Increased Levels of β -catenin, LEF-1, and HPA-1 Correlate with Poor Prognosis for Acral Melanoma with Negative *BRAF* and *NRAS* Mutation in *BRAF* Exons 11 and 15 and *NRAS* Exons 1 and 2

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To determine the expression of β -catenin, lymphoid enhancer-binding protein-1 (LEF-1), and heparanase-1 (HPA-1) and to evaluate these proteins' potential prognostic values in malignant acral melanoma without mutations in *BRAF* exons 11 and 15 and *NRAS* exons 1 and 2, specimens from 90 patients with wild-type *BRAF* and *NRAS* were assessed and analyzed by immunohistochemistry and western blotting. The positive expression of β -catenin, lymphoid enhancer-binding protein-1, and heparanase-1 was observed in 36 (72%), 31 (62%), and 32 (64%) of the detected acral melanomas, respectively. The expression of β -catenin, lymphoid enhancer-binding protein-1, and heparanase-1 was not correlated with gender, age, or diseased body parts ($p > 0.05$), but was significantly positively correlated with the tumor node metastasis (TNM) stage and metastasis (correlation = 0.406 and 0.716, 0.397 and 0.582, 0.353 and 0.579; $p = 0.040$ and 0.0001, 0.0040 and 0.0001, 0.0120 and 0.0001, respectively). We also observed that the increased expression of β -catenin, lymphoid enhancer-binding protein-1, and heparanase-1 was significantly correlated with decreased survival and poor prognosis ($p = 0.001$, 0.010, and 0.023, respectively). A multifactorial analysis using Cox's regression model revealed that β -catenin, lymphoid enhancer-binding protein-1, heparanase-1, and the TNM stage were all independent factors in malignant melanoma (risk ratios were 7.294, 5.550, 5.622, and 4.794; p -values were 0.007, 0.018, 0.018, and 0.029, respectively). This study may provide the basis for the use of β -catenin, lymphoid enhancer-binding protein-1, and heparanase-1 as novel targets in the treatment of malignant invasion and metastasis in acral melanoma cancer. The expression of β -catenin, LEF-1, and HPA-1 was assessed and compared in malignant melanoma with that of peritumoral tissue and benign nevus in the patients with negative mutations in *BRAF* exons 11 and 15 and *NRAS* exons 1 and 2. The study may provide the basis for β -catenin, LEF-1, and HPA-1 as new targets in the treatment of malignant invasion and metastasis in melanoma cancer.

Introduction

MALIGNANT MELANOMA is the most common form of cancer and can be curable in the initial stages, but when the disease is detected at later stages, malignant melanoma can be one of the most lethal malignancies (Mueller and Bosserhoff, 2009; Wolf *et al.*, 2010; Alexiades-Armenakas, 2012; Fernandez-Flores, 2012). In 2010, 114,900 new cases of melanoma were diagnosed in the United States (Purdue *et al.*, 2008; Pacheco *et al.*, 2011). Although melanoma rep-

resents only ~5% of all diagnosed cancers in the United States, 15% of these cases prove to be fatal (Ricotti *et al.*, 2009). From a gender-based analysis, ~38,870 men and 29,260 women were diagnosed with malignant melanoma in 2010, and ~8700 people were predicted to die from the disease (Jemal *et al.*, 2010; Ma *et al.*, 2012; Mitra *et al.*, 2012). Acral melanoma is an uncommon subtype of cutaneous melanoma and is usually detected in non-Caucasians (Aviles-Izquierdo *et al.*, 2010). The disease occurs on non-hairy or acral skin, including the palms, soles and nail beds,

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and exhibits a notably worse prognosis than the more common cutaneous melanoma (Bradford *et al.*, 2009). Recently, the distinct histological and phenotypic characteristics of cutaneous melanomas have suggested that acral melanoma differs from other types of cutaneous melanomas by having a higher frequency of chromosomal aberrations, especially amplifications of particular chromosome regions (Turajlic *et al.*, 2012). Therefore, acral melanoma has become a major public health problem worldwide. Due to the increase in the incidence of malignant melanoma, its resistance to chemotherapy, its difficulty to cure at a later stage, and its high potential for metastasis, the prognosis is still poor for most patients, and malignant melanoma has received increasing attention (Garber, 2009; Hodi *et al.*, 2010; Day and Merlino, 2011; Smyth *et al.*, 2011).

The Wnt/ β -catenin signaling pathway has been reported to play an important role in melanoma progression (Reya and Clevers, 2005; Espada *et al.*, 2009). β -catenin and lymphoid enhancer-binding protein-1 (LEF-1) are the key components of the Wnt signaling pathway (Rubinfeld *et al.*, 1997; Takahashi *et al.*, 2008). Under physiological conditions, molecular changes such as gene mutations in β -catenin increase the stability of β -catenin in the cytoplasm (Friedlander and Hodi, 2010; Gartner *et al.*, 2012). Next, the protein translocates from cytoplasm to the nucleus and binds with LEF-1. β -catenin is a positive regulator of Wnt, and LEF-1 plays an important role in engaging the target gene, which in turn leads to malignant melanoma (Nelson and Nusse, 2004; Eichhoff *et al.*, 2011). Based on previous studies, heparanase-1 (HPA-1) was also found to be a risk factor in skin cancer development (Lewis *et al.*, 2008; Chen *et al.*, 2012; Liu *et al.*, 2012). HPA-1 is reported to be upregulated in metastatic cancers and involved in the invasion and metastasis of cancer cells (Rivera *et al.*, 2008; Orgaz and Sanz-Moreno, 2013). Mutations in NRAS and BRAF were detected in 14.5% and 16.0% of the ALMs, respectively. In our study, we evaluated malignant acral melanoma patients without BRAF and NRAS mutations and investigated the expression of β -catenin, LEF-1, and HPA-1 in malignant melanoma, peritumoral tissue, and pigmented nevus, with the additional analysis of the important roles of β -catenin, LEF-1, and HPA-1 in the progression or prognosis of malignant melanoma.

Materials and Methods

Patients

A total of 180 patients (100 men and 80 women) were included in the study. The median age of the patients was 56.32 years (range 21–80 years). The patients were randomly divided into two groups: group A and group B (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/dna), and every group contained 90 patients. There were 50 malignant melanomas, 20 pigmented nevus, and 20 paratumor tissue specimens in group A. There were 30 malignant melanomas, 30 pigmented nevus, and 30 paratumor tissue specimens in group B. The samples of pigmented nevus and paratumor tissue specimens were used as negative controls. The specimens of group A were processed for immunohistochemical analysis, and the samples of group B were treated for western blot analysis. The study was conducted over a period of 24 months in the ICU of the general hospital of the Tumor Hospital of Yunnan

Province (March 2009 to May 2011). Our research study was in compliance with the Helsinki Declaration, and we obtained approval for the study from the Ethics Committee of Tumor Hospital of Yunnan Province (project no. 2010NS095). The subjects and their families were well informed of the details and signed the relevant contracts before the study.

Immunohistochemical analysis

Immunohistochemical staining was performed as previously described (Adhami *et al.*, 2004; Saleem *et al.*, 2008). Briefly, paraffin-embedded sections were dewaxed, rehydrated, blocked, and incubated overnight with a primary antibody. Next, the specimens were incubated with a secondary antibody for 1 h. All of the antibodies were purchased from Santa Cruz Biotechnology. The stained slides were dehydrated and cleared, and the stained slides were mounted in Permount and visualized using a Nikon Eclipse Ti microscope. Positive and negative controls were included, and all of the controls yielded satisfactory results. The images were captured with a camera attached to the microscope. The positivity of the immunostaining was determined in 10 different fields at 400 $\times$ magnification. We utilized comprehensive standards to evaluate the staining intensity and cell counts, as described by Potti and Mamyama, respectively. Briefly, we randomly selected four nonconsecutive high magnification fields and counted the cells that were imaged. The scores were calculated according to the staining intensity and number of positive cells. (1) Staining intensity: no staining was marked as 0; yellow, brown, and tan colors were marked as 1, 2, and 3, respectively. (2) The counts of positive cells: sample sections with negative staining were marked as 0; less than 10% positive cells were marked as 1; 10% to 50% of positive cells were marked as 2; and more than 50% positive cells were marked as 3.

The final score was calculated as the sum of (1) and (2). A score of 0–1 indicated a negative (–) sample; 2–3 represented weakly positive results by immunohistochemical staining (+); 4–5 indicated moderately positive staining (++); and 6 indicated strongly positive staining (+++).

Western blot analysis

The specimens were washed by phosphate buffered saline and lysed with RIPA (Tris 50 mM, NP-40 1%, NaCl 150 mM, EDTA 1 mM, SDS 0.1%, SDC 0.25%). The protein concentrations were quantified using the Bradford assay. The samples were separated using SDS-PAGE and transferred electrophoretically onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in TBST (Tris-HCl 50 mM, NaCl 150 mM, 0.1% Tween) and then incubated with mouse monoclonal anti-human β -catenin, LEF-1, or HPA-1 antibody. A rabbit anti-mouse IgG antibody coupled with horseradish peroxidase was used as a secondary antibody. The resulting bands were detected using an enhanced chemiluminescence western blotting detection system according to the manufacturer's protocol. β -actin was used as an internal reference, and three independent experiments were performed. The LEF-1 antibody (cat. 2230) and β -catenin antibody (cat. 9582) was obtained from Cell signaling. The HPA-1 antibody was obtained from Santa (cat. Sc-25825). Goat anti-rabbit secondary antibody was obtained from Nuobei biotechnology Co. Ltd. (Kunming, China).

BRAF and NRAS genotype analysis

Primers were designed to amplify exons 11 (G loop region) and 15 (activation segment) of *BRAF* and exons 1 and 2 of *NRAS*. The primer sequences were as follows:

BRAF exon 11F: 5'-TTCTGTTTGGCTTGACTTGA CTT-3'

BRAF exon 11R: 5'-ACTTGTCACAATGTCACCAC ATT-3'

BRAF exon 15F: 5'-TCATAATGCTTGCTCTGATA GGA-3'

BRAF exon 15R: 5'-GGCCAAAAATTTAATCAGT GGA-3'

NRAS exon 1F: 5'-GGTTTCCAACAGGTTCTTGC-3'

NRAS exon 1R: 5'-CACTGGGCCTCACCTCTATG-3'

NRAS exon 2F: 5'-CACACCCCAGGATTCTTAC-3'

NRAS exon 2R: 5'-TGGCAAATACACAGAGGAAGC-3'.

Polymerase chain reactions (PCRs) were performed in 20 μ L reactions containing 0.5 μ M of each primer, 2.5 mM Mg^{2+} , 0.25 mM dNTPs, and 1 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) in 1 $\times$ PCR buffer. In total, 40 amplification cycles (95°C for 30 s, 55°C for 30 s, and 72°C for 45 s) were performed, with an initial denaturation at 95°C for 10 min and a final extension at 72°C for 10 min. The PCR products were purified with 1 μ L ExoI/SAP (37°C for 15 min and then 85°C for 15 min) and were then sequenced directly on both strands using the BigDye[®] Terminator v1.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's protocol and analyzed using the Applied Biosystems 3730 DNA analyzer. All mutations were reconfirmed by independent PCR reactions and direct sequencing.

Statistical analysis

Basic statistical analyses were performed using the statistical software SPSS 20.0 (SPSS, Inc., Chicago, IL). The comparisons between β -catenin (e.g., LEF-1, HPA-1) and the

clinical indicators were performed using Pearson's χ^2 tests. The Kaplan–Meier survival estimates and log-rank tests were used to evaluate the survival data. A value of $p < 0.05$ was considered statistically significant. The multifactorial Cox regression model was used for independent factor analysis.

Results

Patient characteristics

We studied a total of 180 patients in the Tumor Hospital of Yunnan Province, with a median patient age of 56.32 years (range 21–80 years). The patients were randomly divided into two groups: group A and group B (Supplementary Table S1), and every group contained 90 patients. The specimens of group A were processed for immunohistochemical analysis, and the samples of group B were treated for western blot analysis. Some of the specimens were photographed, and the clinical features of the malignant acral melanoma are shown in Supplementary Figure S1 and Supplementary Table S1.

BRAF and *NRAS* mutations are typically found to be abnormally expressed in malignant melanoma. However, malignant tumors negative for *BRAF* and *NRAS* mutations were also observed. Exons 11 was located in G loop region and exon 15 was the activated segment of *BRAF*. Mutations in exons 1 or exon 2 of *NRAS* was obviously seen in patients with malignant melanomas. We detected and selected the malignant acral melanoma specimens with negative mutations in *BRAF* exons 11 and 15 and *NRAS* exons 1 and 2. The sequences of exons and codons of *BRAF* exons 11 and 15 and *NRAS* exons 1 and 2 are shown in Supplementary Figure S2.

Evaluation of β -catenin and LEF-1 staining in acral malignant melanoma

The Wnt/ β -catenin pathway has been reported to be associated with melanoma prognosis. β -catenin and LEF-1 are two common genes in the Wnt/ β -catenin pathway and were

FIG. 1. Paraffin-embedded samples were analyzed by histochemical staining for β -catenin. (A) Normal expression of β -catenin in the cell nucleus and membranes of peritumoral tissue; (B, C) membrane-associated and cytoplasmic expression of β -catenin in malignant melanoma; (D) the expression of β -catenin in benign nevi (β -catenin stain photographed at 400 $\times$ original magnification).

FIG. 2. Paraffin-embedded samples were analyzed by immunohistochemical staining for LEF-1. (A) Normal expression of LEF-1 in peritumoral tissue; (B, C) cytoplasmic expression of LEF-1 in malignant melanoma; (D) the expression of LEF-1 in benign nevi (LEF-1 stain photographed at 400 $\times$ original magnification).

detected by immunohistochemistry in the acral malignant melanoma samples, the results of which were further confirmed by western blot analysis. The results demonstrated that the positive staining for β -catenin appeared mainly as a cytoplasmic brown-colored reaction and was occasionally membrane associated. However, immunopositivity for LEF-1 appeared as a cytoplasmic reaction. The membrane-associated and cytoplasmic localization of β -catenin was observed in the majority of the examined samples (Fig. 1B, C), as 72% of malignant melanomas demonstrated different levels of positive immunostaining. The cytoplasmic expression of LEF-1 (Fig. 2B, C) and HPA-1 (Fig. 3B, C) in

acral melanoma was also observed in the majority of the specimens. The paracancer tissue staining for β -catenin, LEF-1, and HPA-1 was shown in Figures 1A, 2A, and 3A, respectively. The expression levels of β -catenin, LEF-1, and HPA-1 in benign nevi were shown in Figure 1D, Figures 2D, and 3D, respectively. The entire panel of primary and metastatic lesions was examined, and an increase in the number of β -catenin-positive cells was observed in 36 of 50 (72%) malignant melanomas compared with the peritumoral tissue and pigmented nevus. Similarly, the numbers of LEF-1- and HPA-1-positive cells increased to 31 of 50 (62%) and 32 of 50 (64%), respectively (Table 1).

FIG. 3. Paraffin-embedded samples were analyzed by immunohistochemical staining for HPA-1. (A) Normal expression of HPA-1 in peritumoral tissue; (B, C) cytoplasmic expression of HPA-1 in malignant melanoma; (D) the expression of HPA-1 in benign nevi (HPA-1 stain photographed at 400 $\times$ original magnification).

TABLE 1. EXPRESSION OF β -CATENIN, LEF-1, AND HPA-1 IN MALIGNANT MELANOMA COMPARED WITH NORMAL TISSUES

Specimen	Sample no.	β -catenin				LEF-1				HPA-1			
		–	+	++	+++	–	+	++	+++	–	+	++	+++
MM	50	14	6	14	16	19	10	14	7	18	4	16	12
NT	40	34	1	3	2	32	1	2	5	36	1	3	0
χ^2				29.801				20.267				28.664	
<i>p</i>				<1e-06				0.002				<1e-06	

MM, malignant melanoma; NT, normal tissues (peritumoral tissue and pigmented nevus).

Western blot analysis of the expression of β -catenin, LEF-1, and HPA-1 in malignant acral melanomas

We utilized western blotting to further confirm the expression of β -catenin, LEF-1, and HPA-1 in acral melanomas and normal tissues. β -actin was used as an internal reference, and the data from three independent experiments demonstrated that the expression of β -catenin, LEF-1, and HPA-1 in acral melanoma was obviously increased relative to that in pigmented nevus and peritumoral tissue (Fig. 4A). Then, the data were normalized to β -actin expression, followed by t-test analysis, and are presented as the mean $\pm$ SD. The expression of β -catenin, LEF-1, and HPA-1 in malignant melanomas was significantly different from that in peritumoral tissues (Fig. 4B), and the *p*-values were 0.00001, 0.00001, and 0.0010, respectively (Table 2).

Immunohistochemical expression of β -catenin, LEF-1, and HPA-1 in relation to clinical parameters

The expression of β -catenin, LEF-1, and HPA-1 in the malignant melanoma samples, combined with clinical parameters, is detailed in Supplementary Table S2. The data revealed that no correlation existed between the expression of the three proteins (β -catenin, LEF-1, and HPA-1) and gender, age, or diseased body part (Table 3). However, the immunohistochemical staining of β -catenin, LEF-1, and HPA-1 was analyzed in relation to the clinical parameters of acral melanoma progression, relapse, and metastasis. The data demonstrated that the expression of β -catenin exhibited a significantly positive correlation with tumor node metastasis (TNM) stage and relapse and metastasis (correlation=0.4060 and 0.7160, *p*=0.0400 and 0.0001, respectively). A positive correlation was also found between the expression of LEF-1 and the TNM stage and relapse and metastasis (correlation=0.397 and 0.582, *p*=0.0040 and 0.0001, respectively); similarly, HPA-1 was positively correlated with TNM stage and relapse and metastasis (correlation=0.3530 and 0.5790, *p*=0.0120 and 0.0001, respectively) (Table 3).

Recurrence and survival rates were negatively correlated with the expression of β -catenin, LEF-1, and HPA-1, and the TNM stage

To understand the relevance of clinical parameters to the prognosis of human acral melanomas, we analyzed the relationship between the clinical indicators such as age, gender, diseased body part, TNM stage and metastasis, and the data for the 1-, 3- and 5-year survival rates of patients with metastatic melanoma (Supplementary Table S3). The data revealed that the profiles of 3- and 5-year survival were

similar, although the 5-year survival rate was lower. The 3- and 5-year survival rates were slightly higher in women than they were in men, and the survival rate of patients <30 years was slightly higher than that of patients $\geq$ 30 years, although there were no significant differences between the age groups (Supplementary Fig. S3A, B). The data also revealed that there was no significant correlation between

FIG. 4. (A) Western blot analysis of β -catenin, LEF-1, and HPA-1 expression in malignant melanoma, pigmented nevus, and control tissues. β -actin was used as an internal reference, and the data shown were representative of three independent experiments with similar results. (B) β -catenin, LEF-1, and HPA-1 expression in peritumoral, pigmented nevus, and malignant melanoma tissue. The data were normalized to the β -actin levels and were expressed as the mean $\pm$ SD. The data for the peritumoral tissue were used as controls. **p*<0.01, ***p*<0.01 compared with the control.

TABLE 2. EXPRESSION OF β -CATENIN, LEF-1, AND HPA-1 IN MALIGNANT MELANOMA, PIGMENTED NEVUS, AND PERITUMORAL TISSUES

Groups	Sample no.	β -catenin	LEF-1	HPA-1
Malignant melanoma	30	7.91 ± 0.492^a	3.28 ± 0.066^a	3.00 ± 0.134^a
Pigmented nevus	30	2.14 ± 0.187^b	1.66 ± 0.078^b	2.56 ± 0.130^b
Peritumoral tissues	30	2.02 ± 0.189	1.05 ± 0.041	2.13 ± 0.086

The data are presented as the means $\pm$ SD.

^aThe comparison between the malignant melanoma group and the peritumoral tissues group, the p -values for β -catenin, LEF-1, and HPA-1 were $< 1e-06$, $< 1e-06$, and 0.001, respectively. $p < 0.001$: statistically significant.

^bA comparison between the pigmented nevus group and the peritumoral tissues group; the p -values for β -catenin, LEF-1, and HPA-1 are 0.752, 0.020, and 0.014, respectively.

the body parts affected by the disease and the prognosis for the malignant melanoma patients ($p=0.345$) (Supplementary Fig. S3C).

The survival rate for acral melanoma was significantly correlated to the clinical stage and the expression of β -catenin, LEF-1, and HPA-1. Melanomas in stage I and II were more likely to provide a better prognosis (5-year relative survival 25.64%) than those in stage III and IV (5-year relative survival 9.76%) (Supplementary Fig. S3D). We observed that the increased expression of β -catenin, LEF-1, and HPA-1 significantly correlated with decreased survival and poor prognosis ($p=0.001$, 0.010, and 0.023, respectively) (Fig. 5).

Based on the result that several indicators significantly correlated with relapse, metastasis, and the 5-year survival rate for malignant acral melanoma, we analyzed the risk ratios of the clinical factors. As observed in Table 4, the multifactorial Cox regression analysis indicated that the β -catenin, LEF-1, and HPA-1 expression levels and TNM stage were all independent factors in malignant melanoma (risk ratios were 7.294, 5.550, 5.622, and 4.794; p -values were 0.007, 0.018, 0.018, and 0.029, respectively). The results revealed that β -catenin, LEF-1, and HPA-1 expression and TNM stage were significant factors that can aggravate malignant melanoma, especially β -catenin expression.

Discussion

The prevalence of malignant acral melanoma has been increasing rapidly throughout the world (Klit *et al.*, 2011). The disease's incidence has been rising by 30–50% every five years, and the increases affect people in all age groups (Jemal *et al.*, 2010; Reed *et al.*, 2012). The number of patients more than doubled between 1971 and 1990. The Wnt signaling pathway was reported to play an important role in the development and prognosis of malignant melanoma (Murakami *et al.*, 2001; Maeldandsmo *et al.*, 2003). In our study, the cytoplasmic localization of β -catenin and LEF-1, two potential indicators of Wnt/ β -catenin pathway activation, were obviously observed in human malignant acral melanoma. The expression levels of β -catenin and LEF-1 were significantly higher in malignant acral melanoma than they were in benign tumors ($p < 0.05$). The expression of β -catenin and LEF-1 were positively correlated with lymph node metastases, TNM stage, and relapse and metastasis. We also observed that the increased expression of β -catenin and LEF-1 significantly correlated with a decreased survival rate and poor prognosis ($p=0.001$ and 0.010, respectively). Thus, β -catenin and LEF-1 both are risk factors and might participate in the malignant conversion and progression of human melanoma (Widlund *et al.*, 2002; Damsky *et al.*, 2011).

TABLE 3. CORRELATIONS BETWEEN CLINICAL PARAMETERS AND THE EXPRESSION OF β -CATENIN, LEF-1, AND HPA-1 IN MALIGNANT MELANOMA

Clinical parameters	Gender	Age (years)	Diseased parts	TNM stage	Relapse and metastasis
HPA-1					
χ^2	0.3310	4.7950	4.7240	12.9920	18.1380
p	0.9540	0.1840	0.8580	0.0430	0.0001
r	0.0770	−0.1690	0.0300	0.3530	0.5790
p	0.5970	0.2400	0.8380	0.0120	0.0001
LEF-1					
χ^2	1.2150	2.5940	5.7890	15.4340	25.4760
p	0.7490	0.4580	0.7610	0.0170	0.0001
r	0.1000	−0.0340	−0.0150	0.3970	0.5820
p	0.4880	0.8150	0.9150	0.0040	0.0001
β -catenin					
χ^2	5.6680	2.9030	13.7780	21.1970	26.3190
p	0.1200	0.4070	0.1370	0.0200	0.0001
r	−0.0160	−0.2350	0.2160	0.4060	0.7160
p	0.9130	0.1010	0.1310	0.0400	0.0001

$p \leq 0.001$, statistically significant; r , Spearman's rank correlation coefficient.

TNM, tumor node metastasis.

FIG. 5. Kaplan–Meier analysis. Sixty samples were analyzed in every group. (A) Survival analysis of primary and metastatic tumors. Malignant melanomas ($n=50$) were stratified based on membrane-associated and cytoplasmic β -catenin levels. The patients with the membrane-associated and cytoplasmic β -catenin(+) levels exhibited a significantly lower survival probability by Kaplan–Meier analysis as compared with β -catenin(–) individuals (log-rank test). (B) Survival analysis of primary and metastatic tumors. The Kaplan–Meier analysis demonstrated a significantly decreased survival probability in patients with higher cytoplasmic LEF-1 levels (log-rank test). (C) Primary and metastatic tumors were separated into those with the higher cytoplasmic HPA-1 levels/HPA-1(+) and those with lower HPA-1 levels/HPA-1(–). The Kaplan–Meier analysis demonstrated a significantly decreased survival probability in patients with the higher cytoplasmic HPA-1 levels (log-rank test).

HPA-1 has been reported to degrade the extracellular matrix and facilitate the process of the invasion and metastasis of carcinoma (Liu *et al.*, 2008; Yingying *et al.*, 2009; Wagner *et al.*, 2012). A predominant correlation was observed between HPA-1 expression and tumor invasion and lymphatic metastasis in gastric carcinoma (Endo *et al.*, 2001; Takaoka *et al.*, 2003), and this finding is consistent with the results of our study. In our study, the results revealed that HPA-1 was positively correlated with the TNM stage and relapse and metastasis (correlation=0.353 and 0.579; $p=0.012$ and 0.000, respectively), and the high expression of HPA-1 was correlated with decreased survival rate and poor prognosis ($p=0.023$). In our study, a positive correlation was observed between HPA-1 expression and lymphatic metastasis. Taken together, the results indicate that HPA-1 can be considered a marker of invasion and metastasis in acral melanoma.

The risk ratios of the clinical factors were analyzed as shown in Table 4. The multifactorial Cox regression analysis obviously demonstrated that β -catenin, LEF-1, and HPA-1 expression levels and TNM stage were all inde-

pendent factors in malignant melanoma. The risk ratios were 7.294, 5.550, 5.622, and 4.794 and p -values were 0.007, 0.018, 0.018, and 0.029, respectively. However, age, gender, and the diseased parts of the malignant melanoma is not the relatively independent factor. All of the results suggested that β -catenin, LEF-1, and HPA-1 expression and TNM stage were risk factors. Especially, β -catenin was the most dangerous factor to aggravate malignant melanoma.

In conclusion, our data demonstrated that the high expression of β -catenin, LEF-1, and HPA-1 was related to poor prognosis and malignant metastasis in acral melanoma. To our knowledge, this is the first time that an association between the three proteins has been reported, and no attempt has been made to study this further. This study may provide the basis for the use of β -catenin, LEF-1, and HPA-1 as novel targets in the treatment of the malignant invasion and metastasis of acral melanoma.

Acknowledgment

This research was supported, in part, by grants (no. 81260322/H1606, no. 81372322/H1606 and no. 81460440/H1606) from the National Natural Science Foundation of China, a grant (no. 2012FB163) from the Natural Science Foundation of Yunnan Province, a grant (no. 2011FB201) from the Joint Special Funds for the Department of Science and Technology of Yunnan Province-Kunming Medical University, and a grant (no. D-201242) from the specialty fund of high-level talents medical personnel training of Yunnan province.

Disclosure Statement

The authors have declared no conflicts of interest.

References

Adhami, V.M., Siddiqui, I.A., Ahmad, N., Gupta, S., and Mukhtar, H. (2004). Oral consumption of green tea polyphenols inhibits

TABLE 4. RISK RATIOS FOR THE FACTORS THAT AFFECTED THE RECURRENCE AND TISSUE METASTASIS OF MALIGNANT MELANOMA

Factors	Standard error	Risk ratios	p-Value	95% confidence interval
β -catenin	1.266	7.294	0.007	2.554–365.261
LEF-1	1.286	5.550	0.018	1.652–238.403
HPA-1	1.177	5.622	0.018	0.006–0.616
TNM stage	0.430	4.794	0.029	0.168–0.906
Age	0.915	0.260	0.610	0.265–9.590
Parts of the body	0.769	0.162	0.687	0.302–6.159
Gender	1.528	2.094	0.148	0.457–182.434

- insulin-like growth factor-I-induced signaling in an autochthonous mouse model of prostate cancer. *Cancer Res* **64**, 8715–8722.
- Alexiades-Armenakas, M.R. (2012). Melanoma-detecting technologies and standard of care. *J Drugs Dermatol* **11**, 1270–1271.
- Aviles-Izquierdo, J.A., Longo-Imedio, M.I., and Lazaro-Ochaita, P. (2010). Acral cutaneous melanoma in a Spanish Caucasian population. *Melanoma Res* [Epub ahead of print]; DOI: 10.1097/CMR.0b013e3282f1d2b6
- Bradford, P.T., Goldstein, A.M., McMaster, M.L., and Tucker, M.A. (2009). Acral lentiginous melanoma: incidence and survival patterns in the United States, 1986–2005. *Arch Dermatol* **145**, 427–434.
- Chen, Y., Huang, L., and Yu, J. (2012). Evaluation of heparanase and matrix metalloproteinase-9 in patients with cutaneous malignant melanoma. *J Dermatol* **39**, 339–343.
- Damsky, W.E., Curley, D.P., Santhanakrishnan, M., Rosenbaum, L.E., Platt, J.T., Gould Rothberg, B.E., *et al.* (2011). beta-catenin signaling controls metastasis in *Braf*-activated *Pten*-deficient melanomas. *Cancer Cell* **20**, 741–754.
- Day, C.P., and Merlino, G. (2011). Illuminating progression: tracking melanoma metastasis in a gene therapy setting. *Pigment Cell Melanoma Res* **24**, 260–261.
- Eichhoff, O.M., Weeraratna, A., Zipser, M.C., Denat, L., Widmer, D.S., Xu, M., *et al.* (2011). Differential LEF1 and TCF4 expression is involved in melanoma cell phenotype switching. *Pigment Cell Melanoma Res* **24**, 631–642.
- Endo, K., Maejara, U., Baba, H., Tokunaga, E., Koga, T., Ikeda, Y., *et al.* (2001). Heparanase gene expression and metastatic potential in human gastric cancer. *Anticancer Res* **21**, 3365–3369.
- Espada, J., Calvo, M.B., Diaz-Prado, S., and Medina, V. (2009). Wnt signalling and cancer stem cells. *Clin Transl Oncol* **11**, 411–427.
- Fernandez-Flores, A. (2012). Prognostic factors for melanoma progression and metastasis: from hematoxylin-eosin to genetics. *Rom J Morphol Embryol* **53**, 449–459.
- Friedlander, P., Hodi, F.S. (2010). Advances in targeted therapy for melanoma. *Clin Adv Hematol Oncol* **8**, 619–627.
- Garber, K. (2009). Cancer research. Melanoma drug vindicates targeted approach. *Science* **326**, 1619.
- Gartner, J., Davis, S., Wei, X., Lin, J.C., Teer, J.K., Rosenberg, S.A., *et al.* (2012). Comparative exome sequencing of metastatic lesions provides insights into the mutational progression of melanoma. *BMC Genomics* **13**, 505.
- Hodi, F.S., O'Day, S.J., McDermott, D.F., Weber, R.W., Sosman, J.A., Haanen, J.B., *et al.* (2010). Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* **363**, 711–723.
- Jemal, A., Siegel, R., Xu, J., and Ward, E. (2010). Cancer statistics, 2010. *CA Cancer J Clin* **60**, 277–300.
- Klit, A., Drejoe, J.B., and Drzewiecki, K.T. (2011). Trends in the incidence of malignant melanoma in Denmark 1978–2007. Incidence on the island of Bornholm compared with the whole country incidence in Denmark. *Dan Med Bull* **58**, A4229.
- Lewis, K.D., Robinson, W.A., Millward, M.J., Powell, A., Price, T.J., Thomson, D.B., *et al.* (2008). A phase II study of the heparanase inhibitor PI-88 in patients with advanced melanoma. *Invest New Drugs* **26**, 89–94.
- Liu, X., Fang, H., Chen, H., Jiang, X., Fang, D., Wang, Y., *et al.* (2012). An artificial miRNA against HPSE suppresses melanoma invasion properties, correlating with a down-regulation of chemokines and MAPK phosphorylation. *PLoS One* **7**, e38659.
- Liu, X.Y., Fang, H., Yang, Z.G., Wang, X.Y., Ruan, L.M., Fang, D.R., *et al.* (2008). Matrine inhibits invasiveness and metastasis of human malignant melanoma cell line A375 *in vitro*. *Int J Dermatol* **47**, 448–456.
- Ma, M.W., Qian, M., Lackaye, D.J., Berman, R.S., Shapiro, R.L., Pavlick, A.C., *et al.* (2012). Challenging the current paradigm of melanoma progression: brain metastasis as isolated first visceral site. *Neuro Oncol* **14**, 849–858.
- Maelandsmo, G.M., Holm, R., Nesland, J.M., Fodstad, O., and Florenes, V.A. (2003). Reduced beta-catenin expression in the cytoplasm of advanced-stage superficial spreading malignant melanoma. *Clin Cancer Res* **9**, 3383–3388.
- Mitra, D., Luo, X., Morgan, A., Wang, J., Hoang, M.P., Lo, J., *et al.* (2012). An ultraviolet-radiation-independent pathway to melanoma carcinogenesis in the red hair/fair skin background. *Nature* **491**, 449–453.
- Mueller, D.W., and Bosserhoff, A.K. (2009). Role of miRNAs in the progression of malignant melanoma. *Br J Cancer* **101**, 551–556.
- Murakami, T., Toda, S., Fujimoto, M., Ohtsuki, M., Byers, H.R., Etoh, T., *et al.* (2001). Constitutive activation of Wnt/beta-catenin signaling pathway in migration-active melanoma cells: role of LEF-1 in melanoma with increased metastatic potential. *Biochem Biophys Res Commun* **288**, 8–15.
- Nelson, W.J., and Nusse, R. (2004). Convergence of Wnt, beta-catenin, and cadherin pathways. *Science* **303**, 1483–1487.
- Orgaz, J.L., and Sanz-Moreno, V. (2013). Emerging molecular targets in melanoma invasion and metastasis. *Pigment Cell Melanoma Res* **26**, 39–57.
- Pacheco, I., Buzea, C., and Tron, V. (2011). Towards new therapeutic approaches for malignant melanoma. *Expert Rev Mol Med* **13**, e33.
- Purdue, M.P., Freeman, L.E., Anderson, W.F., and Tucker, M.A. (2008). Recent trends in incidence of cutaneous melanoma among US Caucasian young adults. *J Invest Dermatol* **128**, 2905–2908.
- Reed, K.B., Brewer, J.D., Lohse, C.M., Bringe, K.E., Pruitt, C.N., and Gibson, L.E. (2012). Increasing incidence of melanoma among young adults: an epidemiological study in Olmsted County, Minnesota. *Mayo Clin Proc* **87**, 328–334.
- Reya, T., and Clevers, H. (2005). Wnt signalling in stem cells and cancer. *Nature* **434**, 843–850.
- Ricotti, C., Bouzari, N., Agadi, A., and Cockerell, C.J. (2009). Malignant skin neoplasms. *Med Clin North Am* **93**, 1241–1264.
- Rivera, R.S., Nagatsuka, H., Siar, C.H., Gunduz, M., Tsujigiwa, H., Han, P.P., *et al.* (2008). Heparanase and vascular endothelial growth factor expression in the progression of oral mucosal melanoma. *Oncol Rep* **19**, 657–661.
- Rubinfeld, B., Robbins, P., El-Gamil, M., Albert, I., Porfiri, E., and Polakis, P. (1997). Stabilization of beta-catenin by genetic defects in melanoma cell lines. *Science* **275**, 1790–1792.
- Saleem, M., Maddodi, N., Abu Zaid, M., Khan, N., bin Hafeez, B., Asim, M., *et al.* (2008). Lupeol inhibits growth of highly aggressive human metastatic melanoma cells *in vitro* and *in vivo* by inducing apoptosis. *Clin Cancer Res* **14**, 2119–2127.
- Smyth, E.C., Flavin, M., Pulitzer, M.P., Gardner, G.J., Costantino, P.D., Chi, D.S., *et al.* (2011). Treatment of locally recurrent mucosal melanoma with topical imiquimod. *J Clin Oncol* **29**, e809–e811.
- Takahashi, Y., Nishikawa, M., Suehara, T., Takiguchi, N., and Takakura, Y. (2008). Gene silencing of beta-catenin in melanoma cells retards their growth but promotes the formation of pulmonary metastasis in mice. *Int J Cancer* **123**, 2315–2320.

- Takaoka, M., Naomoto, Y., Ohkawa, T., Uetsuka, H., Shirakawa, Y., Uno, F., *et al.* (2003). Heparanase expression correlates with invasion and poor prognosis in gastric cancers. *Lab Invest* **83**, 613–622.
- Turajlic, S., Furney, S.J., Lambros, M.B., Mitsopoulos, C., Kozarewa, I., Geyer, F.C., *et al.* (2012). Whole genome sequencing of matched primary and metastatic acral melanomas. *Genome Res* **22**, 196–207.
- Wagner, M., Suarez, E.R., Theodoro, T.R., Machado Filho, C.D., Gama, M.F., Tardivo, J.P., *et al.* (2012). Methylene blue photodynamic therapy in malignant melanoma decreases expression of proliferating cell nuclear antigen and heparanases. *Clin Exp Dermatol* **37**, 527–533.
- Widlund, H.R., Horstmann, M.A., Price, E.R., Cui, J., Lessnick, S.L., Wu, M., *et al.* (2002). Beta-catenin-induced melanoma growth requires the downstream target Microphthalmia-associated transcription factor. *J Cell Biol* **158**, 1079–1087.
- Wolf, A.M., Wender, R.C., Etzioni, R.B., Thompson, I.M., D'Amico, A.V., Volk, R.J., *et al.* (2010). American Cancer Society guideline for the early detection of prostate cancer: update 2010. *CA Cancer J Clin* **60**, 70–98.
- Yingying, X., Yong, Z., Zhenning, W., Xue, Z., Li, J., Yang, L., *et al.* (2009). Role of heparanase-1 in gastric carcinoma invasion. *Asian Pac J Cancer Prev* **10**, 151–154.

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Received for publication July 5, 2014; received in revised form August 17, 2014; accepted September 17, 2014.