Chapter 4

Nuclear expression of histone deacetylases and their histone modifications predicts clinical outcome in colorectal cancer

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Abstract

Aims: Epigenetic changes are of crucial importance in cancer development and are potentially reversible, thus presenting as interesting targets for anti-cancer therapy. We investigated the clinical prognostic value of histone deacetylases SIRT1, HDAC1 and HDAC2 and histone modifications H4K16Ac and H3K56Ac in colorectal cancer.

Methods and results: The epigenetic markers were immunohistochemically stained on tissue microarrays containing colorectal tumor (n=254) and normal colorectal tissues (n=50). Nuclear expression was assessed on the semi-automated Ariol system. Multivariate trend survival analyses of the combined markers showed better patient survival and less tumor recurrence when more markers showed high nuclear expression. For the combination of the histone deacetylases and H3K56Ac, the hazard ratio (HR) for overall survival (OS) was 0.82 (0.72-0.94; p=0.005) and for distant recurrence-free survival (DRFS) was 0.77 (0.64-0.92; p=0.003) per additional marker showing high expression. Similarly, for the combination of histone deactylases and H4K16Ac, a HR of 0.86 (0.76-0.97; p=0.01) for OS and 0.79 (0.68-0.93; p=0.006) for DRFS were observed per additional marker showing high expression.

Conclusions: The studied epigenetic markers showed clinical prognostic value in colorectal cancer, both as individual markers and when combined into multi-marker analyses. These results indicate that epigenetic mechanisms play an important role in colorectal carcinogenesis.

Introduction

There is a need to identify new biomarkers in colorectal cancer in order to better stratify patients for treatment based on their individual tumor characteristics. For TNM stage I-III colorectal tumors, patient survival and tumor recurrence vary widely among patients, indicating that the current TNM staging system needs further refinement. New biomarkers may be found by unraveling the underlying biology of individual tumors. Epigenetics is a promising field for biomarker research, since changes in epigenetic status have been frequently reported in tumor tissues compared to their normal counterparts (1). In addition, epigenetic mechanisms are potentially reversible, which makes them suitable targets for the development of new therapies (1).

Epigenetic mechanisms include DNA methylation and histone modifications, which directly influence chromatin structure and thereby accessibility of the DNA for transcription factors (2). Several research groups have found global expression of histone modifications to have prognostic value in different cancers, including prostate (3), lung and kidney (4), breast cancer (5), and colorectal cancer (6). In addition to histone modifications, expression of histone-modifying enzymes, including histone deacetylases (7), have also been shown to have prognostic value in colorectal cancer.

Specific histone modification patterns are associated with regions of the genome that are either actively transcribed or repressed (8). One of the histone modifications that is strongly linked to gene activation and can by itself prevent chromatin compaction is H4K16Ac (9). The major H4K16 deacetylase in mammalian cells is SIRT1 (Sirtuin 1), a class III histone deacetylase (10). Human SIRT1 has been shown to be involved in many (disease) processes (11) and altered expression of SIRT1 has been described in several cancers, including colorectal cancer (12). Global levels of H4K16Ac are dependent on the balance between SIRT1 and class I histone deacetylases HDAC1 and HDAC2. Both HDACs have been shown to contribute to the process of non-homologous end-joining (13), which is important for error-free repair of DNA double strand breaks, through deacetylation of histone modification H3K56Ac. Dysregulation in these cellular processes could facilitate carcinogenesis.

Using immunohistochemistry (IHC), we analyzed nuclear expression of histone deacetylases SIRT1, HDAC1 and HDAC2 and histone modifications H3K56Ac and H4K16Ac in tumor specimens of 254 TNM stage I-III colorectal cancer patients from a consecutive patient cohort with extensive clinical follow-up data. We analysed the correlations of expression of the individual markers and combinations of the histone deacetylases with each of the respective histone modifications with clinical outcome.

Materials and methods

Patient selection

Tumor tissues were collected from a consecutive series of 409 (TNM-stage I-IV) patients who underwent surgery at the Leiden University Medical Center (LUMC) of their primary tumor

between 1991 and 2001 and of whom tumor tissue was available. All specimens were handled with a standard protocol for fixation, dissection and histopathological reporting. Patients with pre-operative treatment, multifocal tumors, or a history of cancer (other than basal cell carcinoma or *in situ* tumors) were excluded from analyses. We included only patients with a histologically proven colorectal adenocarcinoma and TNM tumor stage I-III cancer, as determined by an experienced pathologist. Complete clinicopathological data were available for 259 TNM stage I-III patients, and complete covariate and study marker data were available for 254 patients (Table 1), with a mean follow-up of 8.6 years. Clinicopathological parameters of patients in the study cohort were representative for the complete patient cohort. Data were censored when patients were alive or free of recurrence at their last follow-up date. Patient records information was anonymized and de-identified prior to analysis according to national ethical guidelines ("Code for Proper Secondary Use of Human Tissue", Dutch Federation of Medical Scientific Societies), and approved by the Medical Ethical Committee of the Leiden University Medical Center (LUMC). MSI status was tested using the PCR-based MSI Analysis System, Version 1.2 (Promega, Mannheim, Germany), as described previously (14). This study was performed according to the REMARK guidelines (NCI-EORTC) (15).

Immunohistochemistry and scoring

Formalin-fixed paraffin-embedded (FFPE) tissues from each of the patients in this retrospective study were used to construct a tissue microarray (TMA) with 0.6 mm tissue cores, as described previously (16). Sections of 4μm were cut from each of the TMA blocks including 254 colorectal tumor tissues and 50 histopathologically normal colorectal tissues and used for IHC (manual protocol). TMA sections were incubated overnight (16 hours) using primary antibodies at predetermined optimal dilutions. Antibodies used in this study were: anti-H3K56Ac (ab76307, Abcam, Cambridge, UK), anti-H4K16Ac (ab61240, Abcam, Cambridge, UK), anti-SIRT1 (ab32441, Abcam, Cambridge, UK), anti-HDAC1 (ab19845, Abcam, Cambridge, UK) and anti-HDAC2 (ab39669, Abcam, Cambridge, UK), using a standard IHC protocol (17). Briefly, antigen retrieval was performed by heating the sections for 10 min at 95°C in a citrate buffer (pH 6.1; pH Low Target Retrieval Solution, Dako, Glostrup, Denmark) after deparaffinization. Endogenous peroxidase was blocked by incubating the sections in a 0.3% solution of hydrogen peroxide (in PBS) for 20 min. Staining was visualized using the Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse (Dako, Glostrup, Denmark). In each TMA block, control tissues (colon, spleen and liver) were included serving as positive controls across TMA sections for nuclear staining. A no-antibody control section was used as negative control. Stained tissue microarrays were scanned using a 20x magnification and nuclear expression of all markers was assessed using the semi-automated Ariol system (Leica Microsystems, Wetzlar, Germany). Tumor areas (tumor tissues) and colon epithelium (normal tissues) were marked on the computer screen upon visual inspection. The semi-automated Ariol system is specifically designed to recognize cells, nuclei, cell membranes and pixel intensity and was trained carefully for each individual staining. For each TMA section, several random cores were evaluated by visual inspection after automatic analysis in order to verify correct identification of positively stained nuclei.

TABLE 1. Patient characteristics of the study cohort (n=254)

		n	(%)
Age at operation			
	<50	32	12.6
	50-75	161	63.4
	>75	61	24
Gender			
	Male	128	50.4
	Female	126	49.6
TNM stage			
	I	53	20.9
	II	113	44.5
	III	88	34.6
pT stage			
	T1	19	7.5
	T2	38	15.0
	T3	166	65.4
	T4	31	12.2
pN stage			
	N0	168	66.1
	N1	54	21.3
	N2	32	12.6
Histological Subtype			
	Adenocarcinoma	190	74.9
	Mucinous	34	13.6
	Cribriform	14	5.5
	Tubulovillous	5	2.0
	Undifferentiated	10	3.9
	Signet ring cell	1	0.1

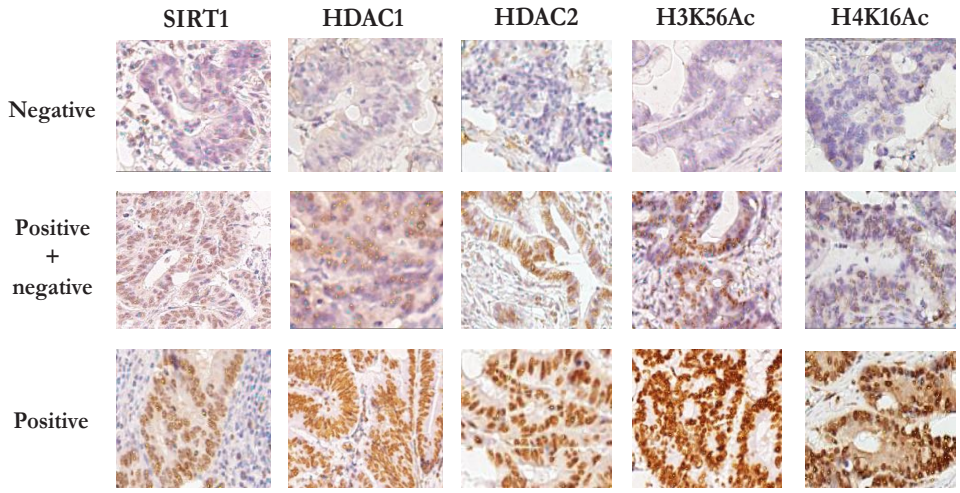
		n	(%)
MSS status			
	MSI	34	13.4
	MSS	175	68.9
	Unknown	45	17.7
Tumor location			
	Colon	187	73.6
	Rectum	67	26.4
Tumor size			
	Mean	4.69	
	Standard error	2.32	
Number of lymph nodes retrieved			
	Mean	8.09	
	Median	8	
	<12	250	98.4
	≥12	4	1.6
Location in the colon			
	Proximal	94	37.0
	Distal	160	63.0
Tumor in follow-up *			
	No	215	84.6
	Yes	39	15.4
Adjuvant therapy			
	No	206	81.1
	Yes	48	18.9

*Patient characteristics are shown for the study cohort (n=254). Patients with unknown status for any of the covariates are not reported in this table, except for MSS status. * = second primary tumor during follow-up period.*

Statistical analysis

Data were analyzed in consultation with a statistician (H.P.) using SPSS 20.0 for Windows (SPSS Inc, Chicago, USA). The Cox proportional hazard model was used for univariate and multivariate survival analysis. Covariates included in all multivariate analyses were age at operation, gender,

Figure 1

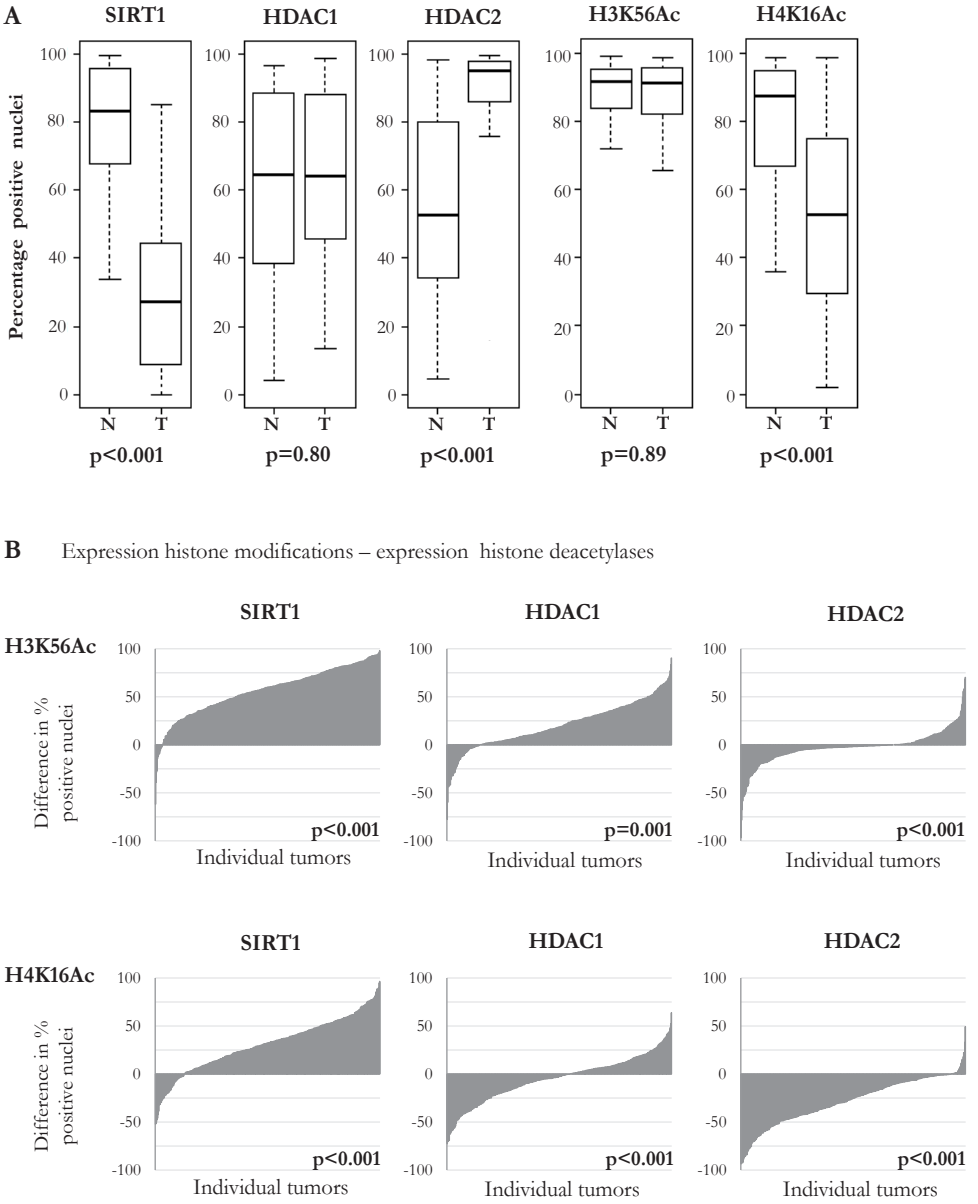


Identification of positively stained and negative nuclei by the Ariol system. *The Ariol system trainer overlay shows correct identification of positive (indicated by yellow dots) and negative (blue dots) nuclei in tumor tissues using immunohistochemistry. TMA slides were scanned using a 20x magnification. Shown for all markers are negative tumor cores (top row), tumor cores with both positive and negative cells (middle row) and highly positive tumor cores (bottom row). The Ariol system was trained to identify positive and negative cells for each marker individually.*

TNM tumor stage (tumor stages I-III), tumor location, tumor size and microsatellite stability (MSS) status. Covariates "tumor in the follow-up" (second primary tumor) and "adjuvant therapy" were entered as time-dependent covariates. Normality of the data was tested using the Shapiro-Wilk test, and non-parametric Wilcoxon signed rank tests were performed to test for statistical differences in expression between normal and tumor samples and between the expression levels of histone modifications and histone deacetylases in individual tumors. Spearman's rank correlation coefficient was used to test the correlation between the expression of histone deacetylases and the respective histone modifications. Median expression was used as a cut-off value to divide patients into high or low expression groups. Kaplan-Meier curves or cumulative incidence plots were generated to visualize the differences in patient survival or tumor recurrence. We performed trend analyses using combined markers with the group numbers as continuous variables. Cox regression analyses were performed using the combined markers as categorical variables to assess the hazard ratios for each of the individual patient

Figure 2 (see next page) Paired analyses of normal versus tumor and histone deacetylases versus histone modifications. ***A***. *Boxplots showing mean expression (indicated as the percentage of immunohistochemically stained positive nuclei). Normal samples (n=50) are shown on the left of each figure (labeled "N") and mean expression of the tumor samples (n=254) is shown on the right (labeled "T"). P-values indicate the results of the non-parametric Wilcoxon signed rank test.* ***B***. *Histograms showing the difference in expression (percentage of positive nuclei as determined after immunohistochemistry) between the histone modifications and histone deacetylases are displayed for H3K56Ac and H4K16Ac against each of the individual histone deacetylases. The difference in expression (y-axis) was calculated for each individual patient (x-axis), according to the following formula: expression difference = expression histone modification – expression histone deacetylase. Negative values indicate higher expression of the histone deacetylase, positive values indicate higher expression of the histone modification. P-values represent the results of the Spearman's correlation analysis.*

Figure 2



groups. Competing risk analyses were performed to assess disease-specific survival. Kaplan-Meier curves or cumulative incidence plots were generated to visualize the differences in patient survival and tumor recurrence between the five groups. For individual marker analyses, the low expression group was used as the reference group, and for combined analyses group 1 (all low)

was used as reference group. Overall survival (OS) was defined as the time from surgery until death (by any cause). Disease-specific survival (DSS) was defined as the time from surgery until death by colorectal cancer, and was based on autopsy reports, where available, and otherwise on death certification. Loco-regional recurrence-free survival (LRRFS) was defined as the time from surgery until the occurrence of a (loco)regional recurrence or death by cancer. Distant recurrence-free survival (DRFS) was defined as the time from surgery until the occurrence of a distant recurrence or death by cancer. For all statistical analyses, a two-sided p-value of 0.05 or less was considered statistically significant.

Results

Expression in normal versus tumor tissues

To minimize observer bias, nuclear expression of all markers in both tumor cells (tumor tissues) and colon epithelium (normal tissues) was scored using the semi-automated Ariol system (Figure 1). We analyzed expression of SIRT1, HDAC1, HDAC2, H3K56Ac and H4K16Ac in normal and tumor tissues. The expression data were not normally distributed for any of the markers (data not shown). SIRT1 and H4K16Ac showed lower nuclear expression in tumor samples compared to normal tissue samples (both $p<0.001$), whereas HDAC2 showed higher nuclear expression in tumor samples compared to normal tissue samples ($p<0.001$) (Figure 2A). The expression of HDAC1 ($p=0.80$) and H3K56Ac ($p=0.89$) did not differ between normal and tumor samples when analyzing the whole patient cohort (Figure 2A). However, within individual tumors, differences between normal and tumor samples were observed for both HDAC1 and H3K56Ac (Figure 3A). For both markers, approximately 50% of the tumor tissues showed higher expression compared to paired normal tissues, and the other 50% showed lower expression in tumor tissues compared to normal tissues. This also translated into survival differences between patients with higher expression and patients with lower expression in the tumor as compared to the normal tissues (Figure 3B).

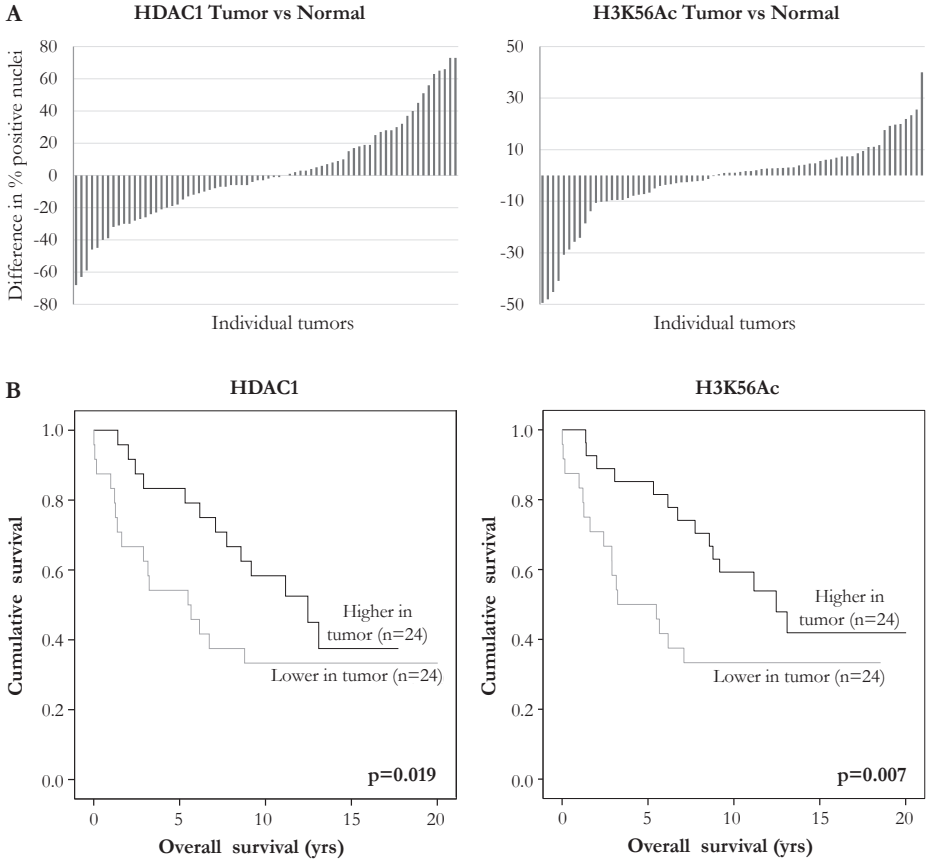
Paired analyses of expression of histone deacetylases and histone modifications

The differences in expression levels between each of the histone deacetylases and either of the histone modifications were plotted for the whole study cohort (Supplementary Figure 1) and for each individual tumor (Figure 2B). In individual tumors, high expression of the histone modifications correlated to low expression of the histone deacetylases (positive values), and *vice versa* (negative values). Spearman's correlation analyses showed that for all three histone deacetylases, there was a significant inverse correlation with the respective histone modifications ($p\leq0.001$).

Survival analyses of individual markers

The median expression for each of the markers, used to divide patients into high and low expression groups, was as follows: SIRT1 (30%), HDAC1 (68%), HDAC2 (95%), H3K56Ac (93%) and H4K16Ac (63%). Median survival was 9.3 years (for both OS and DSS), median recurrence-free survival was 8.8 years for LRRFS and 9.2 years for DRFS. All markers showed

Figure 3



Expression of HDAC1 and H3K56Ac in tumor and normal tissues of individual patients. A*. Histograms showing the difference in expression (indicated as the percentage of immunohistochemically stained positive nuclei) between paired normal and tumor tissues are displayed for HDAC1 and H3K56Ac. The difference in expression (y-axis) was calculated for each individual patient (x-axis), according to the following formula: expression difference = expression in tumor tissue – expression in normal tissue. Negative values indicate higher expression in normal tissues, positive values indicate higher expression in tumor tissues.* ***B****. Kaplan-Meier curves were made to visualize differences in overall survival between patients with higher expression and patients with lower expression in tumor tissues as compared to normal tissues. P-values represent the results of multivariate Cox proportional hazards survival analyses.*

highly significant correlations with patient survival and tumor recurrence, in both univariate and multivariate analyses (Table 2). Patients with high nuclear expression of either of the markers showed better survival and a lower chance of tumor recurrence, which was confirmed by plotting Kaplan-Meier curves or cumulative incidence plots for both patient survival (OS and DSS) and tumor recurrence-free survival (LRRFS and DRFS) (data not shown).

Survival analyses of combined markers

As we know that most of these markers work together in multi-protein complexes in order to remodel the (local) chromatin structure, we performed combined analyses of histone

TABLE 2. Univariate and multivariate survival analyses individual markers

			SIRT1	HDAC1	HDAC2	H3K56Ac	H4K16Ac
OS	Univariate	p-value	0.8	0.07	0.3	**0.004**	**0.03**
		HR	1.0	0.7	0.8	0.6	0.7
		(95% CI)	(0.69-1.33)	(0.53-1.02)	(0.60-1.16)	(0.45-0.86)	(0.50-0.97)
	Multivariate	p-value	0.2	**0.03**	0.1	**0.02**	**0.02**
		HR	0.8	0.7	0.7	0.7	0.7
		(95% CI)	(0.58-1.15)	(0.49-0.97)	(0.54-1.07)	(0.47-0.94)	(0.47-0.94)
DSS	Univariate	p-value	**0.05**	**0.02**	**0.05**	0.1	**0.009**
		HR	0.6	0.6	0.6	0.7	0.5
		(95% CI)	(0.36-0.99)	(0.34-0.92)	(0.36-1.003)	(0.42-1.11)	(0.31-0.84)
	Multivariate	p-value	**0.01**	**0.009**	**0.03**	0.2	**0.02**
		HR	0.5	0.5	0.6	0.7	0.5
		(95% CI)	(0.29-0.87)	(0.29-0.84)	(0.33-0.95)	(0.44-1.21)	(0.31-0.89)
LRRFS	Univariate	p-value	0.2	**0.01**	**0.03**	0.2	**0.03**
		HR	0.7	0.6	0.6	0.7	0.6
		(95% CI)	(0.48-1.15)	(0.37-0.88)	(0.39-0.95)	(0.49-1.16)	(0.40-0.95)
	Multivariate	p-value	**0.05**	**0.008**	**0.009**	*0.07*	**0.03**
		HR	0.6	0.5	0.5	0.6	0.6
		(95% CI)	(0.41-1.01)	(0.34-0.85)	(0.34-0.86)	(0.41-1.03)	(0.38-0.95)
DRFS	Univariate	p-value	0.1	**0.03**	*0.09*	0.2	*0.06*
		HR	0.7	0.6	0.7	0.8	0.7
		(95% CI)	(0.45-1.10)	(0.39-0.94)	(0.43-1.06)	(0.50-1.19)	(0.42-1.01)
	Multivariate	p-value	**0.04**	**0.02**	**0.04**	0.1	*0.07*
		HR	0.6	0.6	0.6	0.7	0.7
		(95% CI)	(0.38-0.98)	(0.35-0.89)	(0.38-0.98)	(0.44-1.11)	(0.41-1.04)

Shown are the results of the univariate and multivariate analyses of all individual markers, with all p-values and hazard ratios (HR) with their 95% confidence intervals (95% CI). OS = overall survival, DSS = disease-specific survival, LRRFS = locoregional recurrence-free survival, DRFS = distant recurrence-free survival. For each marker, the low expression group (below median expression) was used as reference group. Differences in clinical outcome between patient groups are presented as hazard ratios compared to the reference group. Significant p-values are indicated in ***bold****, p-values showing a trend (between 0.05 and 0.1) in Italic.*

deacetylases SIRT1, HDAC1 and HDAC2 together with either H3K56Ac or H4K16Ac. We divided the patients into five groups, based on the number of markers with "high expression" for this specific group of patients, i.e. all low (group 1), one high (group 2), two high (group 3), three high (group 4) and all high (group 5). All multivariate trend analyses showed significant

differences in patient survival and tumor recurrence (Table 3). For the combined analyses of the histone deacetylases and H3K56Ac, each increase of one unit (one additional marker showing high expression) compared to the "all low" patient group resulted in a hazard ratio (HR) of 0.82 (0.72-0.94; p=0.005) for OS, 0.72 (0.59-0.88; p=0.001) for DSS, 0.74 (0.62-0.88; p=0.001) for LRRFS and 0.77 (0.64-0.92; p=0.003) for DRFS. Similarly, for the combination of the histone deactylases and H4K16Ac, a HR of 0.86 (0.76-0.97; p=0.01) for OS, 0.73 (0.60-0.88; p=0.001) for DSS, 0.77 (0.66-0.90; p=0.001) for LRRFS and 0.79 (0.68-0.93; p=0.006) for DRFS were observed per unit of increase. Competing risk analyses showed that the more markers showed high expression, the lower the cumulative incidence (Figures 4A and 4B). For each of the individual patient groups, a decrease in hazard ratio was observed when more markers showed high expression (Figure 4C and Supplementary Table 1). The lowest hazard ratio was observed for patients with high expression of all markers (group 5) as compared to the reference group with low expression of all markers (group 1). A similar stratification of patient groups was observed for overall survival.

Discussion

It is becoming increasingly clear that epigenetics plays an important role in tumor development. Increasing knowledge about the role of epigenetic mechanisms in cancer has guided the development of new epigenetic anti-cancer therapies, often combined with existing therapies (1). To date, however, such epigenetic therapies have only been proven effective in hematological diseases and treatment of solid cancers has proven challenging. For solid tumors, epigenetic therapies may require the development of therapies that for example target multi-protein complexes. In ongoing research an increasing number of such multi-protein complexes are being identified (18). In this study, we investigated three histone deacetylases that act together to remodel the chromatin in response to DNA damage and are important regulators of gene expression during embryonic development (19) and play a role in tumor initiation and progression (20). Deregulation of these histone deacetylases could result in tumor development and progression (12,21). In this study, we demonstrated an increased nuclear expression of HDAC2, and decreased nuclear expression of SIRT1 and H4K16Ac in tumor cells as compared to normal cells. Other groups also reported similar changes in expression between normal and tumor tissues in literature.(7,12) Loss of H4K16Ac has been described to be a common hallmark of human cancers (22), which was mostly linked to hypomethylation of DNA repetitive sequences during tumor progression. This might be correlated to LINE-1 hypomethylation, which we previously showed to correlate with shorter patient survival and higher chances of tumor recurrence in early-stage rectal cancer (23).

***Table 3 (see next two pages)**. Shown are the results of the univariate and multivariate trend analyses of the combined markers using the group numbers as continuous variables, with all p-values and hazard ratios (HR) with their 95% confidence intervals (95% CI). Displayed hazard ratios reflect the hazard ratio with an increase of 1 unit, meaning an increase in the number of markers showing high expression (reflected in a higher group number). OS = overall survival, DSS = disease-specific survival, LRRFS = locoregional recurrence-free survival, DRFS = distant recurrence-free survival. Significant values are shown in **bold**, trends in Italic. * = second primary tumor during follow-up period.*

TABLE 3. Univariate and multivariate trend analyses combined markers SIRT1, HDAC1, HDAC2 and H3K56Ac

		OS			DSS		
Univariate		p-value	HR	(95% CI)	p-value	HR	(95% CI)
Combined markers		**0.02**	0.86	(0.75-0.98)	**0.003**	0.75	(0.62-0.91)
Multivariate							
Combined markers		**0.005**	0.82	(0.72-0.94)	**0.001**	0.72	(0.59-0.88)
Age at operation		**<0.001**	1.86	(1.55-2.23)	**0.009**	1.37	(1.08-1.73)
Gender		0.86	1.03	(0.73-1.46)	0.9	1	(0.59-1.68)
TNM stage	*1*	**0.005**			**<0.001**		
	2	0.13	1.5	(0.89-2.55)	*0.07*	2.47	(0.91-6.68)
	3	**0.002**	2.43	(1.38-4.29)	**<0.001**	8.02	(2.93-21.98)
Tumor location		0.24	1.26	(0.85-1.87)	*0.08*	1.67	(0.94-2.94)
Tumor size		**0.01**	1.09	(1.02-1.18)	**0.05**	1.11	(1.00-1.24)
MSS status	*MSS*	0.79			0.3		
	MSI	0.5	0.84	(0.49-1.41)	0.2	0.55	(0.23-1.32)
	Unknown	0.89	0.97	(0.59-1.58)	0.6	1.2	(0.59-2.41)
Tumor in follow-up *		**0.002**	2.18	(1.32-3.59)	0.2	1.71	(0.69-4.20)
Adjuvant therapy		0.69	1.1	(0.67-1.81)	0.1	0.57	(0.29-1.13)

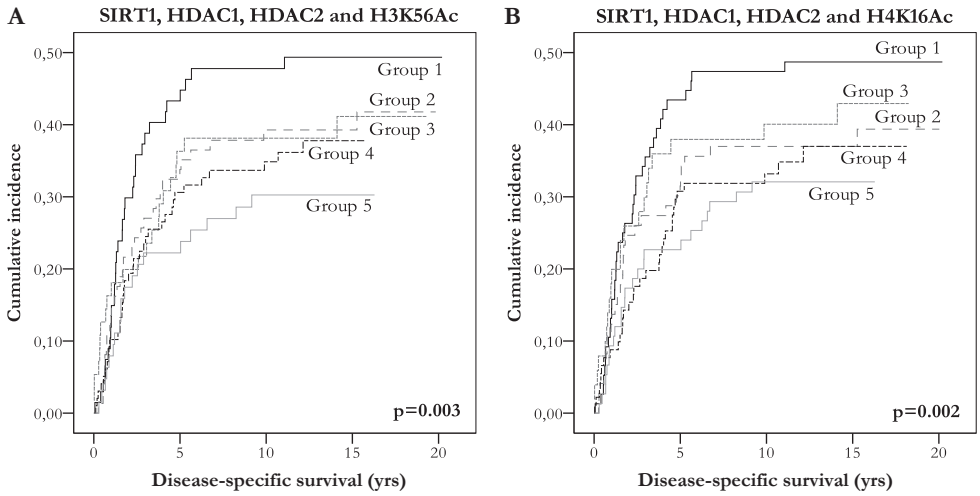
		LRRFS			DRFS		
Univariate		p-value	HR	(95% CI)	p-value	HR	(95% CI)
Combined markers		**0.004**	0.78	(0.66-0.93)	**0.01**	0.81	(0.68-0.95)
Multivariate							
Combined markers		**0.001**	0.74	(0.62-0.88)	**0.003**	0.77	(0.64-0.92)
Age at operation		**0.05**	1.23	(1.00-1.50)	**0.02**	1.29	(1.04-1.59)
Gender		0.67	0.91	(0.58-1.42)	0.7	0.93	(0.58-1.48)
TNM stage	*1*	**<0.001**			**<0.001**		
	2	0.3	1.51	(0.72-3.18)	0.2	1.62	(0.72-3.67)
	3	**0.001**	3.63	(1.69-7.80)	**0.001**	3.99	(1.73-9.21)
Tumor location		**0.03**	1.71	(1.05-2.78)	0.2	1.4	(0.84-2.35)
Tumor size		**0.004**	1.14	(1.04-1.25)	**0.004**	1.15	(1.04-1.26)
MSS status	*MSS*	0.36			0.6		
	MSI	0.33	0.69	(0.34-1.44)	0.4	0.71	(0.34-1.48)
	Unknown	0.37	1.32	(0.72-2.39)	0.8	1.07	(0.56-2.02)
Tumor in follow-up *		**<0.001**	3.39	(1.84-6.27)	**<0.001**	3.89	(2.07-7.30)
Adjuvant therapy		0.13	0.62	(0.33-1.16)	0.3	0.69	(0.36-1.32)

SIRT1, HDAC1, HDAC2 and H4K16Ac

		OS			DSS		
Univariate		p-value	HR	(95% CI)	p-value	HR	(95% CI)
Combined markers		**0.06**	0.89	(0.79-1.003)	**0.002**	0.75	(0.63-0.90)
Multivariate							
Combined markers		**0.01**	0.86	(0.76-0.97)	**0.001**	0.73	(0.60-0.88)
Age at operation		**<0.001**	1.91	(1.59-2.29)	**0.006**	1.39	(1.01-1.77)
Gender		0.8	1.05	(0.74-1.47)	0.8	0.93	(0.56-1.55)
TNM stage	*1*	**0.005**			**<0.001**		
	2	*0.07*	1.61	(0.95-2.73)	*0.07*	2.54	(0.94-6.86)
	3	**0.002**	2.6	(1.42-4.14)	**<0.001**	7.72	(2.82-21.13)
Tumor location		0.3	1.26	(0.85-1.86)	*0.09*	1.62	(0.92-2.88)
Tumor size		**0.01**	1.1	(1.02-1.18)	**0.02**	1.14	(1.03-1.27)
MSS status	*MSS*	0.9			0.4		
	MSI	0.6	0.88	(0.53-1.47)	0.2	0.59	(0.24-1.42)
	Unknown	0.9	0.96	(0.59-1.56)	0.6	1.23	(0.61-2.48)
Tumor in follow-up *		**0.005**	2.02	(1.24-3.29)	0.2	1.72	(0.80-4.24)
Adjuvant therapy		0.8	1.06	(0.65-1.73)	0.1	0.57	(0.29-1.13)

		LRRFS			DRFS		
Univariate		p-value	HR	(95% CI)	p-value	HR	(95% CI)
Combined markers		**0.003**	0.79	(0.68-0.93)	**0.01**	0.81	(0.69-0.95)
Multivariate							
Combined markers		**0.001**	0.77	(0.66-0.90)	**0.006**	0.79	(0.68-0.93)
Age at operation		**0.03**	1.25	(1.02-1.54)	0.01	1.32	(1.06-1.63)
Gender		0.5	0.85	(0.54-1.33)	0.6	0.87	(0.54-1.39)
TNM stage	*1*	**0.001**			0.001		
	2	0.2	1.57	(0.75-3.29)	0.2	1.66	(0.73-3.76)
	3	**0.001**	3.49	(1.62-7.53)	0.001	3.94	(1.70-9.12)
Tumor location		**0.04**	1.67	(1.02-2.72)	0.2	1.37	(0.81-2.31)
Tumor size		**0.001**	1.16	(1.06-1.27)	0.002	1.17	(1.06-1.28)
MSS status	*MSS*	0.4			0.7		
	MSI	0.4	0.76	(0.36-1.57)	0.5	0.76	(0.36-1.58)
	Unknown	0.3	1.34	(0.74-2.45)	0.8	1.08	(0.57-2.06)
Tumor in follow-up *		**<0.001**	3.22	(1.76-5.90)	<0.001	3.75	(2.01-6.99)
Adjuvant therapy		0.1	0.62	(0.33-1.17)	0.3	0.69	(0.36-1.32)

Figure 4

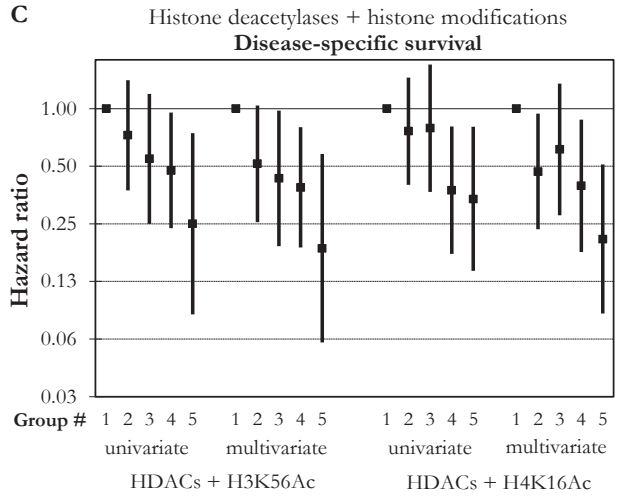


Numbers at risk

Year	0	5	10	15	20
Group 1	42	20	14	13	0
Group 2	48	33	24	13	0
Group 3	34	24	19	7	0
Group 4	62	43	27	3	0
Group 5	31	23	15	2	0

Numbers at risk

Year	0	5	10	15	20
Group 1	48	25	18	10	1
Group 2	50	34	26	14	1
Group 3	26	18	14	7	0
Group 4	55	39	27	4	0
Group 5	42	33	17	2	0



***Survival analyses of the combined marker groups**. Shown are cumulative incidence curves after competing risk analyses for multi-marker analyses using histone deacetylases SIRT1, HDAC1 and HDAC2 combined with either H3K56Ac (**A**) or H4K16Ac (**B**). Group numbers 1-5 indicate the patient groups based on the number of markers showing high expression, with group 1 (all low), group 2 (one high), group 3 (two high), group 4 (three high), and group 5 (all high). In panel **C**, the hazard ratios (HR; y-axis) related to disease-specific survival (DSS) are shown for the combined HDACs (SIRT1, HDAC1 and HDAC2) with each of the histone modifications (H3K56Ac or H4K16Ac) compared to the reference group (group 1) for both univariate and multivariate analyses. HRs are indicated with ■, the corresponding 95% confidence intervals are indicated with protruding black lines.*

It seems paradoxical that although both HDAC1 and H3K56Ac showed no significantly different overall nuclear expression levels in normal versus tumor tissues in the whole study cohort, within individual tumors an inverse correlation was observed. Several studies have suggested different roles for HDAC1 in early stage versus advanced tumors (21,24), which suggests a qualitative difference rather than a quantitative difference between normal and tumor tissues. For both markers, survival differences were observed between patients with high and low nuclear expression in tumor cells of the respective markers, indicating that in individual tumors, aberrant expression of these markers could contribute to the tumorigenic process.

We have shown in this study that by combining multiple histone-modifying enzymes and histone modifications, distinct patient groups can be identified, stressing the importance of analyzing multi-protein complexes together. A higher number of markers showing high nuclear expression correlated with better patient survival and a lower chance of tumor recurrence. This finding can be explained by regarding the cellular functions of the histone deacetylases and the histone modifications. Higher expression of the histone deacetylases might prevent aberrant activation of oncogenes and DNA repetitive sequences. Higher levels of H4K16Ac, as discussed above, could be associated with silenced (methylated) repetitive sequences, which may result in less genomic events such as retrotransposition (LINE-1), translocations, or DNA double strand breaks. High levels of H3K56Ac are necessary for proper non-homologous end-joining, resulting in less error-prone repairs of double strand breaks and hence lower chances of developing rapidly mutating and aggressive tumors.

The immunohistochemical stainings presented in this study can be easily implemented in a clinical setting, as all stainings are performed on paraffin-embedded tissues. With the present-day technological advances using computer-based recognition software, the semi-automated scoring we used might be a first step towards automated scoring of nuclear staining in a clinical setting, thereby reducing the influence of subjectivity of human interpretation of color and color-intensity. Future studies could address the differences in epigenetic regulation between the tumor center and the tumor invasive front on whole tumor sections, as many studies have already reported differential expression of various proteins at different sites within the tumor (25). In addition, comparing tumor cells and the tumor microenvironment might provide useful information in understanding the role of epigenetic changes in colorectal cancer development and/or progression.

In conclusion, we have shown in this study that global nuclear expression of histone modifications and histone deacetylases were correlated to clinical outcome in colorectal cancers. Combining multiple markers gives us more insight into the complex interplay between histone modifiers and histone modifications. These results are a first indication that combining multiple epigenetic markers results in identification of distinct patient groups, and provide insight in the involvement of epigenetic mechanisms in colorectal cancer growth. More research is needed to study the exact functions of the studied histone deacetylases and their associated histone modifications, and to identify other combinations of epigenetic markers that play a role in colorectal cancer.

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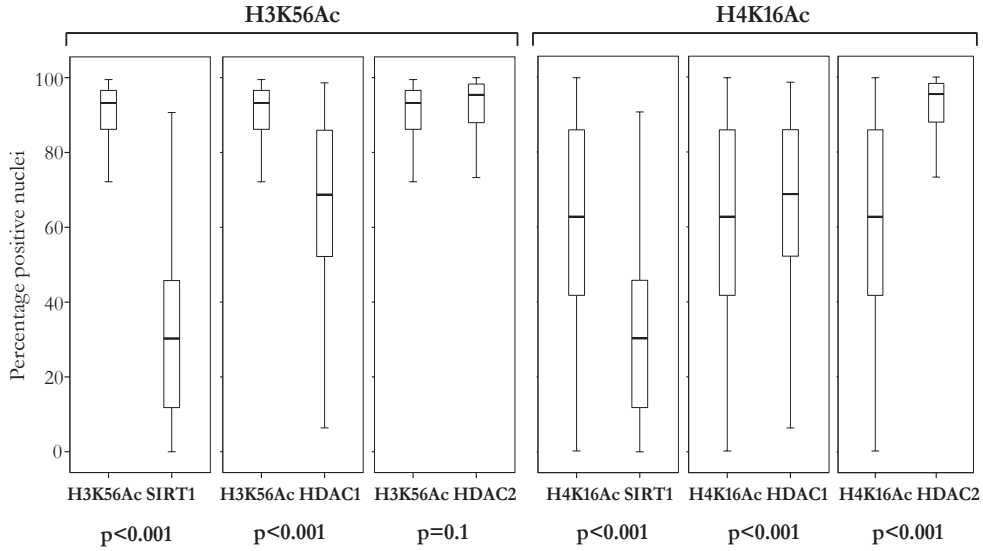
Supplementary files

Supplementary Figure 1



Expression of histone modifications versus histone deacetylases. *Shown are boxplots indicating mean nuclear expression levels (as determined using immunohistochemistry) in tumor cells of the histone modifications versus each of the individual enzymes. P-values represent paired students t-test results.*

Supplementary TABLE 1 (see next two pages)

Shown are the results of the univariate and multivariate analyses of the combined markers using the patient groups as categorical variables, with all p-values and hazard ratios (HR) with their 95% confidence intervals (95% CI). OS = overall survival, DSS = disease-specific survival, LRRFS = locoregional recurrence-free survival, DRFS = distant recurrence-free survival. Patients groups were made based on the number of markers showing high (above-median) expression: all low (group 1), 1 high (group 2), 2 high (group 3), 3 high (group 4) and all high (group 5). Significant values are shown in ***bold****, p-values showing a trend (between p=0.05 and p=0.1) in Italic. * =second primary tumor during follow-up period.*

Supplementary TABLE 1. Univariate and multivariate survival analyses individual groups combined markers

SIRT1, HDAC1, HDAC2 and **H3K56Ac**		**OS**			**DSS**			**LRRFS**			**DRFS**		
		Univariate			Univariate			Univariate			Univariate		
		p-value	HR	(95% CI)	p-value	HR	(95% CI)	p-value	HR	(95% CI)	p-value	HR	(95% CI)
Group 1		0.1			*0.07*			**0.05**			0.1		
Group 2		0.2	0.71	(0.44-1.15)	0.3	0.73	(0.37-1.41)	0.2	0.66	(0.36-1.19)	0.6	0.85	(0.46-1.58)
Group 3		0.2	0.67	(0.39-1.16)	0.1	0.55	(0.25-1.19)	**0.04**	0.48	(0.24-0.98)	0.2	0.63	(0.30-1.31)
Group 4		*0.08*	0.65	(0.40-1.06)	**0.04**	0.48	(0.24-0.95)	**0.04**	0.54	(0.29-0.98)	0.2	0.67	(0.36-1.26)
Group 5		**0.01**	0.44	(0.23-0.84)	**0.01**	0.25	(0.08-0.75)	**0.007**	0.29	(0.12-0.71)	**0.01**	0.29	(0.11-0.77)
		Multivariate			Multivariate			Multivariate			Multivariate		
		p-value	HR	(95% CI)	p-value	HR	(95% CI)	p-value	HR	(95% CI)	p-value	HR	(95% CI)
Group 1		**0.005**			**0.02**			**0.001**			**0.02**		
Group 2		**0.003**	0.45	(0.27-0.76)	*0.06*	0.52	(0.26-1.04)	**0.006**	0.39	(0.21-0.77)	*0.07*	0.54	(0.28-1.05)
Group 3		**0.03**	0.54	(0.31-0.94)	**0.04**	0.43	(0.19-0.97)	**0.009**	0.37	(0.18-0.78)	*0.09*	0.53	(0.25-1.13)
Group 4		**0.003**	0.47	(0.29-0.78)	**0.01**	0.38	(0.19-0.80)	**0.005**	0.41	(0.22-0.77)	0.1	0.59	(0.31-1.13)
Group 5		**0.001**	0.33	(0.17-0.65)	**0.004**	0.19	(0.06-0.58)	**<0.001**	0.16	(0.06-0.41)	**0.001**	0.16	(0.06-0.45)
Age at operation		**0**	1.89	(1.57-2.27)	**0.01**	1.36	(1.08-1.72)	**0.05**	1.22	(0.99-1.49)	**0.03**	1.28	(1.03-1.58)
Gender		0.7	1.06	(0.75-1.51)	0.9	1.01	(0.60-1.71)	0.8	0.94	(0.59-1.49)	0.8	0.94	(0.59-1.52)
TNM stage	*1*	**0.001**			**<0.001**			**<0.001**			**<0.001**		
	2	*0.1*	1.57	(0.93-2.67)	*0.06*	2.58	(0.95-7.01)	0.2	1.68	(0.80-3.53)	0.2	1.78	(0.78-4.06)
	3	**0.001**	2.75	(1.55-4.89)	**<0.001**	8.65	(3.13-23.92)	**<0.001**	4.42	(2.02-9.70)	**0.001**	4.47	(1.92-10.42)
Tumor location		0.2	1.28	(0.86-1.89)	0.1	1.62	(0.91-2.89)	**0.04**	1.67	(1.02-2.73)	0.3	1.34	(0.79-2.25)
Tumor size		**0.01**	1.09	(1.02-1.18)	**0.04**	1.12	(1.00-1.24)	**0.005**	1.14	(1.04-1.24)	**0.003**	1.15	(1.05-1.27)
MSS status	*MSS*	0.5			0.2			0.2			0.5		
	MSI	0.2	0.73	(0.43-1.24)	0.1	0.5	(0.21-1.22)	0.2	0.58	(0.28-1.23)	0.2	0.64	(0.30-1.34)
	Unknown	0.9	0.98	(0.59-1.59)	0.6	1.2	(0.59-2.45)	0.3	1.35	(0.73-2.48)	0.9	1.06	(0.55-2.03)
Tumor in follow-up *		**0.001**	2.26	(1.37-3.74)	0.2	1.78	(0.71-4.44)	**<0.001**	3.78	(2.03-7.06)	**<0.001**	4.68	(2.46-8.91)
Adjuvant therapy		0.9	0.97	(0.59-1.61)	*0.07*	0.53	(0.26-1.06)	**0.04**	0.5	(0.26-0.97)	0.1	0.57	(0.29-1.11)

SIRT1, HDAC1, HDAC2 and **H4K16Ac**		**OS**			**DSS**			**LRRFS**			**DRFS**		
		Univariate			Univariate			Univariate			Univariate		
		p-value	HR	(95% CI)	p-value	HR	(95% CI)	p-value	HR	(95% CI)	p-value	HR	(95% CI)
Group 1		0.3			**0.04**			**0.05**			**0.03**		
Group 2		0.1	0.68	(0.43-1.09)	0.4	0.76	(0.40-1.45)	0.3	0.71	(0.39-1.27)	0.5	0.83	(0.45-1.52)
Group 3		0.3	0.74	(0.42-1.28)	0.5	0.79	(0.37-1.70)	0.4	0.77	(0.39-1.51)	0.7	1.12	(0.58-2.17)
Group 4		*0.07*	0.64	(0.40-1.04)	**0.01**	0.37	(0.17-0.81)	**0.009**	0.42	(0.22-0.80)	**0.01**	0.41	(0.20-0.84)
Group 5		**0.05**	0.58	(0.34-0.99)	**0.01**	0.34	(0.14-0.80)	**0.02**	0.42	(0.21-0.86)	*0.06*	0.49	(0.24-1.03)
		Multivariate			Multivariate			Multivariate			Multivariate		
		p-value	HR	(95% CI)	p-value	HR	(95% CI)	p-value	HR	(95% CI)	p-value	HR	(95% CI)
Group 1		**0.04**			**0.008**			**0.02**			**0.009**		
Group 2		**0.03**	0.56	(0.34-0.94)	**0.03**	0.47	(0.23-0.94)	0.2	0.64	(0.35-1.18)	0.3	0.73	(0.39-1.39)
Group 3		*0.06*	0.58	(0.33-1.03)	0.2	0.61	(0.28-1.35)	0.6	0.81	(0.40-1.63)	0.4	1.32	(0.66-2.64)
Group 4		*0.09*	0.65	(0.39-1.06)	**0.03**	0.39	(0.18-0.88)	**0.03**	0.46	(0.23-0.92)	**0.04**	0.46	(0.22-0.98)
Group 5		**0.003**	0.43	(0.25-0.75)	**0.001**	0.21	(0.09-0.51)	**0.002**	0.3	(0.14-0.63)	**0.006**	0.34	(0.16-0.74)
Age at operation		**<0.001**	1.89	(1.58-2.27)	**0.005**	1.39	(1.10-1.77)	**0.04**	1.24	(1.01-1.52)	**0.02**	1.29	(1.05-1.60)
Gender		0.9	1.02	(0.72-1.44)	0.6	0.87	(0.51-1.47)	0.3	0.79	(0.50-1.27)	0.3	0.79	(0.49-1.28)
TNM stage	*1*	**0.001**			**<0.001**			**0.001**			**0.001**		
	2	*0.07*	1.63	(0.96-2.78)	*0.07*	2.56	(0.93-7.01)	0.3	1.51	(0.71-3.19)	0.4	1.48	(0.65-3.41)
	3	**0.001**	2.87	(1.59-5.19)	**<0.001**	8.9	(3.11-25.51)	**0.002**	3.55	(1.61-7.84)	**0.003**	3.62	(1.53-8.53)
Tumor location		0.21	1.29	(0.86-1.92)	*0.07*	1.73	(0.96-3.09)	**0.05**	1.63	(0.99-2.68)	0.3	1.3	(0.77-2.20)
Tumor size		**0.005**	1.11	1.03-1.19)	**0.007**	1.15	(1.04-1.27)	**0.001**	1.17	(1.07-1.28)	**<0.001**	1.19	(1.08-1.31)
MSS status	*MSS*	0.7			0.4			0.5			0.8		
	MSI	0.4	0.82	(0.49-1.38)	0.2	0.55	(0.23-1.35)	0.4	0.75	(0.36-1.56)	0.5	0.77	(0.37-1.60)
	Unknown	0.8	0.95	(0.58-1.55)	0.7	1.14	(0.56-2.31)	0.4	1.29	(0.71-2.37)	0.9	1.03	(0.54-1.97)
Tumor in follow-up *		**0.01**	1.92	(1.17-3.14)	0.2	1.81	(0.73-4.47)	**<0.001**	3.33	(1.78-6.24)	**<0.001**	4.42	(2.31-8.45)
Adjuvant therapy		0.9	0.99	(0.59-1.64)	*0.08*	0.53	(0.26-1.07)	0.1	0.61	(0.32-1.17)	0.3	0.69	(0.35-1.34)