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ORIGINAL ARTICLE

Clinical significance of histone deacetylases 1, 2, 3, and 7: HDAC2 is an independent predictor of survival in HCC

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Abstract Histone deacetylases (HDAC) are responsible for the transcriptional control of genes through chromatin remodeling and control tumor suppressor genes. In several tumors, their expression has been linked to clinicopathological factors and patient survival. This study investigates HDACs 1, 2, 3, and 7 expressions in hepatocellular carcinoma (HCC) and their correlation with clinical data and patient survival. Tissue microarrays of 170 surgically resected primary HCCs and adjacent uninvolved tissue were evaluated immunohistochemically for the expression of HDACs 1, 2, 3, 7, and Ki-67 and were analyzed with respect to clinicopathological data and patient survival. HDACs 1, 2, 3, and Ki-67 were expressed significantly higher in cancer cells compared to normal tissue (HDAC1: $p=0.034$, HDACs 2 and 3 and Ki-67: $p<0.001$), while HDAC7 expression did not differ between HCC and non-cancerous liver tissue. In tumor tissue HDACs 1–3 expression levels showed high concordance with each other, Ki-67 and tumor grade ($p<0.001$). High HDAC2 expression was associated with poor survival in low-grade and early-stage tumors ($p<0.05$). The expression of the HDACs 1, 2, and 3 (but not HDAC7) isoenzymes correlates with clinicopathological factors, and HDAC2 expression has an impact on patient survival.

Keywords Correlation analysis · Expression analysis · Hepatocellular carcinoma · Histone deacetylases · Overall survival · Tissue microarray

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Introduction

Hepatocellular carcinoma (HCC) belongs to the most common tumor diseases worldwide, and its incidence is increasing in industrial nations [1, 2]. The causes for HCC are diverse and include chronic viral hepatitis, heavy alcohol use, or metabolic diseases such as hemochromatosis. The diversity of the pathophysiological processes that results from these different etiological factors makes HCC a heterogeneous tumor [3]. Curative therapies, such as liver resection and transplantation, are possible in only 15% of patients, but recurrence rates are high. Those not eligible for

surgical intervention benefit from locoregional therapies, such as transarterial chemoembolization and radiofrequency ablation, but these therapies, too, are limited by high recurrence rates. Although several new chemotherapeutic approaches targeting different oncogenic signaling pathways have been introduced into HCC therapy, only the multikinase inhibitor sorafenib showed a beneficial clinical response and improved overall survival of patients [4, 5]. Yet, additional new therapies are urgently needed as many patients experience dose-limiting toxicity and tumor progression.

Recently, epigenetic regulation of gene transcription, e.g., by DNA methylation or histone acetylation, has been shown to be a promising new therapeutic target in oncology [6]. Specifically in tumor cells, inhibition of histone deacetylases (HDACs) leads to cell cycle inhibition, e.g., by upregulation of $p21^{cip1/waf1}$ and various pro-apoptotic effects that are also mediated by non-nuclear signalling of HDAC inhibitors [6–8]. Our own previous data also show that inhibition of HDACs sensitizes HCC cell lines to various chemotherapeutics and therefore provide a rationale for further clinical investigation as a potential therapeutic target in HCC [9, 10].

Different patterns of HDAC overexpression have been shown for various human cancers [11] and these results indicate that HDACs have a prognostic value in renal cell carcinomas [12], tumors of the gastrointestinal tract [13, 14], and prostate cancer [15]. In HCC, out of 18 known HDAC isoforms (HDACs 1–11 and Sirtuins 1–7), only one isoform (HDAC1) has been analyzed in 47 human tumor samples from Asian HCCs and the results indicate a role in patient outcome [16]. We here examined the expression of class I HDACs (1, 2, and 3) and class II HDAC7 in a cohort of 170 European HCC patients along with corresponding uninvolved normal hepatic tissue to correlate their relationship with clinicopathological factors and the impact on patient survival. Specifically with respect to HDAC inhibitor therapy, HDAC expression, determined prior to HDAC inhibitor therapy, could help predict early and overall therapeutic response before treatment initiation [3, 17–19].

Materials and methods

Patients and tissue samples

Archived HCC tumor samples, corresponding adjacent non-tumorous hepatic tissue and clinical data were obtained from 170 patients who underwent curative surgical resection at the Department of Surgery, University Hospital Erlangen, Germany, and at the Department of Surgery, University Hospital, Regensburg, Germany, between 2001 and 2007. Laboratory and clinical data were retrieved from the hospitals' case files, laboratory information systems, and tumor registers. Detailed patient data is presented in Table 1. This study was covered by ethical votes of the medical faculties of the Universities of Regensburg and Erlangen–Nuremberg [20, 21].

Tissue microarray and immunohistochemical staining

The surgical specimens were obtained during routine hepatectomy, fixed in formalin and embedded in paraffin. For each patient, one tumor sample and one sample from adjacent normal tissue uninvolved by the tumor were used in the array. The punches were transferred onto a new paraffin block, forming the tissue microarrays as described previously [20, 21]. Two to three-micrometer slices of these were cut, deparaffinized in xylene, and rehydrated in descending dilutions of ethanol. The following antibody

Table 1 Patient characteristics

			N (percent of total)
Patients	$n=170$	Male	145 (85.3%)
		Female	24 (14.7%)
Age	61.7±11.2 years	Male	63.0±10.4 years
		Female	55.4±12.4 years
pT		1	67 (39.4%)
		2	49 (28.8%)
		3a	47 (27.6%)
		3b	4 (2.4%)
		4	3 (1.8%)
pN		N0	85.7%
		N1	14.3%
pM		M0	91.4%
		M1	8.6%
Tumor grade		G1	13.1%
		G2	59.8%
		G3	27.1%
Child-Pugh classification		No cirrhosis	16.7%
		A	48.4%
		B	27.8%
		C	7.1%
CLIP score		0	75.6%
		1	16.7%
		2	6.4%
		3	1.3%
Etiology	Viral		20%
	Alcohol		35.3%
	Viral and alcohol		2.9%
	Cholangitis		1.8%
	Cryptogenic		9.4%
	No information available		30.6%

labeling was achieved using EnVision+Dual Link System HRP (K4061, Dako GmbH, Hamburg, Germany). Antigen retrieval was done using 1 mM Tris-EDTA buffer in a pressure cooker at 120°C for 5 min. Endogenous peroxidases were blocked by incubating the slices for 5 min with blocking solution (S2023, Dako), followed by 5 min of washing. Primary antibodies were applied for 30 min at room temperature at the given dilutions (using antibody diluent, S2022, Dako): anti-HDAC1 (ready-to-use dilution, ab15316, Abcam plc, Cambridge, UK), anti-HDAC2 (1:500, ab12169, Abcam), anti-HDAC3 (1:500, 612635, BD Biosciences, Heidelberg, Germany), anti-HDAC7 (1:100, ab53101, Abcam), and anti-Ki-67/MIB-1 (1:50, F7268, Dako). Following 5 min of washing, labeled polymer-HRP (goat-anti-rabbit or goat-anti-mouse secondary antibodies linked with horseradish peroxidase, Dako) was applied for 30 min at room temperature. Positive antibody binding was stained using DAB+(K3467, Dako) for 10 min; the nuclei were counterstained using Mayers's hematoxylin (S3309, Dako) for 1 min. Positive controls were run in parallel. In addition, HDAC stainings for random tumors were performed on traditional slides to ensure homogenous distribution of HDAC expression throughout the tumor.

Interpretation of immunohistochemical staining

The sections were investigated by two investigators (AA and KQ), who were blinded to the patient characteristics and outcome. Evaluation of the staining was done in four high-power fields for each case. HDAC expression was evaluated using a semi-quantitative composite categorical score for immunohistochemical intensity and extent (immunoreactivity score, IRS). Intensity of staining (score 0–3: 0, negative; 1, weak; 2, moderate; 3 strong) as well as percentage of cells stained (score 0–3: 0, 0%; 1, 1–25%; 2, 25–50%; 3, >50%) were evaluated separately for each case. The IRS was calculated by adding the intensity and frequency scores, resulting in values ranging from 0 to 6. Inflammatory cells which stained positive were not counted. Ki-67 was quantified by counting the number of positive nuclei per 100 cells in a representative tumor area and scored as follows: 0, negative; 1, 1–5%; 2, 6–10%; 3, 11–20%; 4, 21–30%; 5, 31–40%; and 6, 41–100%. Tumor grade scoring was G1: well-differentiated (WHO grade 1), G2: moderately differentiated (WHO grade 2), and G3: poorly and undifferentiated (WHO grades 3 and 4). Interobserver variability was determined by the κ value (κ=0.946).

Statistical analysis

Statistical analysis was performed using SPSS 17 (SPSS Inc., Chicago, IL, USA). Values for albumin, aspartate transaminase (AST/GOT), alanine transaminase (ALT/GPT), total bilirubin, thromboplastin time (Quick), gamma glutamyl transpeptidase (gamma-GT), lactate dehydrogenase (LDH), cholineesterase (CHE), alpha-1-fetoprotein (AFP), volume of resected tissue, resection weight, and the diameter of the largest tumor were provided as continuous variables, while the categorical variables were defined as follows (as provided by the tumor register database)—carcinoembryonic antigen (CEA): normal (<5 ng/ml), intermediate (5–10 ng/ml), high (>10 ng/ml), carbohydrate antigen 19–9 (CA19–9): normal (<40 IE/ml), intermediate (40–50 IE/ml), high (>50 IE/ml), invasion of lymphatic vessels (yes, no), intrahepatic (yes, no), or extrahepatic blood vessels (no, microscopic, macroscopic), cirrhosis at the time of surgery (yes, no), hepatic steatosis (none: <5% of hepatocytes, slight: 5–33%, strong: >34%, which corresponds with the Brunt steatosis grades 0, 1, and 2/3) [22] and were provided as such by the tumor registers.

Because of the lack of any natural grouping tendencies within the HDAC immunoreactivity scores, patients were stratified into low and high HDAC expression groups based on the median expression levels of each isoenzyme.

Univariate analyses between different subgroups were computed using the Mann–Whitney *U* test (continuous and categorical data) as well as with Pearson's χ^2 test (nominal data). Correlation of continuous variables was analyzed with Spearman's rank-order correlation. Univariate analysis of variance (ANOVA) was used to test for differences between the Child-Pugh groups (using least significant difference test and the Bonferroni's post hoc test to adjust for multiple comparisons). Univariate survival analysis was performed using the Kaplan–Meier method comparing the survival curves with the log-rank test. For multivariate survival analysis, the Cox proportional hazards model was used. All tests were performed two tailed. *P* values of less than 0.05 were considered statistically significant.

Results

The clinicopathological data of the patients are summarized in Tables 1, 2, and 3. Histological samples were available from 170 HCC patients along with corresponding normal hepatic tissue for each patient. Mean age at diagnosis was 61.7±11.2 years with 85.3% male and 14.7% female patients. According to the seventh edition of the TNM classification [23], stage distribution of the primary tumors was 37.2%, 28.8%, 27.6%, 2.4%, and 1.8% for pT1, pT2, pT3a, pT3b, and pT4, respectively. Nodal status was negative (pN0) in 85.7% of patients and positive (pN1) in 14.3%. All HCC were curatively resected (R0). Additionally, most cases (91.4%) lacked distant metastases (M0). Distribution of tumor grades was 13.1%, 59.8%, and 27.1% for G1, G2 and G3, respectively. Of all patients, 48.4% were

Table 2 Laboratory parameters

	Cases with data available	Mean±SD
Albumin (g/l)	99	38.54±7.27
GOT (U/l)	48	61.06±59.01
GPT (U/l)	46	58.87±55.75
Total bilirubin (mg/dl)	114	1.15±1.1
Thromboplastin time (quick,%)	43	85.3±16.84
Gamma-GT (U/l)	43	234.65±412.17
LDH (U/l)	31	223.32±65.59
CHE (U/l)	37	4,306.32±2,015.9
AFP (ng/ml)	124	8,379.87±56,168.74
Volume of resected tissue (cm^3)	82	1,562.33±1,702.29
Resection weight (g)	64	635.63±626.8
Diameter of largest tumor (mm)	143	31.26±36.78

classified as Child A, 27.8% Child B, and 7.1% Child C, while 16.7% did not show cirrhosis at the time of diagnosis. Within the Child-Pugh classification groups, there were no significant differences in the distribution of tumor grade and pT stage (ANOVA, $p>0.1$). Because of the retrospective nature of this study, which spans two centers, not all clinical data were available for all patients. Table 2 indicates mean values along with the number of cases with available data for each value.

Table 3 Additional laboratory and pathologic parameters

	Cases with data available	Category	*N* (percent of total)
CEA	42	Normal	38 (90.5%)
		Intermed	2 (4.8%)
		High	2 (4.8%)
CA19-9	39	Normal	29 (74.4%)
		Intermed	4 (10.3%)
		High	6 (15.4%)
Intrahepatic vessel invasion	78	Yes	14 (17.9%)
		No	64 (82.1%)
Extrahepatic vessel invasion	83	No	67 (80.7%)
		Microscopic	10 (12.0%)
		Macroscopic	6 (7.2%)
Invasion of lymphatic vessels	83	Yes	4 (4.8%)
		No	79 (95.2%)
Number of tumors	134	1	113 (84.3%)
		2	16 (11.9%)
		≥3	5 (3.7%)
Steatosis	52	None	28 (53.8%)
		Slight	7 (13.5%)
		Strong	17 (32.7%)
Cirrhosis	143	Yes	100 (69.9%)
		No	43 (30.1%)

Table 3 summarizes additional categorized pathologic and laboratory findings that were used in the statistical analysis.

Expression pattern of HDACs 1–3 and 7 isoenzymes in HCC and adjacent normal hepatic tissue

In general, all HDAC class I isoenzymes were expressed both in the majority of tumor samples and in the adjacent uninvolved hepatic tissue. Negative status (IRS=0) for the HDACs 1, 2, or 3 isoenzymes in tumor tissue was observed in 15, 9, and 1 cases, respectively (8.8%, 5.3%, and 0.6%), vs. 23, 11, and 12 cases in the corresponding normal hepatic tissue (13.5%, 6.5%, and 7.1%). Complete negative staining for HDACs 1–3 (IRS=0 for all three isoenzymes simultaneously) was observed in three cases (1.8%) within normal hepatic tissue and in no case of the tumors. If present, inflammatory cells were also positive for HDACs 1–3 and were not included in the analysis.

HDACs 1–3 were expressed in the nuclei of cancer cells and in the nuclei of corresponding normal hepatocytes. There was no cytoplasmic expression in either tumor or cancer cells for HDACs 1 and 2, while HDAC3 was occasionally observed as granular cytoplasmic staining. All three isoenzymes exhibited higher IRS in cancer cells compared to uninvolved normal hepatocytes: median HDAC1 IRS in normal uninvolved hepatocytes was 3 (quartiles 2–5) vs. 4 (quartiles 2–5) (Mann–Whitney *U* test, $p=0.034$) in tumor tissue, median HDAC2 IRS 4 (quartiles 3–5) vs. 5 (quartiles 4–6) ($p<0.001$), median HDAC3 IRS 4 (quartiles 3–5) vs. 5 (quartiles 5–6) ($p<0.001$), and median Ki-67 IRS 1 (quartiles 1–1) vs. 1 (quartiles 1–2) ($p<0.001$).

HDAC-positive cancer cells were homogenously distributed throughout the tumor section, as exemplified on traditional slides (Online resource 1). Figure 1 shows representative examples of HDAC and Ki-67 expression from the TMA in normal hepatocytes as well as in grades 1, 2, and 3 tumors. In some cases, we observed differential HDAC expression within the same tumor, in what appeared to be a more malignant progressive cell clone and which corresponded to higher-grade tumor cells (Fig. 1c).

HDAC7 expression was generally lower compared to HDACs 1–3 and stained positive in endothelial cells within tumors and within endothelial cells of uninvolved hepatic tissue, without showing a clear preference for any subcellular compartment (Fig. 1b). Median IRS in endothelial cells within normal hepatic tissue and in tumor endothelium was 2 (quartiles 1–2), without showing any significant differences in expression ($p=0.579$). Weak expression in tumor cells or normal hepatocytes was observed occasionally. Complete negative staining (IRS=0) for HDAC7 was observed in 56 samples (32.9%) in endothelia of normal hepatic tissue and in 60 tumor samples (35.3%).

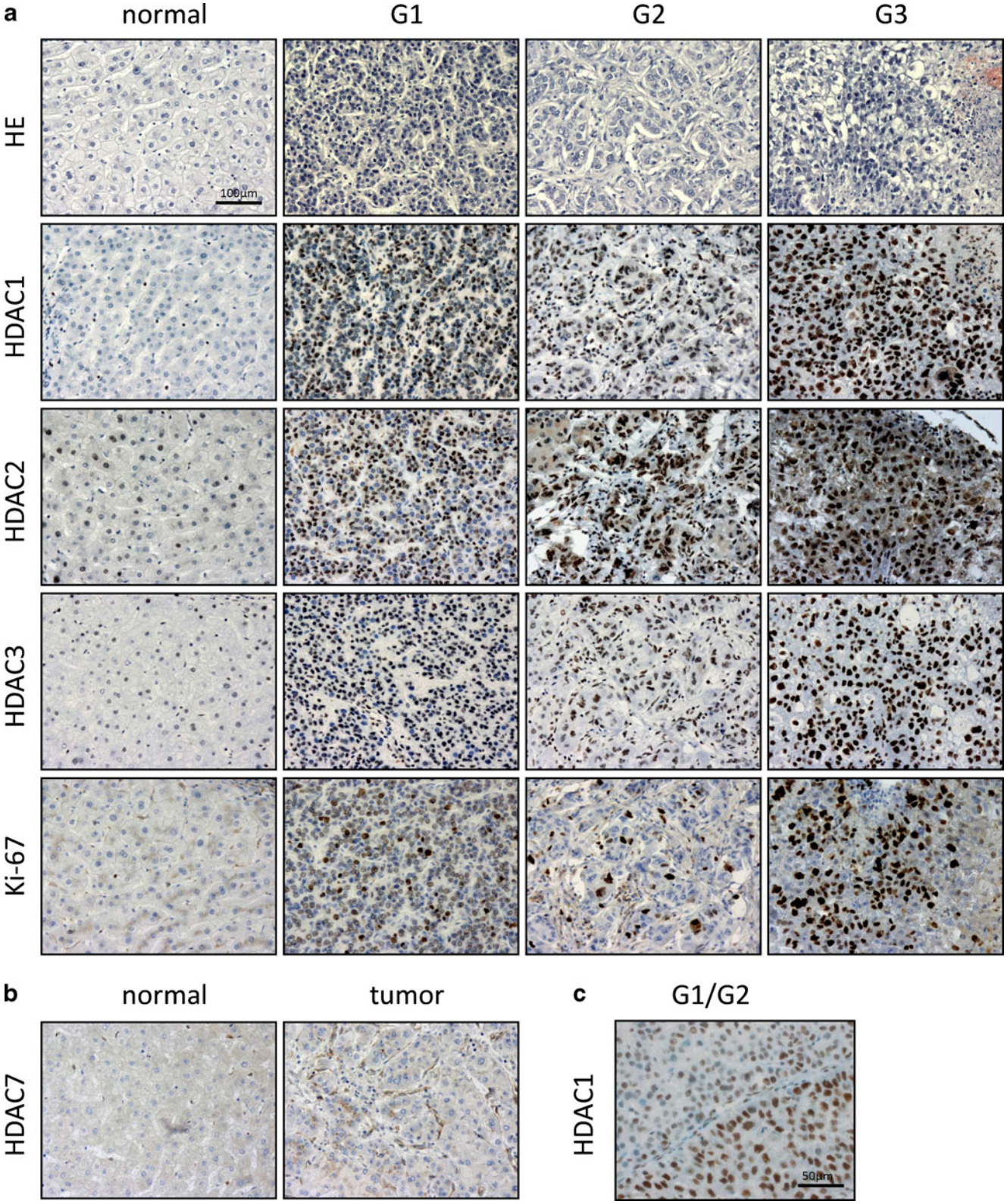


Fig. 1 Representative photomicrographs of HDAC isoenzymes and Ki-67 immunohistochemistry in uninvolved liver tissue and HCCs of different histological grades. **a** Expression of class I HDACs 1, 2, and 3 and of Ki-67. **b** Expression of HDAC7 in normal and HCC tissue. **c** Differential expression of HDAC1 within the same tumor showing higher expression in a G2 subclone than in juxtaposed G1 differentiated tumor areas. Magnification is ×200 for **a** and **b** and ×400 for **c**

As described previously in other solid tumors, expression levels of HDAC isoforms show concordance among themselves [12, 14, 24], with tumor proliferation and tumor dedifferentiation. Using Spearman's correlation coefficient, we compared tumor HDAC immunoreactivity scores between the isoenzymes, with Ki-67 and tumor grades (Table 4). Tumor IRS of HDACs 1–3 correlated significantly with each other and with Ki-67 expression ($p<0.001$). HDACs 1–3 expression in uninvolved and tumor tissue also correlated with each other, showing an overall lower correlation coefficient of maximal $r=0.332$. The expression of HDACs 1–3 and Ki-67 correlated significantly with tumor grade ($p<0.001$). HDAC7 expression did not correlate with any parameter.

Relationship between HDAC isoenzyme expression and clinicopathological factors

In order to assess the relationship between HDAC expression and other clinicopathological parameters, we divided the patients in two groups based on median tumoral HDAC expression of each isoenzyme independently (low vs. high expression, HDAC1 IRS≤4 vs. >4; HDACs 2 and 3 ≤5 vs. >5).

Figure 2 shows the relationship of HDAC expression with pathological parameters which are used to describe the aggressiveness and progression of the tumors and with the clinically prognostic Child-Pugh status. High HDAC expression was associated with tumor dedifferentiation for all three isoenzymes ($p<0.05$, χ^2 test), as indicated by a higher number of G3 and a lower number of G1 tumors in the high HDAC expression groups. High HDACs 1 and 2 isoenzyme expression were associated with pT status, indicated by an increased number of pT3 tumors in the high HDAC2 expression group (HDAC1 $p<0.05$, χ^2 test). Interestingly, we observed an inverse relationship between HDAC isoenzyme expression and Child-Pugh score: high HDAC2 expression was significantly associated with an increased number of child A cases (HDAC2 $p<0.05$, χ^2 test).

Table 4 Correlation analysis showing the Spearman's correlation coefficients (r) for immunoreactivity scores of HDAC isoenzyme and Ki-67 expression and tumor grades

	HDAC2	HDAC3	Ki-67	Grade
HDAC1	0.636	0.530	0.591	0.468
HDAC2		0.544	0.534	0.389
HDAC3			0.515	0.489
Ki-67				0.513

All values were significant at $p<0.001$. HDAC7 did not show a correlation with any other parameter

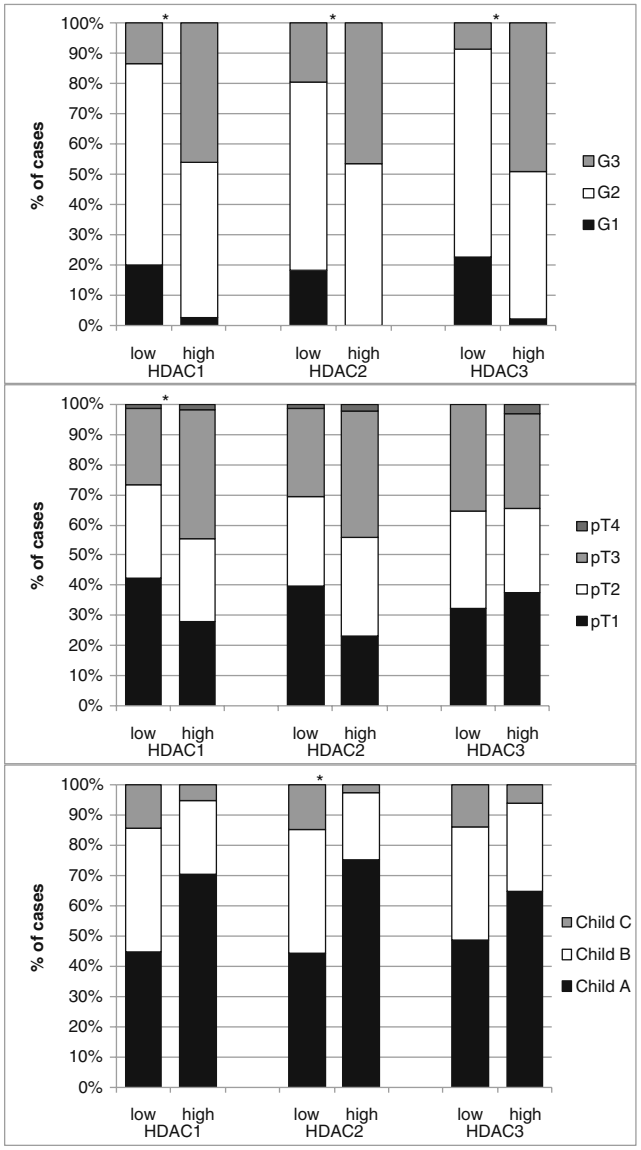


Fig. 2 Tumor differentiation, ungrouped pT, and Child-Pugh status in dependence on HDAC isoform expression levels. Grouped HDAC expression correlates positively with tumor dedifferentiation for all isoenzymes. Significant correlation is also observed for HDAC1 expression with pT status and for HDAC2 with the Child-Pugh classification. *Asterisks* indicate a significance level of $p<0.05$ (χ^2 test)

We further compared the low and high HDAC expression groups with various clinical and pathological data. As illustrated in Table 5, low and high HDAC1 expression groups differed significantly with respect to the volume/weight of the resected tissue, Ki-67 score, tumor grade, steatosis of the hepatocytes, distribution of the patients within the Child-Pugh subgroups, and intrahepatic blood vessel invasion. Low and high HDAC2 expression differed significantly in the AFP levels, volume of the resected tissue, Ki-67 score, tumor grade, and patient distribution in the Child-Pugh scoring. Low and high HDAC3 expression

Table 5 Analysis of continuous and grouped clinicopathological parameters in low and high HDAC expression groups

	Expression level	HDAC1			HDAC2			HDAC3		
		No.	Mean±SD	*p* value	No.	Mean±SD	*p* value	No.	Mean±SD	*p* value
AFP (ng/ml)	Low	51	12,181.55±82,692.61	0.216	68	9,413.23±71,712.22	**<0.001**	55	11,170.3±79,650.45	0.216[a]
	High	47	5,622.72±27,275.57		36	6,838.88±30,665.69		50	54,38.71±26,438.81	
Volume of resected tissue (3rd root of volume, mm)	Low	35	115.8±108.89	**0.002**	41	110.68±108.43	**0.035**	24	106.17±101.06	0.468[a]
	High	23	92.11±100.34		18	92.95±98.43		36	107.38±110.59	
Resection weight (g)	Low	26	700.38±459.74	**0.004**	32	552.81±421.81	0.051	19	536.95±416.73	0.397[a]
	High	18	276.72±311.85		13	311.69±321.84		27	495.93±479.55	
Ki-67 (score)	Low	75	1.13±1.09	**<0.001**	91	1.35±1.52	**<0.001**	74	1.08±0.96	**<0.001[b]**
	High	55	2.93±2.32		46	2.96±2.11		65	2.78±2.24	
Tumor grade (n in groups norm./G1/G2/G3)	Low	62	2/12/40/8	**0.001**	77	1/14/47/15	**0.008**	60	2/13/40/5	**<0.001[b]**
	High	40	1/1/20/18		31	1/0/16/14		50	1/1/24/24	
pT category (pT1/pT2/pT3/pT4)	Low	71	30/22/18/1	0.194	88	35/26/26/1	0.267	68	22/22/24/0	0.435[b]
	High	54	15/15/23/1		43	10/14/18/1		64	24/18/20/2	
Steatosis (none, slight, strong)	Low	25	19/0/6	**0.001**	28	17/2/9	0.222	15	9/0/6	0.219[b]
	High	16	4/5/7		14	5/3/6		28	14/5/9	
Child-Pugh classification (A, B/C)	Low	49	22/27	**0.028**	54	24/30	**0.005**	43	21/22	0.144[b]
	High	37	26/11		36	27/9		48	31/17	
Intrahepatic vessel invasion (no/yes)	Low	33	32/1	**0.048**	39	37/2	0.110	22	0/22	0.059[b]
	High	21	17/4		16	13/3		34	29/5	

The number of cases (*n*) with available retrospective data is given for each parameter. Statistically significant results are printed in *bold*. HDAC low and high groups were divided at the mean HDAC expression in the tumor tissue for each isoenzyme (HDAC1 low IRS≤4, high IRS>4; HDAC2 low IRS≤5, high IRS>5; HDAC3 low IRS≤5, high IRS>5)

[a] Statistical analysis used the Mann–Whitney *U* test

[b] Statistical analysis used the χ^2 -test

differed with respect to tumor grade and Ki-67 expression levels. No differences were found for the low and high HDAC7 groups.

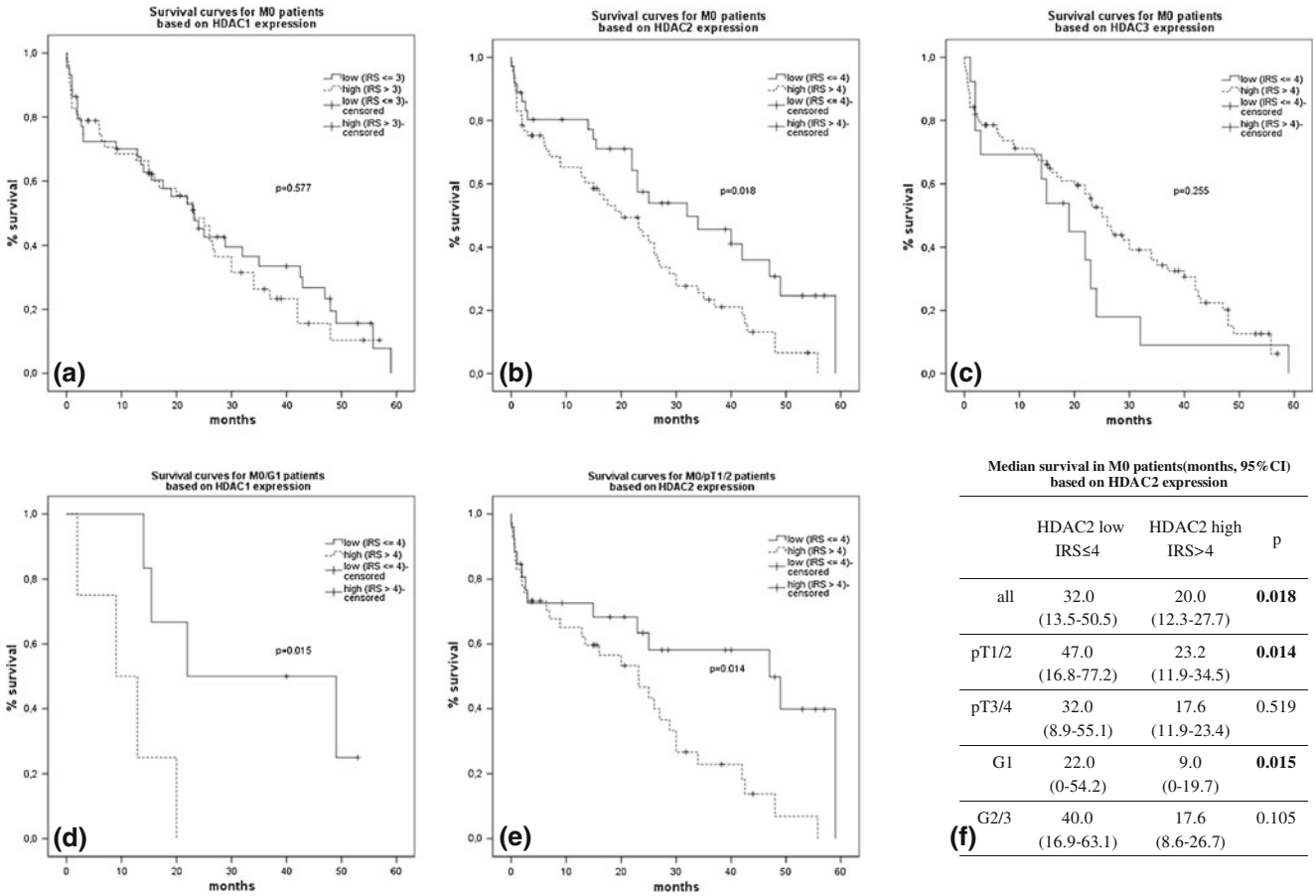
Survival analysis

Using the Kaplan–Meier survival method, we analyzed overall survival after curative surgery in dependence on the HDAC expression status of each of the enzymes and on Ki-67 expression status (Fig. 3). Therefore, the consecutive statistical analysis was performed only for the M0 patient group (91.4% of patients), and then stratified for the pT1/pT2 vs. pT3/pT4 and G1 vs. G2/G3 subgroups.

Patient survival in the whole M0 group was influenced by the HDAC2 expression status: Median survival in the low HDAC2 expression group (IRS≤4) was 32.0 months (standard error 9.4, 95% CI 13.5–50.5) vs. 20.0 months (3.9, 12.3–27.7) in the high HDAC2 expression group (IRS>4, p=0.018, Fig. 3b). HDACs 1 and 3 expression status did not influence survival (Fig. 3a and c) in the whole M0 group.

As expected, patients in the pT1/pT2 group survived longer compared to the pT3/pT4 patients (p=0.038, not shown). In the pT1/2 subgroup, median survival in the low HDAC2 expression group was 47.0 months (15.4, 16.8–77.2) vs. 23.2 months (5.8, 11.9–34.5) in the high HDAC2 expression group (p=0.014, Fig. 3e). In pT3/4 patients, median survival in the low HDAC2 expression group was 32.0 months (11.8, 8.9–55.1) vs. 17.6 months (2.9, 11.9–23.4) in the high HDAC2 expression group (p=0.519, Fig. 3f).

In the low-grade subgroup, median survival in the low HDAC2 expression group was 22.0 months (16.4, 0–54.2) vs. 9.0 months (5.4, 0–19.7) in the high HDAC2 expression group (p=0.015) (Fig. 3d). In the high-grade subgroup, median survival in the low HDAC2 expression group was 40.0 months (11.8, 16.9–63.1) vs. 17.6 months (4.6, 8.6–26.7) in the high HDAC2 expression group (p=0.105,



Median survival in M0 patients(months, 95% CI) based on HDAC2 expression

	HDAC2 low IRS≤4	HDAC2 high IRS>4	p
all	32.0 (13.5-50.5)	20.0 (12.3-27.7)	**0.018**
pT1/2	47.0 (16.8-77.2)	23.2 (11.9-34.5)	**0.014**
pT3/4	32.0 (8.9-55.1)	17.6 (11.9-23.4)	0.519
G1	22.0 (0-54.2)	9.0 (0-19.7)	**0.015**
G2/3	40.0 (16.9-63.1)	17.6 (8.6-26.7)	0.105

(f)

Fig. 3 Comparative overall survival in dependence on HDAC expression levels in tumor tissue. Shown are survival data for HDAC1 (**a**), HDAC2 (**b**), and HDAC3 (**c**) in unstratified patients, for HDAC2 stratified for low-tumor grade (G1, **d**) and early-tumor stage (pT1/pT2, **e**), and the survival times in months along with the 95% confidence intervals (**f**). Significance levels were computed using the log-rank test. The low and high HDAC expression groups were divided at IRS 3 for HDAC1 and IRS 4 for HDACs 2 and 3, which corresponds to the mean expression in uninvolved hepatic tissue

Fig. 3f). There was no significant difference in survival between the G1 vs. G2/G3 patients ($p=0.996$), most probably because of the low number of G1 patients compared to the G2/G3 patients (Table 1).

In multivariate analysis with the inclusion of age, tumor grade, pT category, Ki-67 expression, HDACs 1–3 expression as well as HDACs 1–3 expression groups (low, high), HDAC2 retained its independent statistical significance ($p=0.001$, HR 5.5, 95% CI 2.0–14.9)

Discussion

In this study, we show that class I HDACs, i.e., HDACs 1–3, are expressed significantly higher in HCC than in the normal liver and correlate with tumor dedifferentiation and proliferative activity of the tumor cells. In addition to an impact on clinicopathological factors such as resection weight, AFP levels and an association with the Child-Pugh score, we found that patient survival was negatively affected by high HDAC2 expression levels, especially in the pT1/pT2 subgroup of HCC.

Nuclear expression of class I HDAC isoenzymes is a common feature, shared by most carcinomas analyzed for this marker, including colon, gastric, renal prostate and pancreatic cancers, and endometrioid carcinoma [12, 14, 15, 24, 25]. In addition, there are other common factors shared by the HDAC expression studies performed so far: the first one is the positive correlation of HDAC expression with histological grade, which was reported for prostate [15], renal cell [12], colorectal [14], pancreatic [26] and gastric cancer [14], while this was not observed in some tumors like breast cancer [27]. Second, a correlation between HDAC expression with the respective pT categories was shown in renal cell [12], colorectal [14] and gastric [14], but not in prostate cancer [15]. The third common feature is the correlation of HDACs with one another, which was reported for ovarian and endometrial carcinomas [24], renal cell [12], and colorectal cancer [14], suggesting common regulatory mechanisms. In our study, we found a correlation with grading and with the proliferation marker Ki-67. There was also concordance among class I HDAC expression, but interestingly no correlation with pT categories. In the only study investigating HDAC expression in HCC, Rikimaru et al. reported a poorer differentiation which is associated with high HDAC1 expression [16]. Our results are in accordance with these data, and we additionally report a correlation of HDAC2 and HDAC3 (but not HDAC7) with tumor dedifferentiation in HCC. In the same study, a higher incidence of cancer cell invasion into the portal vein in the high HDAC1 expression group is reported. Concordantly, our data show a correlation between high HDAC1 expression and intrahepatic vessel invasion.

The observed HDAC7 expression pattern in our samples is in line with a study in pancreatic adenocarcinomas [13] showing a cytoplasmic and nuclear staining, which has been described for class II HDACs (HDACs 4, 5, 7, and 9), as these enzymes exhibit nucleocytoplasmic shuttling [6]. Although in this study HDAC7 was associated with pancreatic adenocarcinoma, we could not establish a similar role for HDAC7 in our samples: no statistically different expression between normal and tumor tissue as well as no correlation with either proliferation or any of the other three HDAC isoenzymes was found. This is a novel finding, as the expression of HDAC7 has so far not been investigated in HCC and here we show that it does not seem to play a role in this malignancy. A study investigating the effects of siRNA-mediated knockdown of various HDACs on Hela cancer cell proliferation showed no effect with regard to HDAC7, in contrast to HDACs 1 and 3, a finding which is in line with our data [28]. While most studies agree on the common factors of HDAC expression in tumors, there is diverse data regarding their association with clinicopathological and laboratory data. Our data show an increased percentage of cases with Child A cirrhosis in the high HDACs 1–3 expression groups, reaching significance for HDAC2. While a variety of speculations about this finding are possible, we mainly attribute it to the patient selection bias: Patients in our cohort were selected for curative surgery, most of them (48%) represent Child A cirrhosis and, along with this, a less advanced disease stage. On the other hand, the finding that in our patients, hepatic steatosis occurs more often in the high HDAC1 expression group, suggests a possible regulation of the individual isoenzymes under certain conditions, and possibly also changes in HDAC expression levels prior to tumor initiation.

In previous studies, HDAC expression showed varying correlations with overall survival and tumor stage [12, 14, 15, 27]. This indicates that while the expression of HDACs 1–3 correlates with cellular proliferation and histological grade in many investigated tumors, it does not necessarily translate into other prognostically relevant factors. However, in our patient group, the histopathological correlations with tumor grade and proliferation translate into clinical outcome with regard to HDAC2, for which high expression is associated with poor outcome, both in the general patient population, comparing all cases (HDAC low vs. high expression) as well as in low-grade (G1) and early-tumor stage (pT1/2) patients. This finding corroborates a previous result from prostate cancer patients, where HDAC2 was associated with PSA relapse-free survival [15]. In the first study of HDAC1 expression in HCC, it was shown by Rikimaru et al. that low HDAC1 expression is linked to better overall survival [16]. In comparison to our patient collective, those results were obtained in a smaller cohort of Asian HCC patients who develop HCC on the basis of

chronic viral hepatitis in the majority of cases, while in our cohort only 20% of the patients had a virus-related disease. These differences in etiology could also explain our findings that HDAC2, but not HDAC1 or HDAC3, were prognostic markers in our cases, in contrast to the results reported from Asia. Interestingly, patients with G2/3 tumors showed a better (although not significant) overall survival both in the low (p=0.819) and high (p=0.112) HDAC expression groups compared to G1 tumors. In our cohort, patients with G1 tumor grades are rare compared to patients with G2/G3 (Table 1), and this result might be explained by a disproportionately small G1 group. In contrast, the pT1/2 and pT3/4 groups are more balanced and the results are concordant with what was expected.

Although we show a direct influence of HDAC expression on survival, these findings have to be carefully interpreted, since our retrospective data acquisition did not appreciate the importance of second-line therapies, which themselves could crucially influence survival and thus demand further investigations to evaluate and to compare HDAC expression patterns in resected primary, resected recurrent, or differentially treated HCCs. However, in our cohort, this would lead to small-sized groups and possible selection bias, prompting us to omit such stratification, as done by Rikimaru et al. [16], since we wanted to investigate the expression pattern of HDACs isoenzymes in primary HCC as well as the impact on overall survival.

While most HDAC studies so far have focused on expression analyses in various tissues and tumor entities, very few studies have addressed the specific roles of the HDAC isoenzymes. In endometrial carcinoma, HDAC2, but not HDAC1, was found to be associated with increased proliferation and poor prognosis. HDAC inhibition decreased proliferation and increased apoptosis in vitro, most likely through HDAC2 interaction [29]. In oesophageal adenocarcinomas, HDAC2, but not HDAC1, was associated with tumor dedifferentiation [30]. The role of HDAC2 is further emphasized through a study in which specific knockdown of HDAC2 had the strongest impact on cell viability [14]. These and further studies in endometrial stromal sarcoma cells [31] and in colon and cervical cancers [32] indicate a specific involvement of the HDAC2 isoenzyme in the context of tumor differentiation, proliferation, and impact on survival. However, the exact mechanisms of the isoenzymes and their distinct functions remain elusive.

Considering the fact that several clinical trials are currently ongoing with different pharmacologic inhibitors of HDAC activity (e.g., NCT00823290 indexed at http://clinicaltrials.gov), studies are urgently warranted to provide novel biomarkers for monitoring treatment response and for sensible patient stratification [19]. Based on our data, the most important factor to analyze in subsequent studies is the susceptibility of HDAC expressing tumors to HDACi treatment and the benefit on survival in this patient group, a claim that is based on the fact that class I HDACs (HDACs 1–3) are highly expressed in HCCs, are associated with clinicopathological factors and correlate with patient survival.

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Conflict of interest The authors declare that they have no conflict of interest.

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