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Expression of the class 1 histone deacetylases HDAC8 and 3 are associated with improved survival of patients with metastatic melanoma

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Prior studies have shown that combinations of histone deacetylase (HDAC) and BRAF inhibitors (BRAFi) have synergistic effects on BRAFi-resistant melanoma through enhanced apoptosis and inhibition of the cAMP-dependent drug resistance pathway. However, little is known about the expression of various HDACs and their associations with *BRAF/NRAS* mutation status, clinicopathologic characteristics, and patient outcome. The present study extensively profiled HDAC class 1 and their targets/regulators utilizing immunohistochemistry in human melanoma samples from patients with stage IV melanoma, known BRAF/NRAS mutational status, and detailed clinicopatholgical data. HDAC8 was increased in BRAF-mutated melanoma ($P = 0.016$), however, no association between expression of other HDACs and NRAS/BRAF status was identified. There was also a correlation between HDAC1, HDAC8 expression, and phosphorylated NFκb p65 immunoreactivity ($P < 0.001$). Increased cytoplasmic HDAC8 immunoreactivity was independently associated with an improved survival from both diagnosis of primary melanoma and from first detection of stage IV disease to melanoma death on multivariate analysis (HR 0.992, 95% CI 0.987–0.996; $P < 0.001$ and HR 0.993, 95% CI 0.988–0.998; $P = 0.009$, respectively). These results suggest not only that HDAC8 may be a prognostic biomarker in melanoma, but also provide important data regarding the regulation of HDACs in melanoma and a rational basis for targeting them therapeutically.

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Histone deacetylases (HDACs) are enzymes that remove acetyl groups from lysine residues in the NH2-terminal tails of core histones, resulting in a more closed chromatin structure and repression of gene expression. Hypoacetylation has recently been identified as a common property of many cancers.[1]

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We have reviewed elsewhere evidence that certain oncogenes may mediate their effects by recruiting HDACs to silence important tumor suppressor mechanisms in cancer cells.[2] For example, in neuroblastoma HDAC2 was reported to be upregulated by N-Myc and to target the promoter region of CCNG2 (cyclin G2),[3,4] thus removing the inhibitory effects of cyclin G2 on cell division. In melanoma, several biological effects have been associated with HDACs such as targeting of HDAC1 by T-box 2 (Tbx2) to the promoter of CDKN1 (p21) which is responsible for inhibition of senescence, with inhibition of HDACs leading to the upregulation of p21 expression

resulting in cell cycle arrest or apoptosis.[5] MAGE-A proteins in melanoma were shown to target HDAC3 to p53 thereby inhibiting the latter's transactivating function.[6] In addition, HDACs can target non-histone proteins causing alterations in protein stability, nuclei transportation, protein–protein, and protein–DNA interactions.[7] For example, HDAC3 post-transcriptionally deacetylates NF-κB p65 at lysines 310, 314, and 315, leading to increased association of NF-κB p65 with the promoter region of IL-8 upregulating expression.[8] In addition, the list of non-histone protein targets of HDACs is constantly increasing and current targets include p53, STAT3, c-Myc, HIF-1α, Hsp-90, HMG, E2F, MyoD, Bcr–Abl, and many more.[9]

In addition to their role in inhibition of tumor suppressors, HDACs may also be recruited as mediators of resistance of melanoma to targeted therapies and immunotherapy. We reported previously that the HDAC inhibitor SBHA could reverse resistance of melanoma to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis[10] and that SAHA (vorinostat) could reverse resistance to selective BRAF inhibitors.[2,11] Studies by others have also shown that a resistance pathway against BRAFi driven by G protein-coupled receptors could be reversed with the addition of a HDAC inhibitor.[12] However, little is known about the regulation of HDACs, which oncogenes determine their expression, and in particular in melanoma whether their expression may be related to activating mutations in BRAF or NRAS (which are oncogenic drivers of ~65–70% of all cutaneous melanomas[13–15]). In addition, HDAC regulates the transcription of NF-κB, with increased HDAC-1, -2, and -3 associating with high levels of activated NF-kB and a poor prognosis in patients with pancreatic carcinoma.[16,17] It is unknown whether a similar relationship exists in melanoma.

In the present study, we utilized immunohistochemistry (IHC) to examine the significance of class 1 HDACs and NF-kB protein expression in a cohort of patients who developed stage IV metastatic melanoma and whether their expression was related to mutations of BRAF or NRAS. We found that although the expression of the majority of HDACs was not correlated with BRAF/NRAS mutation status, cytoplasmic HDAC8 and nuclei HDAC3 over expression was associated with improved survival of patients with stage IV metastatic melanoma.

Materials and methods

Patient Selection and Data Collection

A total of 175 stage IV melanoma patients were included in the study as previously described,[14] and consisted of consecutive patients presenting to the Melanoma Institute Australia (MIA) with newly diagnosed metastatic melanoma (stage IV) between 2002 and 2006. All patients included in this cohort were treatment naive to systemic drug therapy with a proven overall survival (OS) benefit, ie, IL-2, ipilimumab, class 1 BRAF inhibitors, or MEK inhibitors. The BRAF/NRAS genotype data were determined in those with available tissue (OncoCarta Panel v1.0)[14] and the most recent archival paraffin-embedded melanoma tissue suitable for tissue microarray construction was utilized. In total, 200 patients were identified utilizing the above criteria, of which 175 had available tissue for further analysis. This study was undertaken at the MIA and Royal Prince Alfred Hospital, Sydney, Australia with human ethics committee approval (X11-0289, HREC/11/RPAH/444).

Patient demographics, primary tumor characteristics (date of primary diagnosis, Breslow thickness, ulceration, mitotic rate, ulceration, and N stage), and clinical details at the time of diagnosis of stage IV melanoma (M site of organ involvement and serum lactate dehydrogenase (LDH)) were determined from review of clinical records and the MIA Melanoma Research database (MRD). For patients with more than one primary melanoma, the primary lesion that led to disease dissemination was designated using a previously described algorithm.[18,19]

Tissue Microarray Construction

Reference sections of the donor tissue block were cut, H&E stains were performed and the slides were marked with a 1-mm circle to identify areas of tumor. Tissue cores 1 mm in diameter were taken from the donor paraffin block using the marked section as a reference and then arranged in a blank paraffin block by a MTA-1 manual tissue arrayer (Beecher Instruments, Sun Prairie, WI, USA). Once constructed, the microarrays were baked for 30 min at 37 °C on a glass slide to even out the surface and fuse the paraffin in the cores and donor block.

Immunohistochemistry

All IHC stainings were performed on a Dako Autostainer Plus (Dako, Glostrup, Denmark) using the EnVision FLEX, high pH visualization kit (K8010, Dako, Glostrup, Denmark) according to the manufacturers' protocols; 4 μm sections of the TMAs were cut and left to dry overnight at room temperature, and then baked at 58 °C for 60 min in a dehydration oven the same day as the IHC was performed. HIER antigen retrieval was performed using Dako target retrieval high pH solution in a Decloaking chamber (Biocare Medical, California, USA) at 125 °C for 30 s for all antibodies other than phosphorylated-NF$\kappa\beta$-p65, which was HIER treated in a Dako PT link (Dako, Glostrup, Denmark) for 20 min at 100 °C. The sections were then washed in TBST and loaded onto the Autostainer. Sections were incubated for 30 min at room temperature with the respective primary antibodies at the following

Table 1 Clinicopathologic patient characteristics and HDAC and NFκB expression

Factor	Value	Total N	Immunoreactivity scores (mean (range))					
			HDAC1 Nuc	*HDAC2 Nuc*	*HDAC3 Nuc*	*HDAC8 Cyto*	*NFκb p65 Cyto*	*p-NFκb p65 Nuc*
Patients	Total	175	18 (0–30)	9 (0–30)	16 (0–30)	16 (0–30)	18 (0–30)	18 (0–30)
Sex	Female	56 (32%)	16 (0–30)	8 (0–30)	15 (0–30)	16 (0–30)	18 (0–30)	17 (0–30)
	Male	119 (68%)	19 (0–30)	9 (0–30)	16 (0–30)	15 (0–30)	18 (0–30)	19 (0–30)
Tissues tested	Local recurrence	9 (5%)	19 (6–30)	6 (1–18)	19 (0–27)	15 (0–20)	20 (0–30)	20 (3–30)
	In-transit metastasis	51 (29%)	**21 (0–30)**	10 (0–30)	17 (0–30)	15 (0–30)	19 (0–30)	20 (0–30)
	Regional lymph node metastasis	66 (38%)	**15 (0–30)**	9 (0–30)	14 (0–30)	15 (0–30)	17 (0–30)	17 (0–30)
	Distant metastasis	49 (28%)	18 (0–30)	9 (0–30)	15 (0–30)	17 (0–30)	17 (0–30)	17 (4–30)
Mutation status	*NRAS* mutant	33 (19%)	17 (0–30)	7 (0–30)	17 (0–30)	*13 (0–30)*	16 (0–30)	16 (0–30)
	BRAF mutant	84 (48%)	19 (0–30)	9 (0–30)	15 (0–30)	*18 (0–30)*	19 (0–30)	19 (0–30)
	NRAS and *BRAF* wild type	58 (33%)	19 (0–30)	10 (0–30)	16 (0–30)	*14 (0–30)*	18 (0–30)	19 (0–30)

Tukey's HSD, $P = 0.04$ (values shown in bold).
$BRAF^{mut}$–$NRAS^{mut}$, $P = 0.017$; $BRAF^{mut}$–$NRAS/BRAF^{wt}$, $P = 0.016$, Tukey's HSD (values shown in italics).

dilutions HDAC1 = 1:200 (SC-7872, Santa Cruz Biotechnology), HDAC2 = 1:50 (SC-9959, Santa Cruz Biotechnology), HDAC3 = 1:150 (SC-11417, Santa Cruz Biotechnology), HDAC8 = 1:50 (NBP1-61892, Novus Biologicals), NF$\kappa\beta$ p65 = 1:800 (CS#8242, Cell Signaling Technology), and phospho-NF$\kappa\beta$-p65 = 1:200 (ab86299, Abcam). HDAC2-treated slides were incubated in the secondary EnVision FLEX+ Mouse (LINKER) antibody for 20 min. Antibody detection was performed using the EnVision FLEX HRP for 30 min and visualized via incubation in 3,3′-diaminobenzidine (DAB) for 5 min. Slides were then counterstained with hematoxylin and mounted.

The slides were examined by a pathologist (AC) who was blind to the clinicopathological data. The percentage of immunoreactive cells was estimated from 0 to 100%. Intensity of staining was judged on a semiquantitative scale of 0 to 3+: no staining (0), weakly positive staining (1+), moderately positive staining (2+), and strongly positive staining (3+). An immunoreactive score (IRS) was derived by multiplying the percentage of positive cells with the staining intensity divided by 10 (IRS range = 0–30). The nucleus and cytoplasm of the tumor cells were separately scored for immunoreactivity using the above parameters.

Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics 21. Correlations of HDAC expression with clinical features were performed using Spearman's rho test. Differences in protein marker immunoreactivity levels between $NRAS^{mut}$, $BRAF^{mut}$, and $NRAS^{wt}/BRAF^{wt}$ patient samples were assessed using a one-way between-subjects ANOVA with a *post hoc* Tukey's HSD test. The ANOVA and *post hoc* Tukey's HSD test was used to test for differences in protein expression between 'local recurrences' (metastases within 5 cm of the primary tumor site), in-transit metastases, regional lymph node metastases, and distant organ or lymph node metastases. Univariate survival analysis was performed using the Kaplan–Meier method together with the log-rank (Mantel–Cox) test to calculate statistical significance. Cox regression analysis was used to determine the factors predictive for patient response and outcome. The distant disease-free interval was measured from the date of culprit primary melanoma diagnosis to the first diagnosis of distant metastatic disease. OS was calculated from the date of diagnosis of primary melanoma and separately calculated from the diagnosis of stage IV melanoma to last follow-up (censored) or death from melanoma (event). Univariate hazard ratios (HRs), 95% confidence intervals (95% CIs), and corresponding *P*-values were obtained using Cox regression. Protein markers found to be significantly associated with clinical outcomes in univariate analysis were then entered into multivariate analysis along with known prognostic features at primary (T stage or Breslow thickness and lymph node status) and at stage IV disease (M-site and serum LHD levels). Statistical significance was defined as a probability level of $P < 0.05$.

Results

Patients and Melanoma Samples

The 175 human melanoma samples comprised of 12 melanoma 'local recurrences' (metastases within 5 cm of the primary tumor site), 51 in-transit metastases, 66 regional lymph node metastases, and 49 distant organ or lymph node metastases (Table 1). Of the 175 melanoma samples that comprise the tissue

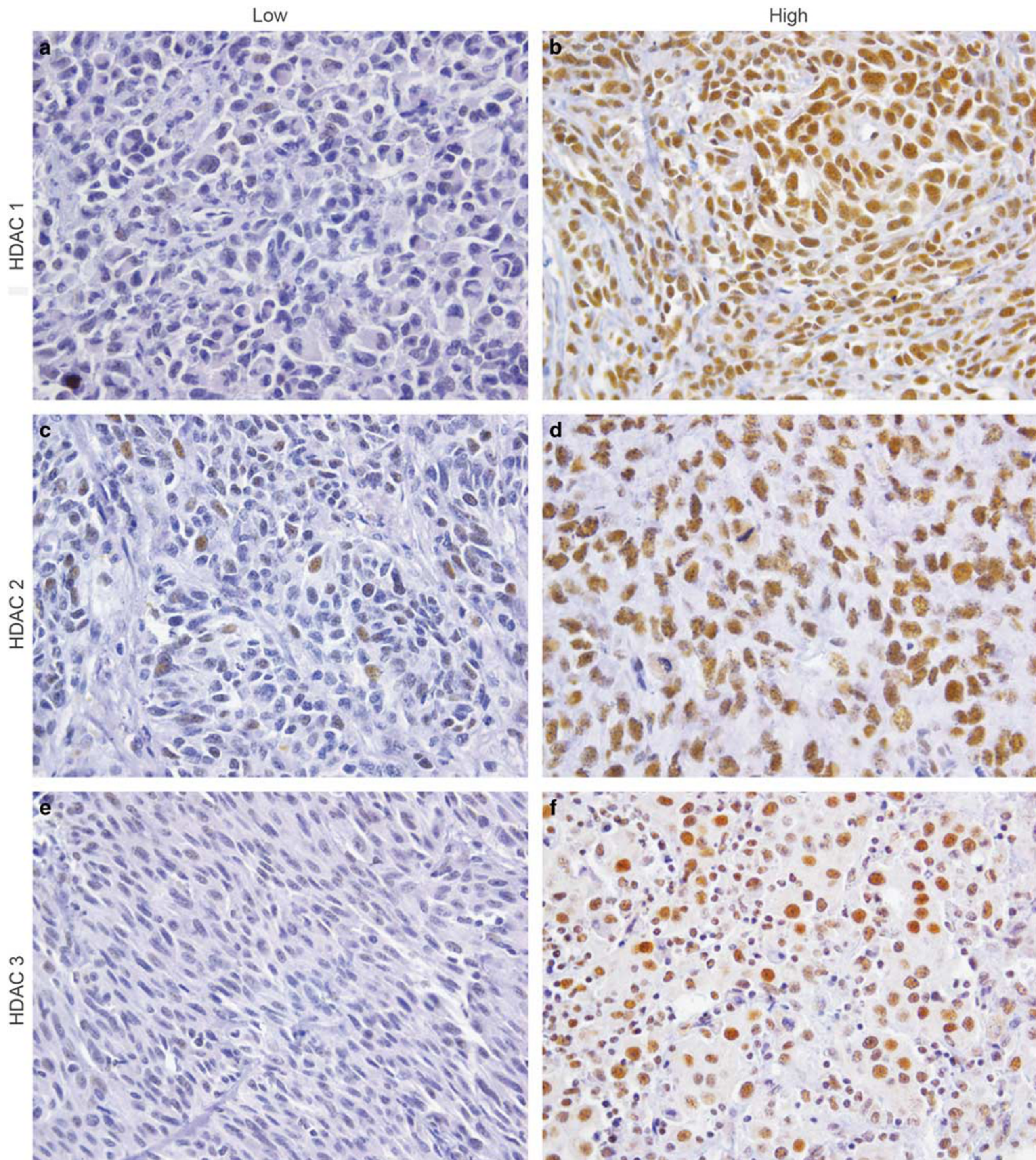

Figure 1 Representative immunohistochemical staining for HDAC and NFκB-p65 in human melanomas. (**a** and **b**) Low and high nuclei immunoreactivity for HDAC1, respectively. (**c** and **d**) Low and high nuclei immunoreactivity for HDAC2, respectively. (**e** and **f**) Low and high nuclei immunoreactivity for HDAC3, respectively. (**g** and **h**) Low and high nuclei and cytoplasmic immunoreactivity for HDAC8, respectively. (**i** and **j**) Low and high nuclei and cytoplasmic immunoreactivity for total NFκB-p65, respectively. (**k** and **l**) Low and high nuclei cytoplasmic immunoreactivity for phospho-NFκB-p65, respectively. All microphotographs were taken with a × 63 objective, Leica DM2000 microscope equipped with a Leica DFC495 digital color camera, and the software Leica Application Suite (Leica, Germany).

microarray, 84 (48%) were *BRAF* mutant, 33 (19%) *NRAS* mutant, and 58 (33%) were both *BRAF* and *NRAS* wild type. The median age at diagnosis of stage IV disease was 58 years with a median OS time from diagnosis of stage IV disease of 11 months.

Cellular Distribution and Expression Levels of Histone Modifiers in Human Melanoma Samples

HDAC-1, -2, and -3 expression was primarily located in the nucleus of tumor cells (Figures 1a–f,

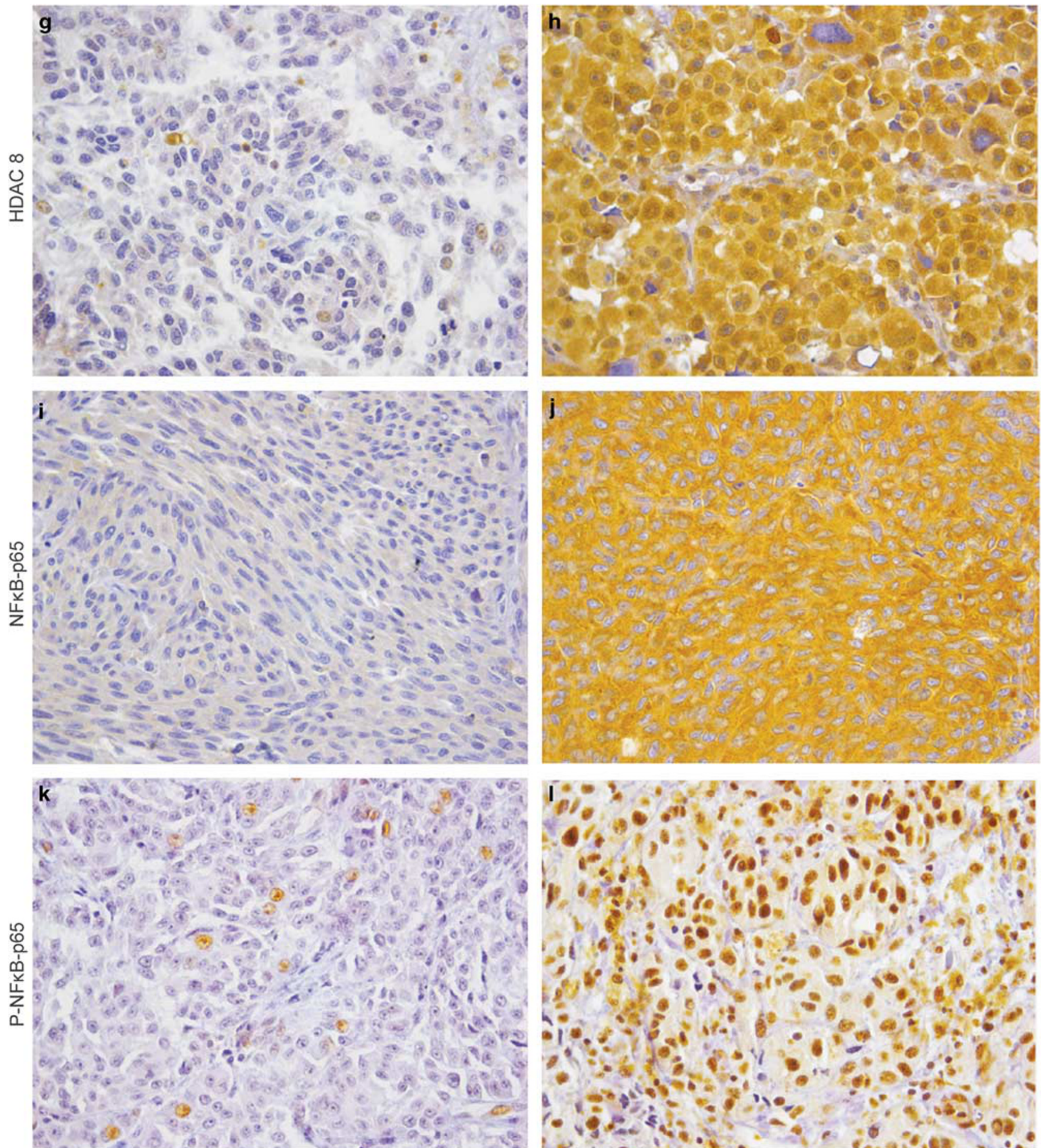

Figure 1 (Continued)

respectively). Conversely, HDAC8 was mainly localized to the cytoplasm of tumor cells, with some moderate to strong nuclei immunoreactivity (Figures 1g and h). The total p65 subunit of NF-kB was expressed primarily in the cytoplasm, with rare cases demonstrating a low degree of nuclei staining (Figures 1i and j). In contrast, expression of the phosphorylated isoform was restricted to the nuclei of tumor cells (Figures 1k and l). The majority of immunoreactivity scores of the above markers did not significantly vary between local recurrence, in transit, regional lymph node, and distant metastases (Figure 2). Although a significant variation was detected between HDAC1 nuclei expression in the four groups ($F(3, 162) = 2.7$; $P = 0.049$), *post hoc* comparisons showed HDAC1 nuclei expression

was higher in in-transit metastases (M = 21, s.d. 10.4) than in regional lymph node metastases (M = 15, s.d. 10.5; $P = 0.04$; Table 1 and Figure 2).

Correlations within Chromatin Modifier Proteins and with NFκB Pathway Activity

Correlations between the various chromatin-modifying proteins and NFκB expression are shown in Table 2. There was a significant and strong positive correlation between nuclei immunoreactivity for HDAC1 and HDAC3 ($r = 0.55$, $P < 0.001$). Similarly, nuclei HDAC3 and cytoplasmic HDAC8 were each significantly correlated with total cytoplasmic NF-kB p65 immunoreactivity ($r = 0.50$, $P < 0.001$ and $r = 0.59$, $P < 0.001$, respectively; Table 2). Likewise, phospho-NF-kB p65 nuclei immunoreactivity was positively correlated with HDAC1, HDAC8, and as expected total NF-kB p65 ($r = 0.31$, $P < 0.001$; $r = 0.36$, $P < 0.001$; and $r = 0.35$, $P < 0.001$, respectively, Table 2).

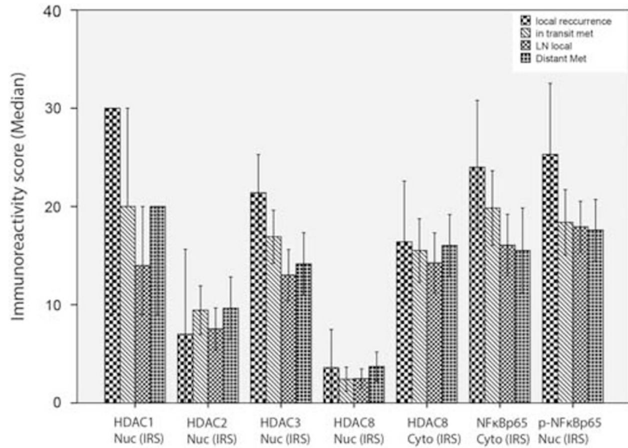

Figure 2 Histograms of HDAC and NFκB-p65 expression during melanoma progression.

Correlations of Chromatin Modifying Proteins with BRAF and NRAS Mutation Status and Clinical Parameters

We analyzed the expression of the above protein markers for associations with *BRAF* and *NRAS* mutation status (Table 1). The expression of cytoplasmic HDAC8 was significantly different between the three groups ($F(2, 156) = 5.742$, $P = 0.004$). *Post hoc* comparisons indicated that the mean IRS of cytoplasmic HDAC8 was higher in *BRAF*-mutant patients (M = 18, s.d. 8.9) compared to both *NRAS* mutant (M = 13, s.d. 9.7; $P = 0.017$) and $NRAS^{wt}$/$BRAF^{wt}$ patients (M = 14, s.d. 9.8; $P = 0.016$). The remaining proteins assessed in the current study were unaffected by *NRAF* and *BRAF* mutation status.

Table 2 Correlations within chromatin modifier proteins and with NFκB pathway activity

	HDAC1 Nuc	*HDAC2 Nuc*	*HDAC3 Nuc*	*HDAC8 Nuc*	*HDAC8 Cyto*	*p65 Cyto*	*p-p65 Nuc*
HDAC1 Nuc							
Rho		*0.39*	**0.55**	0.29	0.24	*0.38*	*0.31*
P		0.00	0.00	0.00	0.00	0.00	0.00
HDAC2 Nuc							
Rho	*0.39*		*0.45*	0.13	0.26	0.25	0.10
P	0.00		0.00	0.12	0.00	0.00	0.25
HDAC3 Nuc							
Rho	**0.55**	*0.45*		*0.31*	*0.34*	**0.50**	0.29
P	0.00	0.00		0.00	0.00	0.00	0.00
HDAC8 Nuc							
Rho	0.29	0.13	*0.31*		*0.45*	*0.43*	0.29
P	0.00	0.12	0.00		0.00	0.00	0.00
HDAC8 Cyto							
Rho	0.24	0.26	*0.34*	*0.45*		**0.59**	*0.36*
P	0.00	0.00	0.00	0.00		0.00	0.00
NFkb-p65 Cyto							
Rho	*0.38*	0.25	0.50	*0.43*	**0.59**		*0.35*
P	0.00	0.00	0.00	0.00	0.00		0.00
p-NFkb-p65 Nuc							
Rho	*0.31*	0.10	0.29	0.29	*0.36*	*0.35*	
P	0.00	0.25	0.00	0.00	0.00	0.00	

Abbreviations: Cyto, cytoplasmic staining; Nuc, nuclear staining; p65, NFκ-p65; p-p65, phosphorylated NFκ-p65; rho, Spearman's coefficient correlation.
Significant correlations ($P < 0.05$) are indicated by underlined numbers.
Spearman's coefficient correlation between 0.5 and 1 are shown in bold.
Spearman's coefficient correlation between 0.3 and 0.49 are shown in italics.

Table 3 Cox regression univariate analysis of HDAC and NFκB expression with survival

Factor	Value	*Disease-free survival from diagnosis of primary melanoma to diagnosis of stage IV disease*				*Overall survival from diagnosis of primary melanoma*				*Overall survival from diagnosis with stage IV metastatic melanoma*			
		HR	*Sig.*	*95% CI*		*HR*	*Sig.*	*95% CI*		*HR*	*Sig.*	*95% CI*	
				Lower	*Upper*			*Lower*	*Upper*			*Lower*	*Upper*
HDAC1 Nuclear	Immunoreactive score	0.992	0.322	0.976	1.008	0.994	0.444	0.977	1.010	0.998	0.790	0.983	1.013
HDAC1 Nuclear	Percentage positive	0.997	0.241	0.993	1.002	0.997	0.274	0.993	1.002	1.000	0.836	0.995	1.004
HDAC2 Nuclear	Immunoreactive score	1.004	0.710	0.982	1.027	1.006	0.628	0.983	1.029	1.005	0.684	0.983	1.027
HDAC2 Nuclear	Percentage positive	1.000	0.997	0.995	1.005	0.999	0.804	0.994	1.004	0.999	0.587	0.994	1.003
HDAC3 Nuclear	Immunoreactive score	0.994	0.596	0.974	1.015	0.993	0.537	0.972	1.015	0.993	0.462	0.975	1.012
HDAC3 Nuclear	**Percentage positive**	0.995	0.091	0.990	1.001	**0.994**	**0.033**	**0.989**	**1.000**	0.996	0.156	0.992	1.001
HDAC8 Cytoplasmic	Immunoreactive score	0.988	0.218	0.969	1.007	0.988	0.237	0.968	1.008	0.995	0.573	0.976	1.013
HDAC8 Cytoplasmic	**Percentage positive**	**0.995**	**0.036**	**0.991**	**1.000**	**0.993**	**0.004**	**0.989**	**0.998**	**0.995**	**0.015**	**0.991**	**0.999**
HDAC8 Nuclear	Immunoreactive score	0.995	0.843	0.950	1.043	0.990	0.699	0.944	1.040	0.983	0.437	0.943	1.026
HDAC8 Nuclear	Percentage positive	0.999	0.640	0.993	1.004	0.998	0.560	0.992	1.004	0.998	0.415	0.993	1.003
NFκB p65 Nuclear	**Immunoreactive score**	**1.301**	**0.039**	**1.013**	**1.671**	**1.369**	**0.017**	**1.058**	**1.771**	1.107	0.296	0.915	1.339
NFκB p65 Nuclear	**Percentage positive**	**1.026**	**0.047**	**1.000**	**1.053**	**1.031**	**0.021**	**1.005**	**1.059**	1.010	0.322	0.990	1.030
Phosho-NFκB p65 Nuclear	Immunoreactive score	0.995	0.647	0.976	1.016	1.00	0.904	0.98	1.02	1.004	0.688	0.985	1.023
Phosho-NFκB p65 Nuclear	Percentage positive	0.999	0.761	0.991	1.006	1.00	0.602	0.99	1.01	1.000	0.919	0.993	1.007

Bold values signify $P < 0.05$.

Distant Disease-Free Interval and OS Analysis

To evaluate the association between the expression of the protein markers and melanoma progression from diagnosis of primary melanoma to diagnosis of stage IV disease and OS times, we used Cox regression univariate analysis. We tested the IRS score and the percentage of immunoreactive tumor cells for each individual marker as a continuous variable for associations with time from the diagnosis of primary melanoma to the diagnosis of stage IV disease, OS from the diagnosis of primary melanoma and separately OS from the diagnosis of stage IV disease to the time of death. The results of these analyses are shown in Table 3 and Figure 3. Finding that increased percentage of cytoplasmic HDAC8 immunoreactive tumor cells correlated with an increased time from the diagnosis of primary melanoma to progression to stage IV disease (HR 0.995, 95% CI 0.991–1.000; $P = 0.0036$) and increased melanoma-specific survival times from diagnosis of both primary and stage IV disease (HR 0.993, 95% CI 0.989–0.998; $P = 0.004$ and HR 0.995, 95% CI 0.991–0.999; $P = 0.015$ respectively; Table 3; Figures 3b and d). Similarly, increased percentage of HDAC3 immunoreactivity nuclei associated with an increased survival time from the diagnosis of primary melanoma to melanoma death (HR 0.994, 95% CI 0.989–1.000; $P = 0.033$; Table 3 and Figure 3a). In contrast increase in expression of NFκb p65 in the nucleus was associated with a reduced time from the diagnosis of primary melanoma to detection of stage IV disease and melanoma death (HR 1.301, 95% CI 0.1.013–1.671; $P = 0.039$ and HR 1.369, 95% CI 1.058–1.771; $P = 0.017$, respectively; Table 3 and Figure 3c). However, this result was dependent upon the few cases ($n = 15$) that displayed nuclear total NFκb p65 immunoreactivity and this result was not reflected in the phosphorylated NFκb p65 isoform of the protein.

In multivariate analysis, initially the known prognostic variables identified by the AJCC staging system were evaluated. The variables included in Cox regression analysis from the diagnosis of primary melanoma to melanoma death included Breslow thickness, ulceration, and N-stage. Finding, as expected, that increased breslow thickness and nodal involvement predicted poor prognosis. However, ulceration was not a significant prognostic factor in this cohort of patients. Mutation status was not entered into the analyses as studies on the identical patient cohort have shown that the *BRAF* and *NRAS* mutation status did not influence survival in this patient cohort.[14] Thereafter, cytoplasmic HDAC8 and nuclear HDAC3 were entered separately into a backward stepwise Cox regression along with the above significant AJCC variables. The percentage of immunoreactive HDAC3 nuclei remained significant in the final model, with increased expression associating with better prognosis at the time of primary diagnosis, along with poor prognostic predictors, Breslow thickness and N-stage (Table 4). Likewise, increased percentage of cytoplasmic HDAC8 immunoreactivity associated with better prognosis from the time of diagnosis of primary melanoma (Table 4).

Similar multivariate analysis was conducted from the time of diagnosis of stage IV disease to melanoma-specific death. M-site and serum LHD were included into the analysis, with increasing M-site and elevated LDH corresponding to poorer survival as expected (Table 4). The percentage of cytoplasmic HDAC8 immunoreative tumor cells was

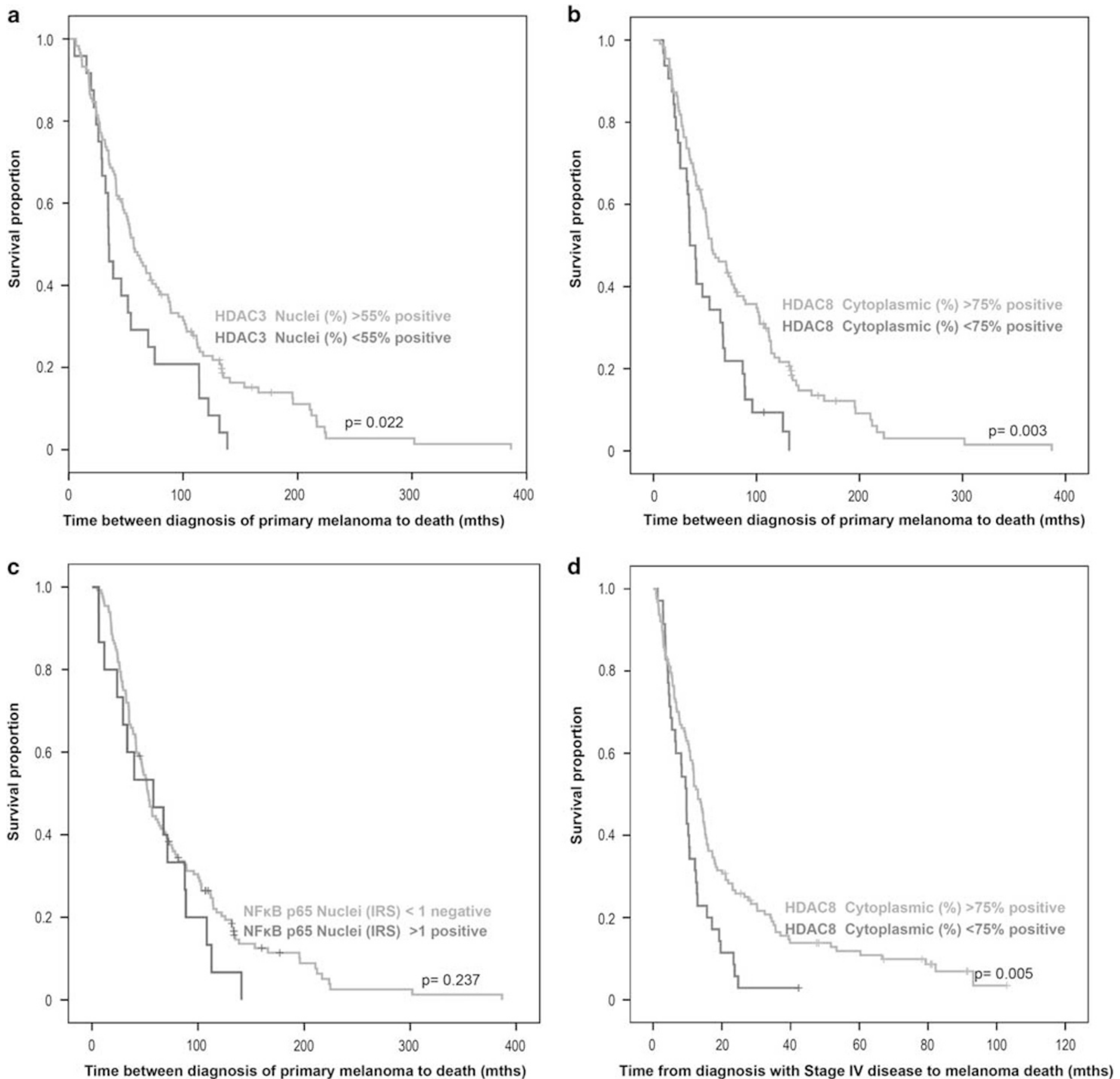

Figure 3 Kaplan–Meier analysis of the protein markers found to correlate with the interval to disease progression and OS in univariant Cox regression analysis. (**a**) Survival from diagnosis of primary melanoma dependent on nuclear HDAC3 immunoreactivity. (**b**) Survival from diagnosis of primary melanoma dependent on cytoplasmic HDAC8 immunoreactivity. (**c**) Survival from diagnosis of primary melanoma dependent on nuclear NFκB-p65 immunoreactivity. (**d**) Survival from diagnosis of stage IV disease dependent on cytoplasmic HDAC8 immunoreactivity. Optimal cut-off thresholds were determined using ROC analysis and are displayed on each figure. Log rank *P*-values are shown with $P < 0.05$ considered significant.

entered into the above model, finding that increased cytoplasmic HDAC8 immunoreactivity significantly associated with improved OS from the diagnosis of stage IV disease (Table 4).

Discussion

Studies over the past few years have shown that class 1 HDACs are usually part of multiprotein complexes that are located at particular sites in the genome that are involved in regulation or suppression of gene expression. The function of such complexes may be subject to modulation by intra- and extracellular signals including those mediated by oncogenes that result in increased growth and survival of cancer cells. The present study examined whether IHC screening studies might identify particular HDACs that were involved in the oncogenic process in melanoma and provide a basis for subsequent analysis of the complexes and oncogenes involved. To our knowledge, this is the first study to relate the

Table 4 Cox regression multivariate analysis of HDAC expression with prognosis

Factor	Value	Hazard ratio	P-value	95% CI for Exp(B) Lower	Upper
Multivariate analysis of overall survival interval from primary melanoma to death according to nuclear HDAC8 immunoreactivity (n = 130)					
Breslow thickness groups	T1		Reference		
	T2	1.335	0.321	0.754	2.364
	T3	1.455	0.144	0.880	2.407
	T4	3.071	< 0.001	1.765	5.343
N Stage	N0		Reference		
	N1	2.473	0.002	1.403	4.358
	N2	1.899	0.057	0.981	3.676
	N3	3.899	0.002	1.631	9.324
HDAC8 (cytoplasmic)	Percentage positive	0.992	< 0.001	0.987	0.996
Multivariate analysis of overall survival interval from diagnosis of stage IV disease to death according to nuclear HDAC8 immunoreactivity (n = 130)					
M-stage site	Subcutaneous or nodal metastasis		Reference		
	Lung	1.366	0.416	0.644	2.895
	All other visceral metastases	1.891	0.072	0.944	3.789
LDH	Normal		Reference		
	Elevated	1.506	0.072	0.964	2.353
HDAC8 (cytoplasmic)	Percentage positive	0.993	0.009	0.988	0.998

expression of HDACs identified in this way with survival of patients with melanoma and their mutational status.

The MAP kinase pathway is an important source of oncogenic signals in melanoma cells due to activating mutations in *BRAF* or *NRAS*. It was therefore of interest that *BRAF* mutations were associated with increased levels of HDAC8 but not with increased levels of the other class 1 HDACs in this study. Although it is well established that HDAC8 is associated with malignant forms of neuroblastoma[20] it has not previously been identified to be of prognostic significance in melanoma. It was shown to have both nuclear and cytoplasmic localization in normal tissues[21] and to be negatively regulated by cyclic AMP-dependent protein kinase A, which phosphorylates the N-terminal end of the protein.[22] This pathway is of particular interest in melanoma as it was implicated in resistance against BRAF inhibitors that was reversible by HDAC inhibitors.[12] Cyclic AMP signaling confers drug resistance to MAPKi via the upregulation of the transcription factors, such as, MITF. However, the HDACi were found to suppress MITF transcription and reverse cAMP-mediated resistance to MAPKi.[12] In addition, other studies have shown that activation of HDAC8 resulted in hyperacetylation of histones 3 and 4 and raised the possibility that it was involved in overall regulation of acetyl levels of histones.

Our study found an association between increased cytoplasmic HDAC8 immunoreactivity and a prolonged survival from the diagnosis of stage IV melanoma that appeared independent of other known prognostic variables in multivariate Cox regression analysis, including M-site and serum LHD levels. However, it must be noted that the HRs indicate a small alteration to risk and the biopsies all come from patients who developed stage IV disease, thus may not reflex that of earlier stages of disease. In addition, the cohort selection based on available archival tissue may bias the cohort to patients who had surgery for stage III or IV disease. Interestingly, the cytoplasmic localization of HDAC8 in the current study contrasts previous reports of subcellular localization to the nucleus of cancer cells.[23] However, cytoplasmic HDAC8 expression has been confirmed via subcellular fractionation experiments,[24] with a recent study suggesting that class I HDACs can bind to cytoplasmic proteins that play a regulatory role in the unfolded protein response.[25] The improved prognosis associated with cytoplasmic HDAC8 expression may reflect as an yet undiscovered cytoplasmic function for the protein or it may result from quarantining of the protein from nuclei and shift the histone acetylation to a more acetylated state.[26]

We examined the potential role of the transcription factor NF-kB in regulation of HDAC expression as previous studies in pancreatic cancer have shown a positive association of HDAC-1, -2, and -3 expression with high levels of activated NF-kB and a poor prognosis.[16] Previously, we showed that activation of NF-kB was associated with resistance of melanoma to the BRAF inhibitor vemurafenib.[27] Our studies support a possible role for regulation by NF-kB, in that HDAC-1 and -8 were correlated with expression of NF-kB. These results appeared clinically significant in that nuclear NF-kB was associated with a shorter duration from the development of primary melanoma to the development of stage IV

metastases and to death. However, NF-kB levels were unrelated to *BRAF* and *NRAS* mutation status. This is contrary to previous studies that show increased NFkb pathway activity associates with mutations to the *BRAF*,[28] *NRAS*[29] in cell lines, and *PI3K* genes in a small cohort of human melanoma tissue samples.[30] The lack of correlation between *BRAF*/*NRAS* mutation status with NFκb expression in the current study may reflect the fact that both *NRAS* and *BRAF* mutations result in MAPK activation, thereby having a similar signaling outcome and that multiple non-MAPK signaling pathways may also induce HDAC and NFκb activation.[30,31]

In conclusion, these IHC studies found that HDAC expression is elevated in metastatic melanoma but only HDAC8 associated with *BRAF* or *NRAS* mutation status. Interestingly, the levels of cytoplasmic HDAC8 are associated with increased survival from onset of stage IV metastatic melanoma. These findings suggest that HDAC8 may not function as a classical class 1 HDAC and that its cytoplasmic functions may play an important role in melanoma biology. Further studies will seek to determine the possible cytoplasmic functions of HDAC8 and whether inhibition of HDAC8 is a possible therapeutic target.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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