The global histone modification pattern correlates with overall survival in metachronous liver metastasis of colorectal cancer

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Abstract. Post-translational histone modifications are known to be altered in cancer tissues, and differences in the histone modification levels have recently been used to predict the clinical outcome in patients with certain types of cancer. In this study, we evaluated the immunohistochemical staining patterns of histone H3 dimethylation and acetylation in metachronous liver metastasis of colorectal carcinomas and examined its correlation with patient prognosis. Double 2 mm core tissue microarrays were made from 54 paraffin-embedded samples of liver metastasis from colorectal adenocarcinoma, and were examined by an immunohistochemical analysis of histone H3 lysine 4 (H3K4) dimethylation, histone, H3 lysine 9 (H3K9) dimethylation and histone H3 lysine 9 (H3K9) acetylation. Positive tumor cell staining for each histone modification was used to classify patients into low- and high-staining groups, which were then examined for correlations with the clinicopathological parameters and clinical outcome. Dimethylation of H3K4 correlated with the tumor histological type (P=0.043), and acetylation of H3K9 correlated with the tumor histological type (P=0.016). In addition, lower levels of H3K4 dimethylation correlated with a poor survival rate (P=0.035). The multivariate survival analysis showed that the H3K4 dimethylation status is an independent prognostic factor for colorectal cancer patients (P=0.011). We suggest that the pattern of histone modification as detected by immunohistochemistry may be an independent prognostic factor for metachronous liver metastasis of colorectal carcinomas.

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Introduction

Colorectal cancer (CRC) is the third most common cancer and the fourth leading cause of cancer-related deaths worldwide (1). In spite of progress made in CRC chemotherapy, the outcomes of CRC with distant metastasis still remain poor. Liver metastasis of CRC is an important prognostic factor, and occurs in 20-25% of CRC patients (2). Hepatic resection is a potentially curative therapy for colorectal liver metastases. However, recurrence develops in approximately 60-70% of all such patients after hepatectomy, thus, suggesting that patients with colorectal liver metastasis often do not benefit from hepatectomy. In addition, the prognostic factors for survival that can be obtained from the resected specimens and the mechanism of tumor progression of the metastases have not yet been fully elucidated. Therefore, it is important to identify the specific biomarker of CRC outcomes, especially for patients with liver metastases.

DNA methylation and histone modification are major epigenetic mechanisms controlling gene regulation, and they are frequently altered in cancer (3). Changes in DNA methylation are closely related to patterns of histone modification (4). Cellular patterns of histone modifications have been reported as providing independent prognostic information for several cancers, including prostate (5,6), kidney (6), lung (6-8), gastric (9), ovarian (10), pancreatic (10,11), esophageal (12,13) and breast cancers (10,14). Modification of histones by methylation and acetylation at lysine residues is generally associated with gene inactivation or silencing (15-19). In CRC patients, it has also been previously reported that reduced H3 lysine 4 methylation and increased H3 lysine 9 methylation play a critical role in the maintenance of promoter DNA methylation-associated gene silencing (15). However, to date, there have been no reports on the prognostic significance of global histone modifications in cases of CRC, including liver metastasis.

In this study, we classified the expression levels of the histone dimethylation in 54 pairs of the liver metastases obtained from patients with metachronous liver metastasis of CRC. To evaluate the clinical significance of histone modification,

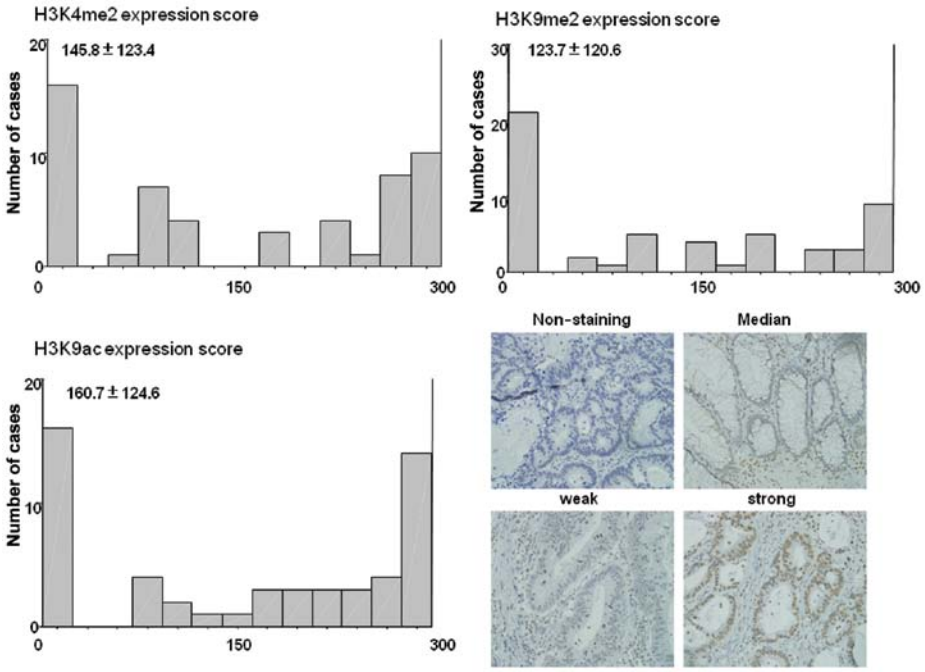

Figure 1. Histograms depicting the detection of histone modifications in CRC by immunohistochemistry. Representative examples of CRC or liver metastasis tissue cores presenting with 4 levels of staining (non-staining, weak, median and strong) of the following histone modifications: H3K4me2, H3K9me2 and H3K9ac. Original magnification, x200. Histograms showing the distribution of H-scores plotted against the number of cases for the histone modifications.

we examined the correlation between the relative expression of global histone modification patterns and the outcomes in patients with CRC.

Materials and methods

Patients and samples. We retrospectively studied the surgical specimens of liver metastasis obtained from 54 patients with metachronous liver metastasis of CRC. All of the patients had undergone curative radical (R0) resection for primary colorectal adenocarcinoma, and none of them were observed to have liver metastasis at the first operation. The metachronous liver metastases were subjected to curative radical resection at a later time. The patients underwent surgery at Kanagawa Cancer Center between January 1992 and December 2007. Primary colorectal tumors and the corresponding liver metastases were obtained from each patient. Informed consent was obtained from each patient. In all cases, archival hematoxylin and eosin-stained (H&E) slides of the respective liver metastasis specimens were retrieved and reviewed to confirm the pathological features, as well as to select suitable tissue blocks for immunohistochemical analysis. The Ethics Committees of the Kanagawa Cancer Center approved the protocol before initiation of the study. No patient had any other malignancies.

Tissue microarrays and immunohistochemistry. Microarrays consisting of cores, each 2 mm in diameter, were prepared from formalin-fixed paraffin-embedded tissue blocks of surgically removed liver metastases, and one tissue core from each liver metastasis that consisted of >80% carcinoma cells was prepared for analysis.

Immunohistochemical staining was performed using commercially-available polyclonal rabbit anti-histone antibodies raised against dimethyl-histone H3 lysine4 (H3K4me2), dimethyl-histone H3 lysine9 (H3K9me2) and acetyl-histone H3 lysine9 (H3K9ac) (Cell Signaling Technology Inc., Danvers, MA). Tissue microarray blocks were sectioned at a thickness of 4 μm and mounted on pre-coated glass slides. The sections were de-paraffinized through a graded series of xylene and rehydrated through a graded series of alcohol to distilled water. Endogenous peroxidase was quenched with 3% hydrogen peroxide in methanol at room temperature. The sections were placed in a 95˚C solution of 0.01 M sodium citrate buffer (pH 6.0) for 40 min for antigen retrieval. Normal goat serum (5%) was then applied for 15 min to block non-specific protein binding sites. Primary rabbit anti-histone polyclonal antibodies were applied for 1 h at room temperature at the following dilutions: anti-H3K4me2 at 1:300, anti-H3K9me2 at 1:300 and anti-H3K9ac at 1:300. Immunoreactive proteins were detected using the Simple Stain MAX PO (R).

All sections were counterstained with Mayer's hematoxylin, and negative controls were included in each staining sequence. The intensity and global level of staining were scored semi-quantitatively for each tissue microarray by an investigator blinded to all of the clinicopathological variables. The global level of staining refers to the percentage of tumor cells that stained positively for an antibody within each tissue microarray at x200 magnification using a light microscope.

Scoring of immunohistochemical reactivity. Immunohistochemical scoring was done by the modified Histo-score (H-score) (20), which involves semi-quantitative assessment of both the intensity of staining (graded as 0, no staining; 1, weak; 2, median; and 3, strong, using adjacent normal mucosa as the median) and the percentage of positive cells. The range

Table I. Relationship between the expression of the histone modifications and the clinicopathological features.

Variables/categories	H3K4me2 expression			H3K9me2 expression			H3K9ac expression		
	Low (n=28)	High (n=26)	P-value	Low (n=30)	High (n=24)	P-value	Low (n=23)	High (n=31)	P-value
Age	61±9	62±9	0.892[b]	59±7	64.±11	0.075[b]	61±7	62±10	0.517[b]
Gender			0.535[c]			**0.015**[c]			0.975[c]
Male	16	17		14	19		14	19	
Female	12	9		16	5		9	12	
Size (cm)			0.554[c]			0.902[c]			0.22[c]
<5	15	16		17	14		11	20	
≥5	13	10		13	10		12	11	
Histological type[a]			**0.043**[c]			0.063[c]			**0.016**[c]
Well/Moderate	24	26		26	24		19	31	
Poor/Mucinous	4	0		4	0		4	0	
Depth of invasion			0.777[c]			0.429[c]			0.554[c]
T1-T3	14	12		13	13		10	16	
T4	14	14		17	11		13	15	
Location			0.151[c]			0.322[c]			0.724[c]
Colon	14	18		16	16		13	19	
Rectum	14	8		14	8		10	12	
Lymph node metastasis			0.171[c]			0.257[c]			0.623[c]
Absent	6	10		7	9		6	10	
Present	22	16		23	15		17	21	
Adjuvant chemotherapy			0.394[c]			0.066[c]			0.902[c]
Absent	14	10		10	14		10	14	
Present	14	16		20	10		13	17	

[a]Well, well differentiated; moderate, moderately differentiated; Poor, poorly differentiated. [b]Wilcoxon test; [c]Pearson's χ^2 test. Bold indicates values that were statistically significant (<0.05).

of possible scores is 0-300, enabling us to categorize our cases into biologically relevant groups depending on different levels of detection, which could potentially be missed using simpler scoring methods. Tumor samples with an H-score <150 for individual chromatin markers were designated as having low detection, where scores ≥150 were designated as high detection. The distribution of staining was assessed in tissue microarray sections.

Statistical analysis. The relationship between histone modification scores and potential explanatory variables, including age, gender, tumor size, histological type, depth of invasion, lymph node metastasis, adjuvant chemotherapy and location were evaluated with the χ^2 test and the Wilcoxon test. The postoperative survival rate and disease free survival rate were analyzed by the Kaplan-Meier method, and differences in survival rates were assessed with the log-rank test. A Cox proportional-hazard model was used for the multivariate analyses. Differences were considered significant when P<0.05. Each statistical analysis was performed using the SPSS II software program, version11.0.1J for Windows (SPSS, Inc., Chicago, IL).

Results

Characteristics of histone modifications. Representative immunostaining results for the three histones are shown in Fig. 1. Only nuclear staining for the three histones was regarded as positive, and cases were scored for each mark using a modified H-score. Histograms showing the staining intensity and distribution of H-scores plotted against the number of cases are shown in Fig. 1.

The expression of histone markers correlates with the clinicopathological factors. The expression scores of the histone modifications were categorized as low or high according to whether they were <150 or ≥150. The relationship between the expression levels of three histone modifications and the patient age, gender, tumor size, histological type, depth of invasion, location of lymph node metastasis and adjuvant chemotherapy after first operation, were assessed. The H3K4me2 status was positively correlated with the tumor histological type of the liver metastasis. The H3K9ac status was also positively correlated with the tumor histological type. However, the H3K9me2 status was found to only significantly correlate with gender (Table I).

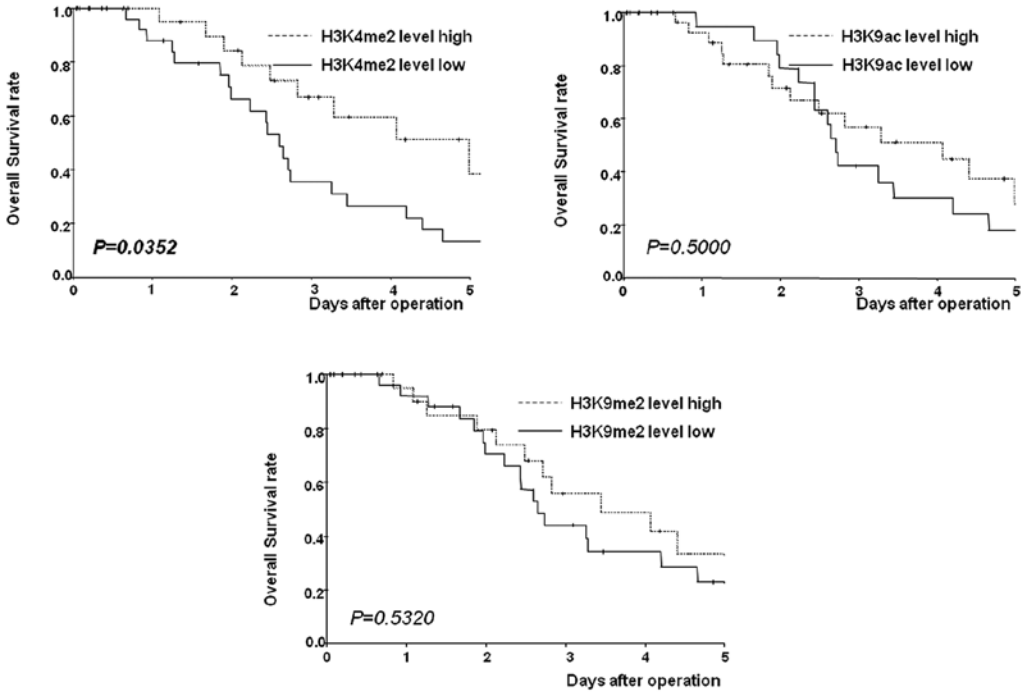

Figure 2. The Kaplan-Meier survival curves with the log-rank test for 54 patients after resection of the liver metastasis. A comparison of the overall survival based on liver metastases between the groups with high H3K4me2, H3K9me2 and H3K9ac expression and low expression, respectively. The group with high expression of H3K4me2 in liver metastases showed significantly better survival than the group with low expression (P=0.0352).

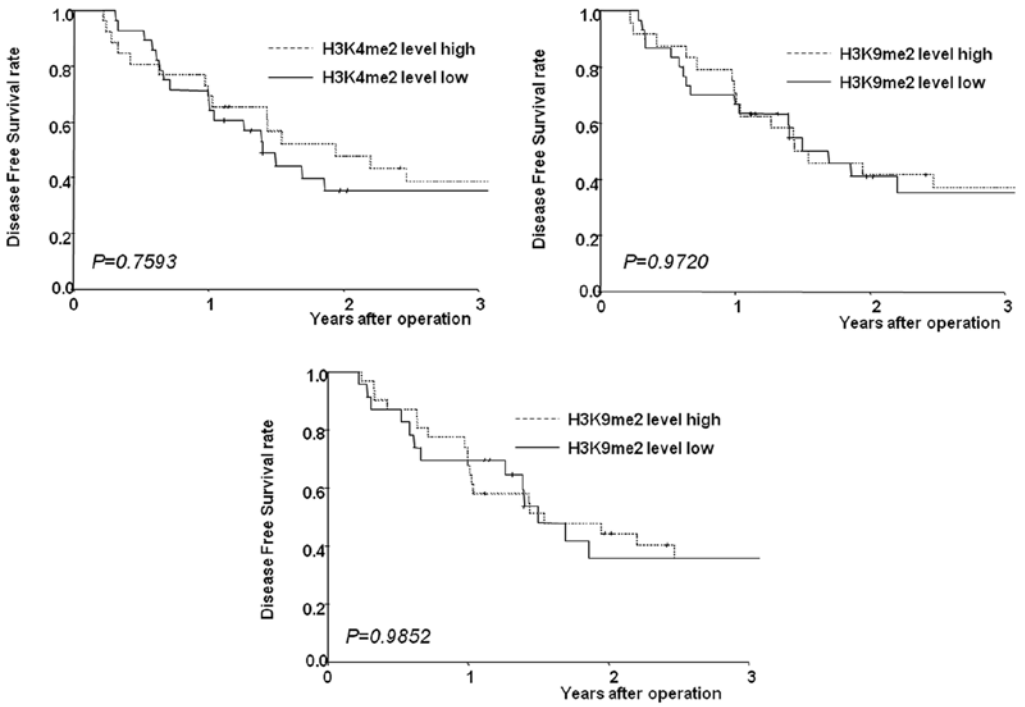

Figure 3. The Kaplan-Meier disease free survival curves with the log-rank test for 54 patients after resection of the liver metastasis. A comparison of the overall survival based on liver metastases between the groups with high H3K4me2, H3K9me2 and H3K9ac expression and low expression of each of these factors. There were no significant differences between the histone levels.

Relationships between histone markers and patient outcomes. With regard to the modification patterns, the group with high expression of H3K4me2 showed significantly better survival from the day of liver resection than those with a low expression level (P=0.0352). The group with high expression of H3K9me2 and H3K9ac showed a better survival than those with low expression, but the difference was not significant (H3K9me2, P=0.5320; H3K9ac, P=0.5000, Fig. 2). The disease free survival between the day of liver resection and the second recurrence did not significantly correlate with any

Table II. The results of a multivariate analysis of the clinicopathological factors for overall survival.

Variables/categories	n	Hazard ratio	95% CI[b]	P-value
Size (cm)				
<5	31	1		
≥5	23	1.919	0.922-3.922	0.081[c]
Histological type[a]				
Well/Moderate	50	1		
Poor/Mucinous	4	1.342	0.335-5.370	0.678[c]
Depth of invasion				
T1-T3	26	1		
T4	28	1.305	0.551-3.091	0.545[c]
Location				
Colon	32	1		
Rectum	22	1.166	0.488-2.787	0.73[c]
Lymph node metastasis				
Absent	16	1		
Present	38	0.51	0.206-1.262	0.145[c]
Preoperative CEA				
Absent	32	1		
Present	21	1.012	0.370-2.774	0.981[c]
Preoperative CA19-9				
Absent	36	1		
Present	16	2.396	1.024-5.604	**0.044**[c]
Adjuvant chemotherapy				
Absent	24	1		
Present	30	1.928	0.852-4.363	0.115[c]
H3K4me2 expression				
Low	28	1		
High	26	0.338	0.146-0.783	**0.011**[c]

[a]Well, well differentiated; Moderate, moderately differentiated; Poor, poorly differentiated. [b]CI, confidence interval. [c]Cox proportional hazard regression. Bold indicates values that were statistically significant (<0.05).

histone modification pattern (Fig. 3). The median follow-up period was 907 days.

Prognostic factors for colorectal cancer. On a multivariate Cox regression analysis including tumor size, histological type, depth of invasion, lymph node metastasis, preoperative (the first colorectal resection) CEA, CA19-9 and a lower level of H3K4me2, H3K4me2 expression and preoperative CA19-9 was an independent predictor of overall survival in patients with CRC (H3K4me2, P=0.011; CA19-9, P=0.044, Table II).

Discussion

Epigenetic alterations, such as DNA methylation and histone modification, play important roles in carcinogenesis by controlling gene activity and nuclear structural design (21,22). Recent studies have suggested that the global patterns of histone modifications can be used to predict patient outcomes for several cancers. The aim of this study was to determine the prognostic significance of histone modification in metachronous liver metastases by using an immunohistochemical analysis.

We first examined the relationship between histone modifications and clinicopathological features. In gastric carcinoma, Park *et al* (9) reported that cases with more H3K9ac-positive cells tended to be poorly differentiated adenocarcinomas. In esophageal squamous cell carcinoma, I *et al* (13) reported that the global levels of H3K9Ac and H3K9me2 in well-differentiated cases showed a tendency to be higher than those in moderately or poorly differentiated cases, but the difference in these levels were not found to be statistically significant. Our present study demonstrated that a high H3K4me2 level in the liver metastasis tended to be present in subjects with poorly differentiated adenocarcinomas, and that a positive H3K9ac status also tended to be associated with poorly differentiated adenocarcinomas.

We then examined the relationship between three histone modification levels and the outcomes of CRC with metachronous liver metastasis. Seligson *et al* (5) previously reported that prostate carcinoma patients with low cellular levels of H3K4me2 had a poorer prognosis, with a significantly increased risk of tumor recurrence compared with patients with higher levels of this modification. In lung cancer patients, a high H3K4me2 level (≥85% of tumor cells) was associated with a significantly better survival of stage I patients with large-cell or squamous cell carcinomas. In addition, low H3K9ac levels (<68% of tumor cells) were also associated with a better survival of stage I patients. In the case of pancreatic carcinoma, low cellular levels of H3K4me2 or H3K9me2 were both significant and independent predictors of poor survival in the univariate and multivariate models (11). In our study, a high level of H3K4me2 modification in liver metastases was associated with a better overall survival than a low level of this histone modification in patients with CRC. According to a univariate Cox regression analysis, a lower level of H3K4me2 modification in the liver metastases was a significant independent predictor of overall survival in these patients.

Histone modifications and DNA methylation seem to play an important role in regulating transcription and other nuclear processes. Previous reports have shown the relationship between histone modifications and DNA methylation in cancer cells. For example, promoter CpG-island hypermethylation in cancer cells has been reported to be associated with a particular combination of histone markers, for example, deacetylation of histones H3 and H4, loss of H3K4 trimethylation, and gain of H3K9 methylation and H3K27 trimethylation (23). In addition, Dnmt3L interacts with unmethylated H3K4 through its N-terminus and with Dnmt3a through its C-terminus, thus linking the DNA methylation machinery to the modification state of histone tails (24). However, while the biochemical mechanism underlying histone demethylation has been deciphered, it is still not clear how methyl groups are removed from DNA (25).

Recently, several groups have reported these epigenomic modifications to predict the clinical outcomes in human cancers, and H3K4 and H3K9 modifications are important

in the epigenetic silencing of tumor suppressor genes. Of interest, there is evidence that epigenomic profiles can predict the responses of cancer to chemotherapy, at least in pancreatic carcinoma. One report showed that the histone levels were predictive of survival specifically for patients with node-negative cancer or for those receiving adjuvant fluorouracil, but not gemcitabine (11). The impact of histone levels on CRC is not clear. If the histone modification proves to be a useful biomarker in the other cancers, then the existence of a ready-made target treatment would be invaluable for future chemotherapy.

Recent and ongoing comprehensive cancer genome studies have been identifying many gene alterations involved in histone modifications (26). Most strikingly, high-resolution SNP genotyping of medulloblastoma identified many previously unknown recurrent gene amplifications and homozygous deletions, and those events converged on genes controlling histone lysine methylation (27). We speculate that the H3K4 or H3K9 hypomethylation status may be caused by multiple genetic alterations of histone methylation modifiers, which may trigger global histone lysine modifications, rather than modification on specific gene regions of limited number, and as a whole, this is associated with the higher malignant behavior of CRC.

In conclusion, our results suggest that the pattern of histone modifications in liver metastasis as detected by immunohistochemistry can be successfully used as an independent prognostic factor for metachronous liver metastasis of colorectal cancer.

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