBP SI: ≤ 16.6 (baseline >16.7)	4.539	1.095 to 14.293	0.0356
Histological differentiation: poor (baseline well/moderately)	7.331	2.696 to 19.940	<0.0001
Lymph node metastasis: positive (baseline negative)	6.346	1.748 to 20.626	0.0036
Stage: III, IV (baseline 0, I, II)	5.326	1.549 to 18.316	0.008
Tumour size (cm): ≥5.0 (baseline <5.0)	4.226	1.238 to 14.923	0.0186
Depth of invasion: T4 (baseline Tis, T1, T2, T3)	6.875	1.066 to 49.579	0.0496
Age (years): ≥65 (baseline <64)	2.026	0.829 to 6.925	0.0605
Gender: male (baseline female)	0.725	0.429 to 1.825	0.4425

HR, hazard ratio; HDAC, histone deacetylase; CBP, CREB-binding protein; SI, staining index.

Table 4 Results of multivariate analysis

Predictive variables	HR	CI	p-Value
p300 SI: >11.9 (baseline <11.8)	4.118	1.049 to 16.170	0.0425
CBP SI: <16.6 (baseline >16.7)	4.026	1.133 to 14.301	0.0313
Lymph node metastasis: positive (baseline negative)	4.693	1.295 to 17.007	0.0186
Age (years): ≥65 (baseline <64)	0.501	0.158 to 1.590	0.2410
Gender: male (baseline female)	0.851	0.284 to 2.556	0.7742

HR: hazard ratio, CI: 95% confidence interval, P: P value, The values of p300 and CBP SI, and lymph node metastasis were adjusted for depth of invasion, age, and gender.

covered with hybridisation solution containing fluorescein isothiocyanate (FITC)-labelled DNA probes (500–2000 ng/ml) and hybridised at 55°C overnight. Two control studies were carried out to test the specificities of the oligonucleotide probes and the stainability as previously described.²⁶

RT-PCR analysis of p300 mRNA in human colorectal cancer

Expression of *p300* mRNA in 17 fresh specimens of colorectal cancer (7 cases of well, 6 of moderately, and 4 of poorly differentiated adenocarcinoma) was examined. RT-PCR was performed with One Step RT-PCR assay kit (Qiagen).²⁶ Primer sets used for RT-PCR amplification were as follows: *p300*-forward, 5'-AAA CCC ACC AGA TGA GGA C-3'; *p300*-reverse, 5'-TAT GCA CTA GAT GGC TCC GCA G-3'. As a control, *hypoxanthine-phosphoribosyl-transferase* (*HPRT*) level was also analysed (forward, 5'-CGG GGG ACA TAA AAG GTT AT-3'; reverse, 5'-CCA CTT TCG CTG ATG ACA C-3').

Statistical analysis

Both intraobserver and interobserver reproducibilities were evaluated simultaneously using the Kruskal–Wallis test. The

Mann–Whitney non-parametric test was used to compare the staining index of pairs of subjects and the Kruskal–Wallis test was used to for categorical data. Correlation between the indexes was determined by a simple linear regression test. Survival curves were calculated using the Kaplan–Meier method and analysed by the log-rank test. The influence of each variable on survival was assessed by the Cox proportional hazards model. A value of $p<0.05$ was considered to be significant.

RESULTS

Expression of HDAC1 protein and mRNA in colorectal cancers

Table 1 summarises the results of the immunohistochemical analyses; tables 2, 3, and 4, respectively, summarise information on intraobserver/interobserver variations and the results of the univariate and multivariate analyses.

Expression of HDAC1 protein was detected in the nuclei of both neoplastic and non-neoplastic cells, with a mean HDAC1 SI of 11.1 (3.2) (range 3.6–15.3) (fig 1A). The mean HDAC1 SI in the carcinomas was significantly higher than that in the corresponding normal mucosae ($p<0.01$), but no relationship

Figure 1 Expression of HDAC1 protein and mRNA in cancer tissue and the corresponding normal mucosa. Immunohistochemical detection of HDAC1 protein in cancer tissue (A) and the corresponding normal mucosa (B). The HDAC1 SI in the carcinoma was significantly higher than in the normal mucosa. (C) In situ hybridisation analysis of *HDAC1* mRNA in well differentiated adenocarcinoma of colon. *HDAC1* mRNA expression in cancer tissue and normal mucosa was detected on the nuclei. HDAC, histone deacetylase.

Figure 2 Expression of CREB-binding protein (CBP) and mRNA in cancer tissue and the corresponding normal mucosa. Immunohistochemical detection of CBP protein in well differentiated adenocarcinoma (A) and the corresponding normal mucosa (B). (C) Immunohistochemical detection of CBP protein in poorly differentiated adenocarcinoma. CBP expression in the medullary tumour (right) was higher than in the pleomorphic tumour (left). (D) In situ hybridisation of CBP mRNA in moderately differentiated adenocarcinoma of colon.

with any of the clinicopathological findings, such as gender, age, tumour size, differentiation, and progression was observed. Similarly, we confirmed that the incidence of high expression levels of *HDAC1* mRNA was greater in the cancer cells than in normal epithelial cells by ISH analysis (fig 1B).

Expression of CBP protein and mRNA in colorectal cancers

The CBP SI ranged from 10.3 to 21.9 (mean 16.1 (4.7)) and was significantly higher in the carcinomas (fig 2A) than in the normal mucosae ($p < 0.01$). Among poorly differentiated adenocarcinomas, the CBP SI for medullary carcinomas (16.8 (2.5),

$n = 13$) was higher than that for pleomorphic carcinomas (13.2 (4.2), $n = 9$) ($p < 0.05$; fig 2B). A significant difference was observed between stages I and IV ($p = 0.05$). We also detected the presence of *CBP* mRNA levels in this series of colorectal carcinomas by ISH analysis (fig 2C).

Expression of p300 protein and mRNA in colorectal cancers

The mean p300 SI of the tumours was 14.6 (4.8) (range 5.1–23.0), which was significantly higher than that in the normal mucosae ($p < 0.01$) (fig 3A). Furthermore, the mean p300 SI in poorly differentiated carcinomas was 18.4 (4.6), indicating an

Figure 3 Expression of p300 protein in cancer tissue and the corresponding normal mucosa. (A) p300 protein was more highly expressed in the carcinoma tissues than in the normal mucosae (inset). (B) Immunohistochemical detection of p300 in poorly differentiated adenocarcinoma. The SIs of the pleomorphic tumour was lower than in the medullary tumour.

Figure 4 RT-PCR analysis of *p300* mRNA in representative seven cases of human colon cancer. As a control, expression levels of *HPRT* mRNA in each case were also shown. Cases 1–5, well and moderately differentiated adenocarcinoma. Cases 6 and 7, poorly differentiated adenocarcinoma. N, normal colon mucosa, T, tumour sample.

Figure 5 Kaplan-Meier survival curves of patients with colorectal carcinoma. (A) Subdivided according to HDAC1 status. No significant difference was shown between the two groups. (B) Subdivided according to CBP status. Overall survival was significantly higher in patients with CBP overexpression. (C) Subdivided according to p300 status. Overall survival was significantly lower in patients with p300 overexpression. HDAC, histone deacetylase; CBP, CREB-binding protein.

increase compared with well and moderately differentiated adenocarcinomas. Among poorly differentiated adenocarcinomas, the p300 SI for medullary carcinomas (20.1 (5.5)) was

Take-home messages

- Overexpression of p300 is an indicator of poor prognosis of patients with colorectal cancer.
- High CREB-binding protein expression correlates with significantly better survival.

higher than that for pleomorphic carcinomas (16.3 (2.7)) ($p<0.05$; fig 3B). A marked increase in p300 mRNA levels was detected in 13 (76%) of 17 specimens by RT-PCR (fig 4). Four cases of poorly differentiated adenocarcinomas showed higher p300 mRNA expression levels than their corresponding normal mucosae.

Relationship between HDAC/HAT protein expression and clinicopathological findings

An univariate Cox regression analysis showed that overexpression (SI >11.9) of p300, reduction of CBP (SI <16.6), poorly differentiated histology, lymph node metastasis, TNM stage, tumour size, and depth of invasion were all significant prognostic factors (table 3). Figure 5 shows the Kaplan-Meier survival curves for subgroups of patients. There was no statistical significance between HDAC1 overexpression and prognosis (fig 5A). However, overall survival was significantly higher in patients with CBP overexpression (fig 5B), and lower in patients with p300 overexpression (fig 5C). A Cox multivariate regression analysis showed that p300 overexpression, reduced CBP expression, and lymph node metastasis were independent negative prognostic factors, after adjustment for the depth of tumour invasion and the age and sex of the patient (table 4).

DISCUSSION

To the best of our knowledge, this is the first report demonstrating the overexpression of HDAC1 at both the transcriptional and translational levels in colorectal carcinomas. Our results are in general agreement with those of a previous study on HDAC1 expression levels in gastric cancers.²⁷ Since overexpression of HDAC1 causes down-regulation of tumour suppressor genes, such as p53 and VHL,^{9, 28} these data imply that raised HDAC1 expression might lead to hypoacetylation of histone and subsequent silencing of several tumour suppressor genes in colorectal cancers. In support of this idea, the physiological consequence of hypoacetylation due to deletion or inactivation of HAT is a high incidence of malignant tumours: our present data also indicate up-regulation of p300 expression in colorectal tumours.

Histone modification due to reversible acetylation by HDAC/HAT is believed to play a significant role in controlling gene expression,²⁸⁻³¹ and HDAC inhibitors including butyrate, trichostatin A, and others have antitumour activity against various human malignancies, such as breast and prostate cancers as well as colon cancer.^{4-6, 32-36} Studies in which human tumour lines are implanted into nude mice also indicate that HDAC inhibitors may be novel therapeutic agents for cancer treatment.⁷ The present study showed the frequent HDAC1-expression in colonic adenocarcinoma tissues compared with corresponding normal mucosae. This evidence indicates the possibility of availability of HDAC inhibitors for colonic cancer.

In the present study both CBP and p300 were overexpressed in adenocarcinoma tissues compared with normal mucosae. Furthermore, overall survival was good in patients with CBP overexpression and poor in those with p300 overexpression. A Cox multivariate regression analysis also showed the significance of the p300 overexpression and reduced CBP expression.

Because there is a high degree of structural homology between CBP and p300, it may seem that the present data cannot be explained. However, accumulating evidence indicates that CBP and p300 are not completely redundant but have unique roles; these functional differences could be due to differential association with other proteins or differences in substrate specificity between these acetyltransferases.^{37,38} For example, CBP and p300 play very distinct roles in *survivin* gene transcription.³⁹ In humans mutant huntington represses CBP, but not p300, by binding and protein degradation.⁴⁰ Interestingly, CBP1 directly associates with p300 by binding to the PDXLS motif in the bromodomain of p300 and blocks the accessibility of p300 to histones, thus repressing p300-mediated histone acetylation and transcriptional activation.⁴¹ Consequently, the evidence of p300 overexpression and reduced CBP expression in colonic cancer in the present study may indicate enhanced p300-mediated histone acetylation and transcriptional activation.

In conclusion, the present study indicated that expression levels of HDAC/HAT-associated proteins HDAC1, CBP, and p300 were up-regulated in human colorectal cancers. Overexpression of p300 is an indicator of poor prognosis of these patients; conversely, high CBP expression correlates with significantly better survival. Further studies will be needed to clarify the downstream mechanisms involved in controlling the biological behaviour of colorectal carcinomas via histone-modifying molecules.

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