

Prognostic Impact of *PHIP* Copy Number in Melanoma: Linkage to Ulceration

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Ulceration is an important prognostic factor in melanoma whose biologic basis is poorly understood. Here we assessed the prognostic impact of pleckstrin homology domain-interacting protein (*PHIP*) copy number and its relationship to ulceration. *PHIP* copy number was determined using fluorescence *in situ* hybridization (FISH) in a tissue microarray cohort of 238 melanomas. Elevated *PHIP* copy number was associated with significantly reduced distant metastasis-free survival (DMFS; $P=0.01$) and disease-specific survival (DSS; $P=0.009$) by Kaplan–Meier analyses. *PHIP* FISH scores were independently predictive of DMFS ($P=0.03$) and DSS ($P=0.03$). Increased *PHIP* copy number was an independent predictor of ulceration status ($P=0.04$). The combined impact of increased *PHIP* copy number and tumor vascularity on ulceration status was highly significant ($P<0.0001$). Stable suppression of *PHIP* in human melanoma cells resulted in significantly reduced glycolytic activity *in vitro*, with lower expression of lactate dehydrogenase 5, hypoxia-inducible factor 1 alpha subunit, and vascular endothelial growth factor, and was accompanied by reduced microvessel density *in vivo*. These results provide further support for *PHIP* as a molecular prognostic marker of melanoma, and reveal a significant linkage between *PHIP* levels and ulceration. Moreover, they suggest that ulceration may be driven by increased glycolysis and angiogenesis.

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INTRODUCTION

Melanoma is an important clinical problem, with an estimated 76,250 new cases and 9,180 deaths in 2012 (Siegel *et al.*, 2012). The clinical behavior of melanoma can be unpredictable, making prognostic predictions difficult in individual patients. Although tumor thickness consistently emerges as the single most significant prognostic factor determining survival, and while ulceration increases the risk of death within a given thickness range, additional factors are required to refine the prognostic assessment of melanoma patients. Beyond histological factors that are reliably associated with decreased

survival, molecular markers represent the next frontier in the evaluation of melanoma outcome. Despite the identification of numerous putative molecular prognostic factors, no prognostic biomarkers are routinely used in the evaluation of melanoma patients (Gogas *et al.*, 2009). In addition, few biomarkers have been shown to retain prognostic impact, when measured on more than one platform (i.e., measurements at the DNA vs. RNA vs. protein level).

Recent studies performed by our group identified a previously unknown role for the pleckstrin homology domain-interacting protein (*PHIP*) in melanoma progression (De Semir *et al.*, 2012). *PHIP* was identified as the top gene overexpressed in metastatic versus primary melanomas by gene expression profiling analysis (Haqq *et al.*, 2005). Short hairpin RNA (shRNA)-mediated suppression of *PHIP* resulted in significant suppression of melanoma cell invasion and metastatic potential in both murine and melanoma cell lines. High levels of *PHIP* expression were associated with significantly reduced survival in both mouse models and human tissues. Specifically, high *PHIP* expression (as determined by immunohistochemical analysis) was independently predictive of distant metastasis-free survival (DMFS) and disease-specific survival (DSS). In this study, we analyze the role of *PHIP* copy number for its prognostic significance in primary cutaneous melanoma and explore the linkage between *PHIP* levels and ulceration development in melanoma.

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Abbreviations: CD31, cluster of differentiation 31; DMFS, distant metastasis-free survival; DSS, disease-specific survival; FISH, fluorescence *in situ* hybridization; HIF1A, hypoxia-inducible factor 1 alpha subunit; LDH5, lactate dehydrogenase 5; *PHIP*, pleckstrin homology domain-interacting protein; shRNA, short hairpin RNA; TMA, tissue microarray; VEGF, vascular endothelial growth factor

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RESULTS

Given the recent demonstration of a significant prognostic role for PHIP protein expression and the presence of elevated *PHIP* copy number in melanoma (De Semir et al., 2012), we aimed to determine the prognostic impact of *PHIP* copy number in primary cutaneous melanoma. *PHIP* copy number was analyzed using fluorescence *in situ* hybridization (FISH) on a tissue microarray (TMA) cohort of 238 specimens, with known immunohistochemical analysis of PHIP protein expression, by an observer blinded to patient outcomes. Overall, 22% of the cohort possessed a mean copy number of 3 or greater. See Figure 1a for representative examples of low versus high *PHIP* copy number. As the ratio of *PHIP* and probes representing the centromere of chromosome 6 in almost all of the cases was close to 1.0 (Supplementary Table S1 online), the primary attribute assessed was percentage of cells expressing three or more copies of the *PHIP* gene, in which there was a range from 0 to 87%, with a mean of 33.4%. There was a significant correlation between *PHIP* copy number and level of expression of the PHIP protein ($P < 0.0001$, Supplementary Figure S1 online).

Initially, we analyzed the relationship between *PHIP* copy number and melanoma outcome using univariate analysis. High *PHIP* copy number was associated with decreased melanoma survival, when DMFS ($P = 0.01$ by log-rank test) and DSS ($P = 0.009$ by log-rank test) were assessed using Kaplan–Meier analysis (Figure 1b and c). In addition, there was a significantly increased risk of distant metastasis or death due to melanoma with the presence of high *PHIP* FISH scores.

In all, 45.4% of patients with high copy number had distant metastasis, when compared with 25.5% with low copy number (Fisher’s exact test, $P = 0.015$). In total, 42.2% of patients with high copy number died of metastatic melanoma, when compared with 17.7% of patients with low copy number (Fisher’s exact test, $P < 0.002$).

Next, we examined the relationship between *PHIP* copy number and survival using multivariate Cox regression analysis. High *PHIP* FISH scores were significantly predictive of reduced DMFS ($P = 0.03$, Table 1) and DSS ($P = 0.027$, Table 2) in multivariate models that included tumor thickness, ulceration, age, gender, and tumor site. Thus, *PHIP* copy number was independently predictive of survival associated with melanoma, similar to the results obtained for PHIP protein expression (De Semir et al., 2012).

Recently, high PHIP immunohistochemical scores were shown to correlate with ulceration status (De Semir et al., 2012). We aimed to confirm this relationship at the DNA level. High *PHIP* copy number was significantly associated with ulceration status when analyzed by univariate logistic regression analysis ($P = 0.004$). In addition, high *PHIP* copy number was associated with a significantly increased risk of developing ulceration. Thus, ulceration was present in 45.5% of cases with high *PHIP* copy number, when compared with 28.8% of cases with low *PHIP* copy number ($P = 0.013$, Fisher’s exact test).

Previously, we reported that ulceration was associated with increased tumor vascularity in the primary tumor, when assessed morphologically (Kashani-Sabet et al., 2002).

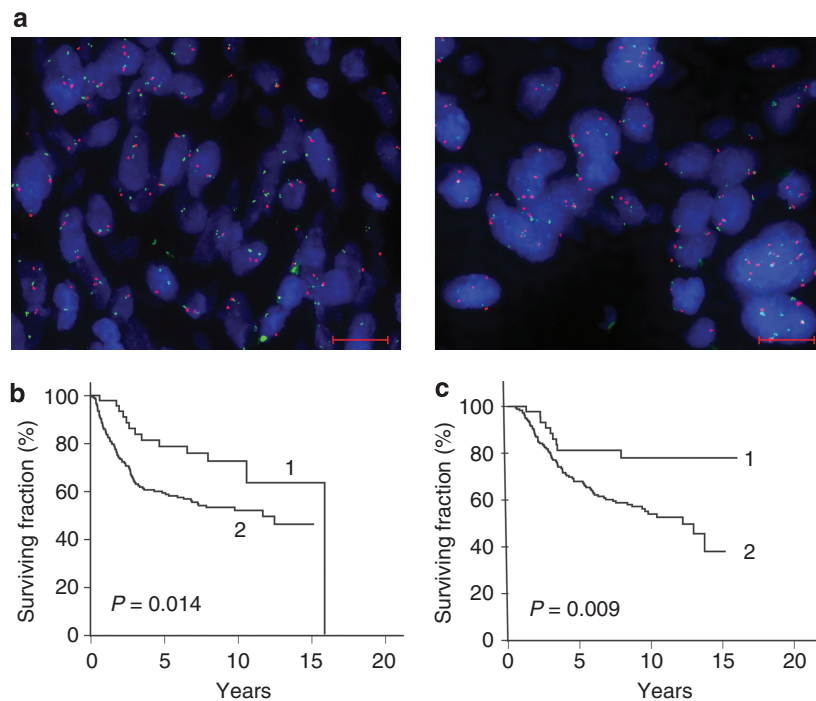


Figure 1. Pleckstrin homology domain-interacting protein (*PHIP*) copy number and its correlation with distant metastasis-free survival (DMFS) and disease-specific survival (DSS) in melanoma patients. (a) Dual color fluorescence *in situ* hybridization (FISH) for *PHIP* locus (red) and centromere of chromosome 6 (green) representative of low *PHIP* copy number (left panel) and high copy number (right panel). Scale bar = 20 μm . (b) Kaplan–Meier analysis of DMFS in patients with low *PHIP* copy number (curve 1) versus patients with high *PHIP* copy number (curve 2). (c) Kaplan–Meier analysis of DSS in patients with low *PHIP* copy number (curve 1) versus patients with high *PHIP* copy number (curve 2).

Table 1. Cox regression analysis of impact of various prognostic factors on DMFS of melanoma cohort (N= 231)

Prognostic factor	χ^2	Risk ratio	P-value
Tumor thickness	6.96	1.38	0.008
High PHIP copy number	4.73	1.99	0.03
Ulceration	3.31	1.51	0.07
Sex	1.41	1.34	0.24
Site	0.92	1.24	0.34
Age	0.61	0.95	0.44

Abbreviations: DMFS, distant metastasis-free survival; PHIP, pleckstrin homology domain-interacting protein.

Table 2. Cox regression analysis of impact of various prognostic factors on DSS of melanoma cohort (N= 231)

Prognostic factor	χ^2	Risk ratio	P-value
Tumor thickness	6.13	1.38	0.013
High PHIP copy number	4.87	2.21	0.027
Ulceration	3.63	1.58	0.057
Sex	1.00	1.30	0.32
Site	0.81	1.24	0.37
Age	0.01	0.99	0.91

Abbreviations: DSS, disease-specific survival; PHIP, pleckstrin homology domain-interacting protein.

Elevated tumor vascularity (as determined by melanomas with prominent vs. all lower degrees of vascularity) was significantly associated with ulceration status. Ulceration was present in 60.3% of the cases with prominent tumor vascularity, when compared with 22.2% of cases with lower degrees of tumor vascularity ($P < 0.0001$, Fisher's exact test). This served to confirm the previously reported results in this TMA cohort. We then explored the combined impact of high PHIP copy number and elevated tumor vascularity on the development of ulceration. Ulceration was present in 20.2% of melanomas with both low PHIP copy number and low tumor vascularity. This increased to 38.8% of cases ulcerated with either prognostic factor elevated, and to 72.2% of melanomas ulcerated, when both factors were elevated ($P < 0.0001$, χ^2 test of percentage differences).

We then assessed the relationship between PHIP copy number and ulceration status, using multivariate logistic regression analysis. Presence of ulceration in melanoma is also known to increase both with increasing tumor thickness and with increasing mitotic rate (Balch *et al.*, 2001b; Thompson *et al.*, 2011). A multivariate logistic regression analysis was performed, including low versus high PHIP copy number, low versus high tumor vascularity, tumor thickness, and mitotic rate. All four factors were significantly predictive of the incidence of ulceration (Table 3). Thus, PHIP FISH scores were independently predictive of

Table 3. Logistic regression analysis of impact of various prognostic factors on incidence of ulceration in melanoma cohort (N= 228)

Prognostic factor	χ^2	Odds ratio	P-value
Mitotic rate	15.24	1.23	0.0001
Tumor vascularity	10.26	3.05	0.001
Tumor thickness	5.99	1.63	0.01
High PHIP copy number	4.10	1.96	0.04

Abbreviation: PHIP, pleckstrin homology domain-interacting protein.

ulceration status, even with the inclusion of these other factors ($P = 0.04$).

Next, we explored cellular pathways regulated by PHIP expression potentially relevant to the development of ulceration. Targeted suppression of PHIP (using an anti-PHIP shRNA) in C8161.9 human melanoma cells (with elevated PHIP copy number) was previously shown to significantly suppress melanoma cell invasiveness and metastatic potential (De Semir *et al.*, 2012). Given that PHIP functions in the IGF1 receptor pathway that is important in regulating glucose metabolism, we assessed whether suppression of PHIP activity resulted in altered glycolytic activity of melanoma cells. C8161.9 cells expressing the anti-PHIP shRNA produced significantly lower amounts of lactate than control cells expressing a shRNA targeting luciferase (anti-luciferase shRNA; Figure 2a). This was associated with decreased expression of lactate dehydrogenase 5 (LDH5), the most efficient isoenzyme of lactate dehydrogenase, which catalyzes the conversion of pyruvate to lactate in the final step of glycolysis (Figure 2b). In addition, culturing anti-PHIP shRNA-expressing cells in the presence of methylpyruvate, which bypasses glycolysis and directly enters the Krebs cycle, resulted in significantly increased invasion (Figure 2c). Thus, the pro-invasive role of PHIP in melanoma is mediated, at least in part, by activating the glycolytic pathway. Moreover, C8161.9 melanoma cells with reduced PHIP expression produced lower levels of vascular endothelial growth factor (VEGF), as determined by an ELISA assay of human VEGF secretion (Figure 2d). As VEGF expression is controlled by hypoxia-inducible factor 1 alpha subunit (HIF1A; Forsythe *et al.*, 1996), melanoma cells with suppressed PHIP expression were also shown to express lower levels of HIF1A by western analysis (Figure 2b).

Finally, we assessed the effects of suppression of PHIP on the angiogenic potential of human melanoma cells *in vivo*. C8161.9 melanoma cells were inoculated subcutaneously into nude mice, and the resultant tumors examined for microvessel density using immunofluorescence of CD31 positivity. Although there was no significant difference in the tumor volume between the two groups (data not shown), tumors in the anti-PHIP shRNA-expressing group (Figure 3a, right panel) exhibited large areas of necrosis when compared with the control group (Figure 3a, left panel). Analysis of CD31 expression revealed that the control tumors were characterized by large, abnormal blood vessels, and

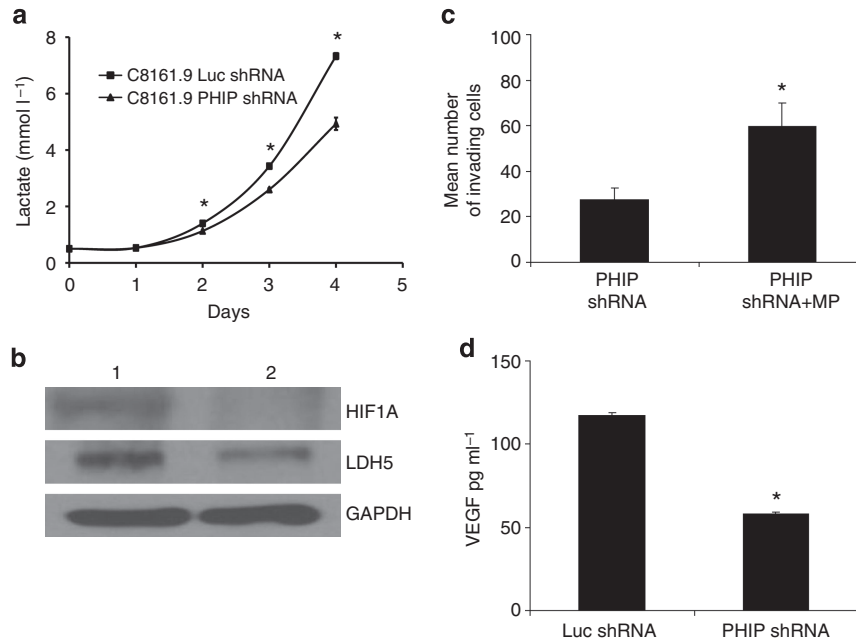


Figure 2. Effects of short hairpin RNA (shRNA)-mediated suppression of pleckstrin homology domain-interacting protein (PHIP) in human melanoma cells. (a) Lactate production in C8161.9 cells expressing anti-*PHIP* shRNA versus control cells expressing anti-*luciferase* (anti-*luc*) shRNA. Mean \pm SE; $N=3$. *Denotes $P<0.003$. (b) Western analysis of lactate dehydrogenase 5 (LDH5) and hypoxia-inducible factor 1 alpha subunit (HIF1A) in C8161.9 cells expressing anti-*luc* shRNA (control cells, lane 1) or anti-*PHIP* shRNA (lane 2). (c) Invasive capacity of C8161.9 stable transformants expressing anti-*PHIP* shRNA with or without the addition of methylpyruvate (MP). Mean \pm SE; $N=3$. *Denotes $P<0.05$. (d) ELISA assay of human vascular endothelial growth factor (VEGF) levels produced by C8161.9 cells expressing anti-*PHIP* shRNA versus control cells expressing anti-*luc* shRNA. Mean \pm SE; $N=3$. *Denotes $P<0.0001$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

hemorrhage, apparent from autofluorescence of erythrocytes and lipofuscin (Baschong *et al.*, 2001; Figure 3b and c, left panels). By contrast, the vessels in the anti-*PHIP* shRNA-expressing tumors were smaller, with little or no hemorrhage evident (Figure 3b and c, right panels). Finally, the anti-*PHIP* shRNA-expressing tumors had significantly reduced microvessel density when compared with the control tumors ($P<0.02$ as assessed both by a *T*-test and by a two-sample randomization test, Figure 3d). Thus, increasing PHIP expression in human melanoma cells promotes angiogenic potential.

DISCUSSION

In this article, we describe a previously unreported role for *PHIP* copy number in the prognosis associated with melanoma. *PHIP* FISH scores were predictive of a significantly increased risk of both distant metastasis and death due to melanoma, and were independently predictive of DMFS and DSS. In addition, elevated *PHIP* FISH scores were associated with a higher incidence of ulceration and were independently predictive of ulceration status. The combined impact of *PHIP* copy number and tumor vascularity was additive, as cases with high levels of both factors had a significantly higher incidence of ulceration than cases with low levels of either or both factors. Finally, C8161.9 human melanoma cells with suppressed PHIP expression exhibited reduced glycolytic activity and angiogenic potential.

These results provide further support for the role of PHIP as a molecular prognostic marker for melanoma, as initially

described using immunohistochemical analysis of PHIP expression. High PHIP protein expression was associated with a significantly increased risk of distant metastasis and death due to melanoma, and was independently predictive of DMFS and DSS (De Semir *et al.*, 2012). Our analysis of *PHIP* copy number parallels the results obtained in human and murine melanoma cell lines, in which suppressed PHIP expression resulted in significantly reduced metastatic potential and increased survival in xenograft models.

To date, few markers have been shown to provide significant prognostic impact in melanoma when assessed on more than one platform. For example, *SPP1* expression has been shown to have independent prognostic significance when assessed at the RNA and protein levels (Rangel *et al.*, 2008; Conway *et al.*, 2009). Our studies have demonstrated a significant prognostic role for PHIP at both the DNA and protein levels. Although the ratio of *PHIP* to chromosome 6 centromeric probe was close to 1.0 in the great majority of cases examined, changes in *PHIP* copy number, ranging from euploidy to three or greater copies, were highly correlated with increased PHIP expression. Additional studies of PHIP levels will be required in distinct cohorts in order to further validate its prognostic role.

Beyond an independent impact on survival, these studies confirmed a significant and independent relationship between PHIP levels and ulceration status. In addition, *PHIP* copy number combined markedly with tumor vascularity to increase the incidence of ulceration in primary melanoma specimens. Ulceration is an important

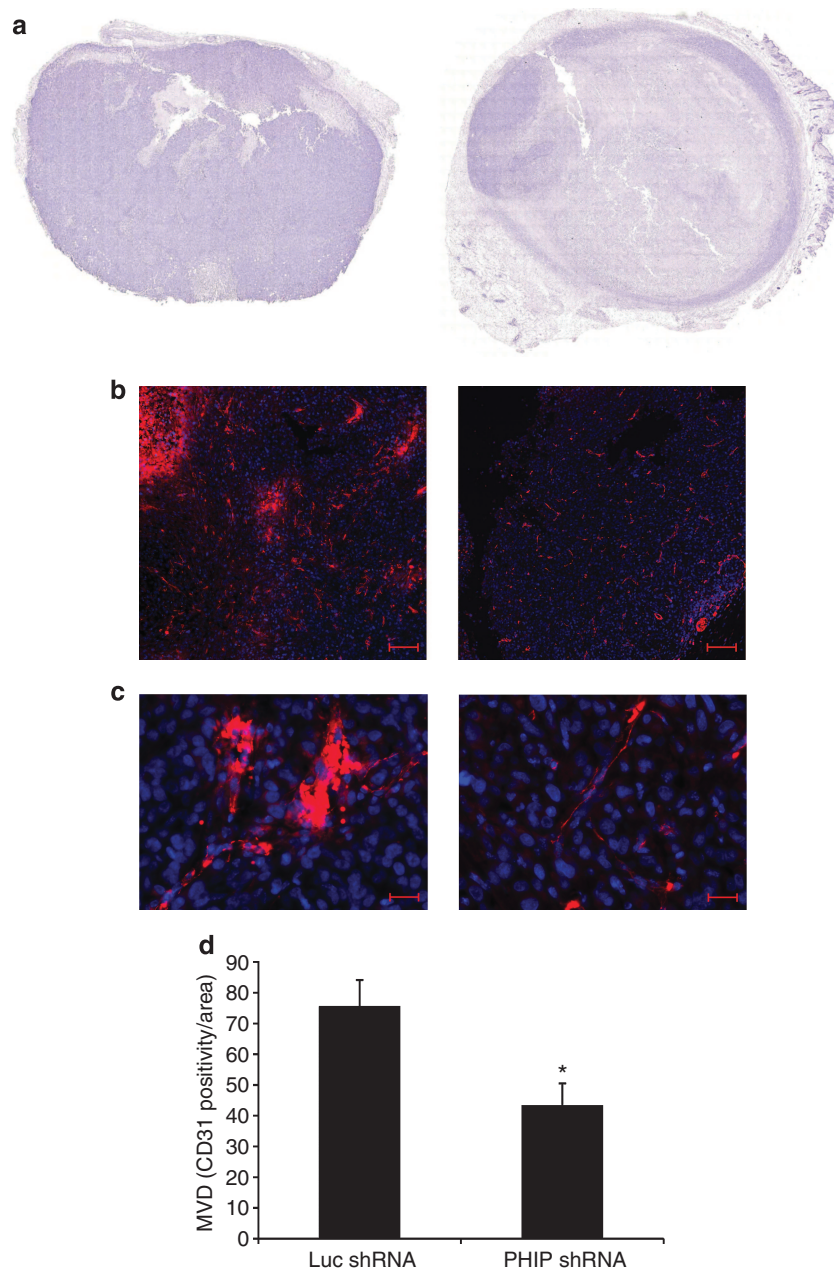


Figure 3. Effects of pleckstrin homology domain-interacting protein (PHIP) expression on the angiogenic potential of C8161.9 human melanoma cells *in vivo*. (a) Representative images of hematoxylin and eosin (H&E) staining of subcutaneous tumors in nude mice. The left panel shows the growth pattern of C8161.9 cells expressing anti-*luciferase* (anti-*luc*) short hairpin RNA (shRNA), compared with C8161.9 cells expressing anti-*PHIP* shRNA (right panel). (b) Composite images of immunofluorescence detection of CD31 (red) and 4,6-diamidino-2-phenylindole (DAPI; blue) as counterstain. The left panel shows the staining pattern of CD31 in subcutaneous tumor with C8161.9 cells expressing anti-*luc* shRNA compared with C8161.9 cells expressing anti-*PHIP* shRNA (right panel). Scale bar = 100 μ m. (c) Composite images of CD31 staining pattern with higher magnification for tumors with C8161.9 cells expressing anti-*luc* shRNA (left panel), or anti-*PHIP* shRNA (right panel). Scale bar = 20 μ m. (d) Quantification of microvessel density (MVD) based on CD31 immunofluorescence positivity in tumors (six per group) with C8161.9 cells expressing either anti-*luc* shRNA or anti-*PHIP* shRNA. Mean \pm SD. *Denotes $P < 0.02$.

prognostic factor that has been incorporated into the American Joint Committee on Cancer staging classification for melanoma for over a decade (Balch *et al.*, 2001a, 2009). More recently, ulceration has been suggested as a potential predictive marker of benefit to adjuvant IFN alpha therapy (McMasters *et al.*, 2010; Eggermont *et al.*, 2012). However,

the biologic basis for the development of ulceration is poorly understood.

Increasing incidence of ulceration in melanoma is associated with increasing tumor thickness, mitotic rate, and tumor vascularity (Balch *et al.*, 2001b; Kashani-Sabet *et al.*, 2002; Thompson *et al.*, 2011). In addition, gene expression

profiling studies have identified a gene signature that correlates with ulceration (Winnepenninckx *et al.*, 2006). However, the specific cellular pathways and mechanisms by which ulceration develops are unclear. The linkage between PHIP levels and ulceration implies a role for the IGF1 receptor pathway in ulceration, given PHIP's involvement in this signal transduction pathway. In addition, our studies in human melanoma cells demonstrating that downregulation of PHIP expression results in significantly reduced glycolytic activity, VEGF secretion, and microvessel density provide mechanistic insights, identifying glucose metabolism and tumor angiogenesis as pathways by which ulceration may develop in melanoma.

Tumor cell growth is promoted by increased glucose consumption, a phenomenon known as the Warburg effect (Warburg 1956). The importance of glycolysis to melanoma progression has been amply reported in melanoma cell lines (Halaban *et al.*, 1997, 2002; Bagheri *et al.*, 2006) and is demonstrated clinically by the utility of positron emission tomography scanning in the radiologic assessment of melanoma both during initial staging (Reinhardt *et al.*, 2006; Falk *et al.*, 2007) and in response to targeted therapy (McArthur *et al.*, 2012). Glycolysis in tumor cells is driven by AKT (Elstrom *et al.*, 2004), and PHIP is a potent activator of AKT in melanoma (De Semir *et al.*, 2012). Moreover, increased glucose metabolism can itself drive tumor angiogenesis, invasion, and metastasis (Lay *et al.*, 2000; Tsutsumi *et al.*, 2004). Thus, our studies on PHIP in melanoma describe a previously unreported link between PHIP, the Warburg effect, tumor angiogenesis, and ulceration development. As PHIP is activated through increased copy number in a subset of melanomas, additional markers of ulceration will need to be identified in melanomas without PHIP activation. However, the mechanistic analyses provided here should facilitate the identification of these additional markers in distinct molecular subsets of melanoma patients.

Finally, our investigation of PHIP in melanoma tissues and cell lines suggests an intriguing link between ulceration and LDH levels. These results imply that a common tumor-promoting pathway (i.e., IGF1 receptor), resulting in increased glycolysis, may underlie the biologic basis of two prominent prognostic markers incorporated into the American Joint Committee on Cancer staging classification, one that refines the prognosis of localized melanoma, the other the prognosis of disseminated melanoma. Elevated LDH5 expression in primary melanoma has been previously shown to be associated with reduced survival on univariate analysis, but was not independently predictive of melanoma outcome (Zhuang *et al.*, 2010). Furthermore, LDH activity has been correlated to HIF1A expression and angiogenic potential (Koukourakis *et al.*, 2003). However, further studies will be required in order to confirm the link between ulceration and LDH levels suggested by this analysis. In addition, high levels of angiopoietin-2 have been reported in multiple cancers, with a possible prognostic role (Bonner and Arbiser 2012), and are accompanied by upregulation of RAS-related C3 botulinum toxin substrate signaling pathways (Felcht *et al.*, 2012). Thus, it is possible that elevated *PHIP* copy number

can modulate angiopoietin levels in melanoma, in part through mitochondrial dysfunction, nicotinamide adenine dinucleotide phosphate oxidase induction, and increased generation of reactive oxygen species (which result in angiopoietin-2 activation). In conclusion, our studies support an important role for PHIP as a molecular marker of melanoma ulceration, metastasis and survival, and provide insights into the cellular pathways through which ulceration can develop.

MATERIALS AND METHODS

Study population

This study used a TMA cohort that was previously described according to REMARK guidelines (Rangel *et al.*, 2006; Kashani-Sabet *et al.*, 2009). The molecular prognostic factor analyses performed herein were approved by the appropriate ethics boards both at CPMC and UCSF.

Fluorescence *in situ* hybridization

FISH was performed as previously described (Wiegant and Raap 2001; Chin *et al.*, 2003; De Semir *et al.*, 2012) using bacterial artificial chromosome clones RP11-767O1, RP11-484L10, RP11-217L13, and CTD-2297E14 to detect the *PHIP* locus and clones RP11-26M18 and RP11-136K2 to detect 6q11.1 and 6p11.1 respectively (February 2009 freeze of the UCSC Genome Browser, <http://genome.ucsc.edu>). Bacterial artificial chromosome DNA's were prepared with the Large-Construct kit (Qiagen, Valencia, CA) and labeled by nick translation with Alexa Fluor 488 and 594 dUTPs (Life Technologies, Grand Island, NY) as described (Wiegant and Raap 2001). The quality and mapping of all probes were verified by hybridization to normal metaphase spreads in combination with a commercially available centromeric probe for chromosome 6 (Open Biosystems, Lafayette, CO) before tissue analysis. Hybridization on TMA's was performed as described previously (Wiegant and Raap 2001; Chin *et al.*, 2003). Images were taken with a Zeiss Axio Imager Z2 controlled by AxioVision software (Zeiss, Jena, Germany). The FISH signals were assessed and counted manually from images with several Z stack layers acquired using a Zeiss Axio Image Z2 microscope controlled by AxioVision software (Zeiss, Jena, Germany). At least 30 nuclei from each case were evaluated, and the signals were interpreted according to guidelines described previously (Bayani and Squire 2004) and recorded as 1 through 5 or greater. Signals from bacterial artificial chromosome clones detecting 6q11.1 and 6p11.1 were interpreted as the centromeric signal for chromosome 6 and the count was used as control.

Cell culture studies

C8161.9 human melanoma cells stably expressing anti-*PHIP* shRNA and anti-*luciferase* shRNA were generated and propagated as described (De Semir *et al.*, 2012). Lactate production and invasion into matrigel were assayed as reported (De Semir *et al.*, 2012). C8161.9 cells were treated with 10 mM methylpyruvate (Sigma, St Louis, MO) for 48 hours before the invasion assay. VEGF secretion was measured by using an ELISA kit following the manufacturer's instructions (DY293B, R&D Systems, Minneapolis, MN). Western blotting was performed as described (De Semir *et al.*, 2012) using antibodies targeting HIF1A (ab16066, Abcam, Minneapolis, MN), LDH5 (ab1015, Abcam), and GAPDH (MAB374, Millipore, Billerica, MA).

Immunofluorescence

Groups of six nu/nu mice were inoculated subcutaneously with 1 million C8161.9 human melanoma cells expressing either control *luc* or anti-*PHIP* shRNA. On day 25, the mice were sacrificed and the tumors were assessed for microvessel density based on CD31 positivity by immunofluorescence, which was performed using a rabbit anti-CD31 antibody (ab28364, Abcam) and a secondary antibody labeled with Alexa Fluor 594 (Life Technologies) as described previously (Jalas et al., 2011). Mosaic images were acquired with $\times 20$ magnification at a fixed exposure with a Zeiss Axio Imager Z2 microscope controlled by AxioVision software. After automatically stitching the images, the number of CD31-positive objects and area were determined using ImageJ software (NIH, Bethesda, MD). The number of CD31-positive vessels was quantitated and the microvessel density reported as the total CD31 count over the entire area of the tumor.

Statistical analysis

Statistical methods used to assess the significance of various prognostic factors on melanoma outcome were previously described (Rangel et al., 2006; Kashani-Sabet et al., 2009). The coding for clinical or pathological attributes was performed as described by the AJCC staging committee for melanoma (Balch et al., 2001b). The correlation between *PHIP* copy number and immunohistochemical score was tested by regression analysis using Data Desk software (Data Description, Ithaca, NY). For both DMFS and DSS, the definition of high *PHIP* FISH scores, as measured by percentage of cells harboring three or more copies of *PHIP*, was established as equal to or greater than 16%. A 16% cutoff served to maximize the average of the sensitivity and specificity in the prediction of both DMFS and DSS, analyzed separately. The same 16% cutoff was then uniformly and consistently used in all subsequent analyses of DMFS and DSS. The association between high *PHIP* copy number and DMFS or DSS was assessed using both Kaplan-Meier analysis and multivariate Cox regression. The association between high *PHIP* copy number and presence or absence of ulceration was assessed using both Fisher's exact test and multivariate logistic regression analysis. The association between a high degree of tumor vascularity and ulceration was also assessed using both Fisher's exact test and multivariate logistic regression. Assessment of the differential impacts of neither, either, and both of these two factors was done using both χ^2 analysis and Fisher's exact test. In predicting ulceration status, high *PHIP* copy number was uniformly defined as $\geq 35\%$ of tumor cells harboring three or more copies of the *PHIP* gene. This cutoff was similarly selected as the one that maximized the average of sensitivity and specificity in predicting ulceration. All *P*-values reported are two sided.

CONFLICT OF INTEREST

MK-S owns stock in Melanoma Diagnostics. Intellectual property covering the marker described in this study was licensed to Myriad Genetics Laboratories. The remaining authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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