

## Shedding of Distinct Cryptic Collagen Epitope (HU177) in Sera of Melanoma Patients

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**Abstract Purpose:** Extracellular matrix remodeling during tumor growth plays an important role in angiogenesis. Our preclinical data suggest that a newly identified cryptic epitope (HU177) within collagen type IV regulates endothelial and melanoma cell adhesion *in vitro* and angiogenesis *in vivo*. In this study, we investigated the clinical relevance of HU177 shedding in melanoma patient sera.

**Experimental Design:** Serum samples from 291 melanoma patients prospectively enrolled at the New York University Medical Center and 106 control subjects were analyzed for HU177 epitope concentration by a newly developed sandwich ELISA assay. HU177 serum levels were then correlated with clinical and pathologic parameters.

**Results:** Mean HU177 epitope concentration was 5.8 ng/mL (range, 0-139.8 ng/mL). A significant correlation was observed between HU177 concentration and nodular melanoma histologic subtype [nodular,  $10.3 \pm 1.6$  ng/mL (mean  $\pm$  SE); superficial spreading melanoma,  $4.5 \pm 1.1$  ng/mL; all others,  $6.1 \pm 2.1$  ng/mL;  $P = 0.01$  by ANOVA test]. Increased HU177 shedding also correlated with tumor thickness ( $\leq 1.00$  mm,  $3.8 \pm 1.1$  ng/mL; 1.01-3.99 mm,  $8.7 \pm 1.3$  ng/mL;  $\geq 4.00$  mm,  $10.3 \pm 2.4$  ng/mL;  $P = 0.003$  by ANOVA). After multivariate analysis controlling for thickness, the correlation between higher HU177 concentration and nodular subtype remained significant ( $P = 0.03$ ). The mean HU177 epitope concentration in control subjects was 2.4 ng/mL.

**Conclusions:** We report that primary melanoma can induce detectable changes in systemic levels of cryptic epitope shedding. Our data also support that nodular melanoma might be biologically distinct compared with superficial spreading type melanoma. As targeted interventions against cryptic collagen epitopes are currently undergoing phase I clinical trial testing, these findings indicate that patients with nodular melanoma may be more susceptible to such targeted therapies.

The increasing incidence of melanoma remains a public health concern, with an estimated 60,000 new cases diagnosed and ~8,000 deaths from this disease in the United States in 2007 (1). Metastatic melanoma has long been refractory to existing therapies; even adjuvant treatment of high-risk melanoma patients with IFN- $\alpha$  has been shown to have only a modest effect on overall survival (2). The identification of melanoma patient populations with distinct molecular alterations will be a

more rational approach in the design of novel treatment modalities and strategies.

The pharmacologic inhibition of extracellular matrix protein fragments is a legitimate target for cancer therapy. The proteolytic remodeling of extracellular matrix components has been shown to be integral to tumor invasion and angiogenesis (3-5). In particular, cleavage of type IV collagen, a major component of the vascular basement membrane, generates

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protein fragments that have been shown to function as endogenous inhibitors of angiogenesis (6, 7). Several of these proteolytic fragments have been identified and evaluated as novel therapeutic targets, and whereas preliminary investigations with these molecules were promising, phase I and II clinical trials concluded with disappointing results (8–12).

Distinct cryptic collagen peptides are among the protein fragments exposed by collagen type IV remodeling, and recent data indicate that these cryptic epitopes may facilitate tumor migration and angiogenesis (3, 5, 13). Cryptic epitope, HUIV26, within denatured type IV collagen, has been identified to exhibit angiogenic properties in both murine and chick embryo animal models.<sup>4</sup> Recently, a second novel cryptic epitope, HU177, has been described and also seems to play a functional role in tumor angiogenesis (14). HU177 was selectively exposed in the interstitial matrix of tumors as well as in the extracellular matrix of angiogenic blood vessels. Furthermore, a monoclonal antibody directed to the HU177 cryptic site inhibited endothelial cord formation *in vitro* and tumor angiogenesis *in vivo* (14). These data suggest that the selective targeting of cryptic collagen epitopes may represent an effective antiangiogenic treatment strategy.

In this study, we tested the hypothesis that melanoma can induce detectable changes in systemic levels of cryptic epitope shedding, specifically the HU177 epitope. We also correlated the levels of HU177 shedding with clinical and pathologic parameters. Our data identified nodular melanoma patients as a subset of patients who may be biologically distinct and may be more responsive to treatment with anti-extracellular matrix agents. Our study supports further investigation of HU177 epitope as a candidate for targeted intervention for treatment of nodular melanoma.

## Patients and Methods

**Study population.** The study cohort consisted of 291 melanoma patients prospectively enrolled in the Interdisciplinary Melanoma Cooperative Group at the New York University School of Medicine between August 2002 and November 2006 [119 females, 172 males; mean age, 59 y (range, 20-96 y)]. Clinicopathologic and demographic data were recorded prospectively for all patients. The New York University Institutional Review Board approved this study, and informed consent was obtained from all patients at the time of enrollment.

Sera samples from 106 control subjects, including patients with nonmelanoma cancer [bladder cancer ( $n = 56$ ), renal cancer ( $n = 10$ ), and testicular cancer ( $n = 10$ ), and normal volunteers ( $n = 30$ )], were included. The mean age was 64 y (range, 23-91 y). All control subjects signed informed consent before inclusion in this study.

**Serum preparation and determination of HU177 concentration by ELISA.** Blood was collected from primary melanoma patients after primary melanoma excisional surgery ( $n = 176$ ). In 33 patients the blood was collected before surgery. Patients presenting to New York University for treatment of metastatic melanoma ( $n = 82$ ) had blood drawn during their first consult at New York University. All serum samples were collected in 10-mL BD serum tubes, stored immediately at 4°C, and then centrifuged at 10°C for 10 min at 1,500 × *g*. The supernatant serum was then aliquoted into 1.5-mL cryovials and stored at -80°C until further use. All samples examined in the ELISA assay detailed below were subjected to only one freeze-thaw cycle.

HU177 cryptic epitope concentration (ng/mL) was quantified by a newly developed "capture" ELISA assay. A 96-well flat-bottomed microtiter plate was coated with monoclonal antibody HU177

(50 µg/mL) and incubated overnight at 4°C. After several PBS washes, plates were blocked with 2.5% bovine serum albumin at 37°C for 2 h. Standards were created using native collagen type IV that was denatured by boiling and then diluted in PBS. Patient samples or standards were added to each well (100 µL) in triplicate. After 2-h incubation, biotinylated anti-collagen IV antibody was added (1:20,000; Southern Biotech). Following 1.5-h incubation, anti-biotin monoclonal antibody conjugated to horseradish peroxidase was added (1:20,000; Sigma-Aldrich). The plate was then incubated with a substrate solution of 3,3',5,5'-tetramethylbenzidine for 5 to 10 min at room temperature. Substrate absorbance was determined at 400 nm (Bio-Rad microplate reader). Known concentrations, in duplicate, of denatured collagen were used to establish a standard curve ranging from 5 to 100 ng/mL, from which the concentration of cryptic epitope within patient samples was extrapolated (Fig. 1).

**Statistical analysis.** Descriptive statistics were calculated for baseline demographic and clinicopathologic characteristics. Associations between epitope HU177 shedding and age, gender, tumor thickness, ulceration, histologic subtype, recurrence, metastatic tumor type, and time of blood draw were evaluated with the *t* test (or Wilcoxon rank-sum test), the ANOVA test (or Kruskal-Wallis test), and the Spearman rank correlation coefficient, as appropriate. Multivariate ANOVA was done to assess the independent effect of histologic subtype on epitope HU177 shedding after adjustment for tumor thickness. For univariate and multivariate analyses, tumor thickness was evaluated both as a continuous variable and as a categorical variable ( $\leq 1$ , 1.01-3.99, or  $\geq 4$  mm in Breslow thickness). This classification was selected as it effectively stratifies patients into prognostic groups (15). For histologic subtype analyses, patients were grouped into three categories: those who were diagnosed with nodular melanoma, those with superficial spreading melanoma, and those with "other" subtypes. Likewise, age was analyzed both as a continuous variable and as a categorical variable ( $\geq 60$  versus  $< 60$  y). Mean epitope concentrations of patients categorized by each stage of melanoma were also calculated. All *P* values were two-sided with statistical significance evaluated at  $\alpha = 0.05$ . All analyses were done in SAS version 9.1 (SAS Institute, Inc.) and SPSS version 15.0 (SPSS, Inc.).

## Results

Table 1 illustrates the clinical and pathologic characteristics of the study population as well as the association with HU177

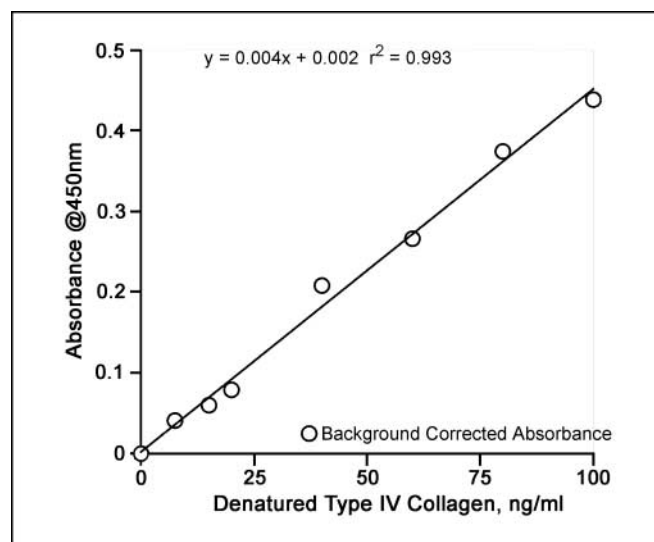


Fig. 1. Typical standard curve generated with denatured type IV collagen showing the linearity of the sandwich ELISA assay over the range of 5 to 100 ng/mL.

**Table 1.** Clinicopathologic characteristics of study population ( $n = 291$ ) and association with HU177 serum concentration

Variable	Patients, $n$ (%)	Mean HU177 conc. $\pm$ SE, ng/mL	Univariate analysis $P$
Sex			
Female	119 (41)	5.34 $\pm$ 0.65	0.47
Male	172 (59)	6.16 $\pm$ 0.93	
Age, y			
Mean $\pm$ SD	59 $\pm$ 16.3	$r = -0.05$	0.39
Primary tumor histologic type			
Superficial spreading melanoma	118 (59)	4.53 $\pm$ 0.45	0.01*
Nodular melanoma	51 (26)	10.27 $\pm$ 2.84	
Acral lentiginous	6 (3)	2.03 $\pm$ 0.78	
Desmoplastic melanoma	6 (3)	13.55 $\pm$ 7.07	
Lentigo maligna melanoma	6 (3)	2.41 $\pm$ 0.68	
Other	8 (4)	5.28 $\pm$ 2.48	
Unknown	4 (2)	8.52 $\pm$ 3.86	
Thickness (mm)			
1.00	113 (54)	3.81 $\pm$ 0.37	0.003
1.01-3.99	73 (35)	8.75 $\pm$ 2.04	
4.00	22 (11)	10.28 $\pm$ 2.23	
Primary tumor ulceration			
Present	35 (18)	8.06 $\pm$ 1.73	0.28
Absent	163 (82)	5.74 $\pm$ 0.93	
Stage at blood draw			
Stage I	140 (48)	5.84 $\pm$ 1.06	0.76
Stage II	40 (14)	7.01 $\pm$ 1.47	
Stage III	61 (21)	6.01 $\pm$ 0.93	
Stage IV	50 (17)	4.65 $\pm$ 1.05	
Primary tumor location			
Axial	113 (55)	5.67 $\pm$ 0.64	0.44
Extremity	94 (45)	6.99 $\pm$ 1.59	
Metastatic tumor type			
Regional lymph node	20 (25)	6.08 $\pm$ 1.62	0.19
Regional skin or subcutaneous	18 (22)	3.33 $\pm$ 0.73	
Distant lymph node	3 (4)	4.33 $\pm$ 4.23	
Visceral organ	19 (23)	7.60 $\pm$ 2.43	
Multiple sites	22 (27)	2.91	

\* $P = 0.01$ , nodular versus superficial spreading versus all others.

serum concentration. Serum HU177 epitope concentration was determined for 209 primary melanoma patients (140 stage I, 40 stage II, 29 initial stage III) and 82 patients with recurrent or metastatic disease (32 recurrent stage III and 50 stage IV). The mean HU177 epitope concentration of the study cohort was 5.8 ng/mL (range, 0-139.8 ng/mL) compared with 2.4 ng/mL in control subjects (range, 0-6.09 ng/mL,  $n = 106$ ). Mean HU177 epitope concentration for primary patients was 6.2 ng/mL (range, 0-139.8 ng/mL) compared with 4.9 ng/mL (range, 0-39.8 ng/mL) in metastatic patients ( $P = 0.09$ , Wilcoxon rank-sum test).

**HU177 epitope shedding correlates with nodular melanoma, primary tumor thickness, and time of blood draw.** Figure 2A displays the mean HU177 epitope concentration for each melanoma histologic subtype. Patients who presented with a primary nodular melanoma ( $n = 51$ ) showed a higher mean HU177 serum concentration compared with those with superficial spreading melanomas ( $n = 118$ ) and other subtypes ( $n = 30$ ; Table 1;  $10.3 \pm 1.69$  versus  $4.5 \pm 1.1$  versus  $6.1 \pm 2.1$  ng/mL (mean  $\pm$  SE), respectively;  $P = 0.01$ , ANOVA test).

Figure 2B shows the mean HU177 epitope concentration for each of the following three subgroups of tumor thickness:  $\leq 1.00$  mm ( $n = 113$ ), 1.01 to 3.99 mm ( $n = 73$ ), and  $\geq 4.00$  mm ( $n = 22$ ). The mean primary tumor thickness was 2.1 mm (range, 0.1-30 mm). Our results indicate that increasing tumor

thickness correlates with higher levels of HU177 epitope shedding (Table 1;  $P = 0.003$ , ANOVA test). Evaluation of HU177 levels with tumor thickness as a continuous variable further supported the association between HU177 epitope shedding and increasing tumor thickness ( $P = 0.005$ , Spearman rank correlation coefficient). Multivariate analysis confirmed the independent correlation between nodular subtype and HU177 epitope concentration after controlling for tumor thickness ( $P = 0.03$ ).

We also considered the possibility that the relationship between HU177 epitope shedding and nodular histologic subtype may be confounded by an association between the time of blood draw in primary patients and HU177 epitope shedding. However, even after controlling for time of blood draw and tumor thickness, multivariate analysis continued to validate the independent correlation between nodular histologic subtype and HU177 epitope concentration ( $P = 0.04$ , multivariate ANOVA). Multivariate analysis also confirmed the independent correlation between tumor thickness and HU177 epitope concentration after controlling for time of blood draw ( $P = 0.007$ , multivariate ANOVA).

**HU177 epitope shedding in patients with ulcerated melanomas and/or recurrences was elevated but not statistically significant.** Primary melanoma patients with ulcerated tumors

showed higher mean HU177 epitope shedding than those whose tumors were not ulcerated (Table 1; 8.06 versus 5.74 ng/mL;  $P = 0.12$ , Wilcoxon rank-sum test).

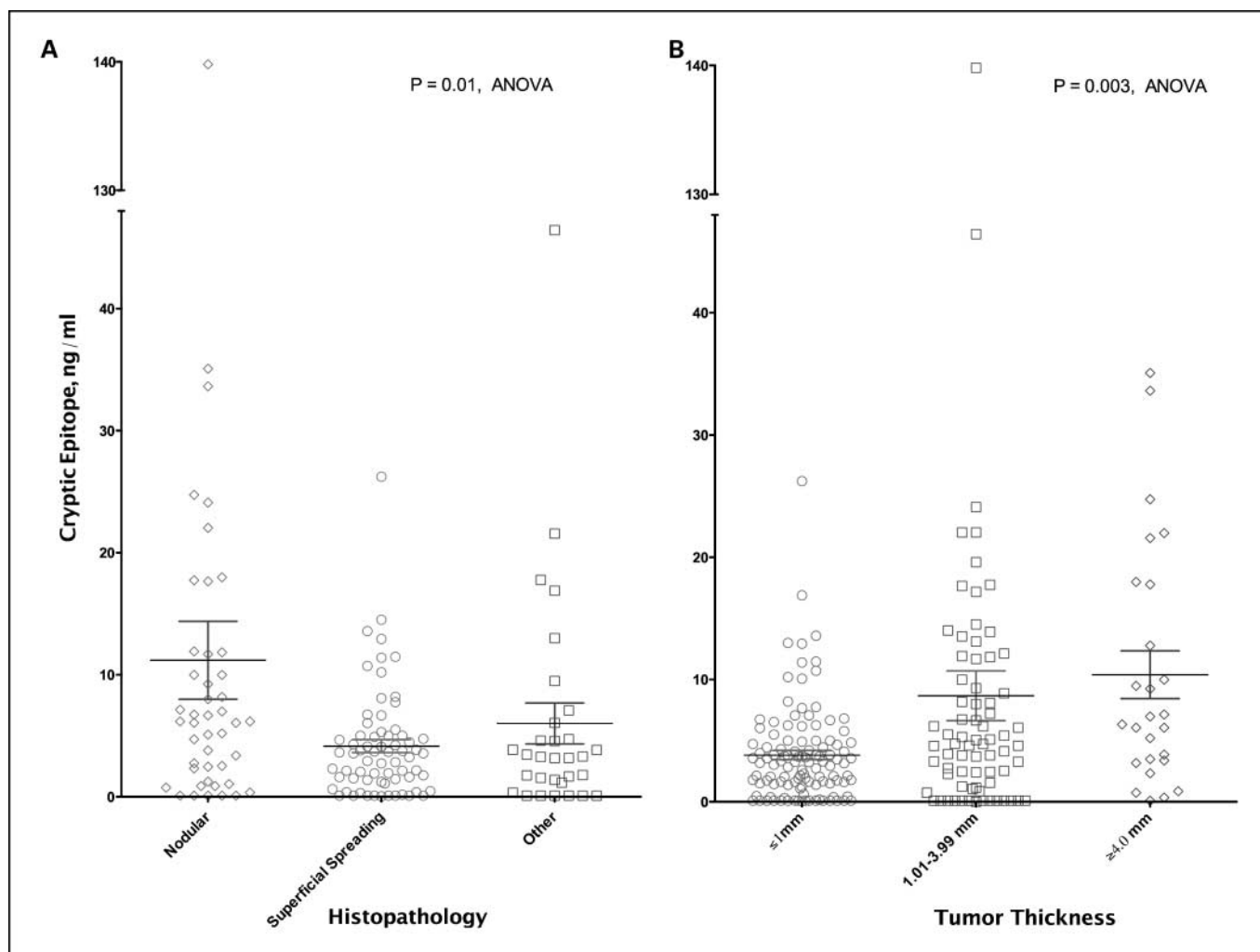
The correlation between HU177 epitope shedding and recurrent or metastatic disease was also evaluated. Twenty-three of 209 (11%) primary melanoma patients developed recurrences or metastases during a median follow-up time of 31.5 months (range, 10.3-45.2 months). These included 7 cases of lymph node metastases, 8 with skin or subcutaneous metastases, and 8 with visceral metastases. There was a trend toward increased HU177 epitope shedding in patients who developed recurrences when the median HU177 concentration was compared with patients without recurrences ( $P = 0.07$ , Wilcoxon rank-sum test). On other hand, mean HU177 epitope concentration did not correlate with age, gender, or stage.

### Discussion

Our study reveals several important observations. First, it shows for the first time the feasibility of detecting and

quantifying circulating levels of cryptic collagen IV epitope (HU177) in the sera of melanoma patients using a newly developed assay. We show that even single primary tumors can induce detectable changes in systemic levels of degraded collagen. Second, our data support a hypothesis that primary nodular melanoma has distinct genetic and/or phenotypic characteristics that can be exploited for targeted therapy. Lastly, the increased HU177 epitope concentration observed in primary, but not metastatic, melanomas supports a restricted, tissue-specific shedding of cryptic collagen epitopes.

Nodular melanoma is characterized by rapid vertical growth and clinical features that are unaccounted for in the ABCD acronym (16). As such, these patients pose a challenge, often presenting with high-risk, thick lesions at the time of diagnosis (17). In fact, reviews of four separate melanoma databases found that approximately two thirds of all thick melanomas ( $\geq 3$  mm) were of nodular histologic subtype (18-20). Furthermore, the median thickness of nodular melanomas has not changed over the last decade (16). In contrast, superficial spreading melanomas, the most common histologic



**Fig. 2.** HU177 correlation with histologic subtype and primary tumor thickness: scatter graphs of shed HU177 collagen epitope. *A*, HU177 and histologic subtype. Patients who presented with a primary nodular melanoma showed a higher mean HU177 serum concentration compared with those with superficial spreading melanomas and other histologic subtypes ( $P = 0.01$ , ANOVA). *B*, HU177 and primary tumor thickness. The mean tumor thickness for primary patients was 2.1 mm (range, 0.1-30 mm). Increasing tumor thickness correlated with higher levels of mean HU177 epitope shedding ( $P = 0.003$ , ANOVA test). Means are indicated for each subgroup; bars, SE.

subtype according to the Surveillance, Epidemiology, and End Results data, are typically diagnosed as thin lesions (median, 0.54 mm; ref. 16). The relative higher frequency of nodular melanoma among thick tumors is particularly alarming considering its poor prognosis for all stages of disease when compared with superficial spreading melanoma (21). The worse prognoses of nodular melanoma have also been shown in cutaneous head and neck melanomas, portending a significantly increased risk of death when compared with all other histologic subtypes (22).

Our data support that nodular melanoma might represent a distinct biological entity. Additional support is found in a recent microarray study of primary and metastatic melanoma specimens (23). These authors identified a characteristic gene expression profile unique to nodular melanomas. Specifically, nodular melanomas displayed up-regulation of genes implicated in tumor invasion and cell adhesion (i.e., matrix metalloproteinase 16, BCL2-related protein A1, intercellular adhesion molecule 1, and carcinoembryonic antigen-related cell adhesion molecule 1; ref. 23). The molecular signature of superficial spreading melanomas showed a differential profile, with up-regulation of genes involved in alternative signaling pathways, specifically cell cycle regulation and cell-cell communication (23). Studies examining alterations in DNA copy number or somatic mutations also support distinct genetic differences between melanoma subtypes. In one study, nodular melanomas were more likely to have loss of p16 expression compared with superficial spreading melanomas (24). There are also differences in *BRAF* mutation frequency among melanoma subtypes, with the largest studies having reported *BRAF* mutation frequencies in the range of 27% to 63% for nodular melanomas (25). This knowledge of the genetic alterations underlying distinct melanoma subtypes may provide opportunities to tailor treatment regimens based on genetic and/or phenotypic profiles. In a limited study subset, we examined serum metalloproteinase activity from patients with high and low HU177 serum collagen levels but did not find a correlation.

Our findings suggest that the HU177 epitope could serve as a novel target for drug development. Previously, studies have shown that treatment with a monoclonal antibody directed against HU177 leads to the significant inhibition of tumor growth and angiogenesis *in vivo* (14). Consequently, a phase I clinical trial was approved to evaluate the safety, tolerability, pharmacokinetics, and antitumor activity of D93 (TRC093), a humanized monoclonal antibody directed to the HU177 site. This study is currently being conducted at several medical centers in patients with advanced cancers (TRACON Pharma). Given the increased shedding of HU177 epitope in nodular melanoma patients and its potential role in angiogenesis, a rational therapeutic approach could be to specifically examine the response of nodular melanoma patients to inhibitors of angiogenesis, allowing for the selection of patients who are more likely to benefit from this particular modality of cancer therapy. In fact, both vascular endothelial growth factor and hypoxia-inducible factor 2 $\alpha$  have been shown to be indepen-

dent negative prognostic variables in nodular melanoma, further supporting the potential efficacy of antiangiogenic strategies on melanomas of this histologic subtype (26).

Given the dismal prognosis of advanced stage nodular melanoma and the lack of available adjuvant treatment modalities, the testing of angiogenesis inhibitors on an adjuvant basis in this population is worth further examination. An increasing number of these agents are currently under clinical investigation, with anti-vascular endothelial growth factor strategies as the most actively pursued. In particular, bevacizumab, a humanized anti-vascular endothelial growth factor monoclonal antibody approved for colorectal cancer in 2005, is now being tested in phase II trials of melanoma (27, 28). The clinical evaluation of antiangiogenic therapies in combination with standard treatments is also ongoing (29).

In this study, we found that primary melanomas, as a whole, had higher levels of HU177 shedding compared with metastatic cases, although this observation did not reach statistical significance. It is possible that exposure of the HU177 epitope is specific to the extracellular matrix of the skin, with minimal expression in extracutaneous sites. We evaluated the shedding of HU177 in patients with other tumor types including renal, bladder, and testicular cancers. In general, we found that the serum levels of HU177 in these patients were lower than that found in either primary or metastatic melanoma patients.

The prognostic relevance of HU177 epitope expression in melanoma requires further investigation. Despite the relatively short median follow-up time of patients enrolled in the New York University-Interdisciplinary Melanoma Cooperative Group (31.5 months), we observed a trend toward increased HU177 epitope shedding in patients who developed recurrences of their disease. Evidence supporting the predictive potential of HU177 epitope shedding has been shown in a phase II trial of sorafenib (BAY 43-9006) in malignant melanoma, where serial serum HU177 levels were shown to correlate with tumor response as monitored by positron emission tomography/computed tomography standardized uptake value changes (30). These findings are promising and support the further exploration of HU177 as a prognostic marker in melanoma.

In summary, we showed that circulating levels of cryptic collagen IV epitope HU177 can be detected in the sera of melanoma patients; primary nodular melanoma may be a candidate for targeted therapy design because it displays distinct genetic and/or phenotypic characteristics; and shedding of cryptic collagen epitopes seems to be restricted and tissue specific. In the context of prior basic and clinical work, this study supports further investigation of HU177 epitope as a candidate for targeted intervention for treatment of nodular melanoma.

### Disclosure of Potential Conflicts of Interest

P. Brooks holds a patent for monoclonal antibody HU177 and is a consultant for TRACON Pharmaceuticals.

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