

Differential Clinical Significance of Individual NKG2D Ligands in Melanoma: Soluble ULBP2 as an Indicator of Poor Prognosis Superior to S100B

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Abstract Purpose: Cytotoxic lymphocytes interact with human tumor cells via the activating immunoreceptor NKG2D, recognizing a variety of stress-associated MIC and ULBP surface molecules. However, tumors can escape from this immunosurveillance by shedding NKG2D ligands (NKG2DL), rendering the soluble products detectable in patients' sera.

Experimental Design: To elucidate the clinical significance of NKG2DL diversity, we studied their expression on melanoma tissues and their presence as soluble molecules in sera from >200 melanoma patients and compared the latter with the well-established serum marker S100B.

Results: Immunohistochemistry revealed a heterogeneous expression of MIC and ULBP2 molecules between and within melanoma metastases. Compared with MIC, ULBP2 was less frequently expressed. Accordingly, elevated levels of soluble ULBP2 (sULBP2) were detected in sera of melanoma patients less frequently than elevated levels of soluble MICA (sMICA), although both soluble NKG2DL (sNKG2DL) were significantly increased compared with sera of healthy controls ($P < 0.0001$). Strikingly, elevated concentrations of sULBP2, but not of sMICA, were strongly associated with disease progression ($P < 0.0001$) and tumor load ($P = 0.0003$). Elevated serum levels of either sNKG2DL correlated with reduced overall survival, albeit considerably stronger for sULBP2 ($P < 0.0001$) than for sMICA ($P = 0.011$). In early-stage (I-III) melanoma patients, only sULBP2 ($P < 0.0001$) but neither sMICA nor S100B revealed prognostic significance. Multivariate analysis identified sULBP2 ($P = 0.0015$) and S100B ($P = 0.013$) but not sMICA as independent predictors of prognosis.

Conclusion: Our data reveal marked differences in the clinical significance of individual sNKG2DL. Only sULBP2 is an independent predictor of prognosis, the significance of which is superior to the well-established and widely used melanoma serum marker S100B. (Clin Cancer Res 2009;15(16):5208–15)

Genetic and epigenetic alterations are hallmarks of cancer. These modifications, on the one hand, are a prerequisite for cancer development and progression but, on the other hand, expose tumor cells as altered self to the immune system. Genetic alterations can initiate an intrinsic DNA damage response that in turn induces the expression of surface ligands binding

to the activating immunoreceptor NKG2D (1). NKG2D is expressed on innate, innate-like, and adaptive immune effectors such as natural killer (NK) cells, $\gamma\delta$ cells, and $\alpha\beta$ T cells, respectively. Whereas NKG2D engagement directly activates NK cells, in T cells it delivers costimulatory signals modulating T-cell receptor-mediated activation (2–4). Studies in different

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Translational Relevance

NKG2D is an activating immunoreceptor of cytotoxic lymphocytes, the ligands of which, MIC and ULBP molecules, are expressed on tumor cells. Tumors can escape from NKG2D immunosurveillance by ligand shedding, rendering the soluble products detectable in patients' sera. We analyzed sera from >200 melanoma patients for the levels of soluble MICA and soluble ULBP2 (sULBP2) in correlation to the clinical course of disease and showed that elevated sULBP2, but not soluble MICA, is a strong indicator of poor prognosis. By comparison of sULBP2 with the widely used melanoma serum marker S100B, we confirmed sULBP2 as an independent prognostic factor, which is superior to S100B. This mainly results from the strong correlation of elevated sULBP2 serum levels with poor clinical outcome in early-stage patients. Thus, our study reveals marked differences in the clinical significance of individual NKG2D ligands and shows the clinical usefulness of sULBP2 as a prognostic indicator in early- and late-stage melanoma.

mouse models emphasized the importance of NKG2D for tumor immune surveillance (5–9). When grafted into mice, tumor cells modified to express NKG2D ligands (NKG2DL) were rejected in contrast to nonmodified tumor cells (7, 8). Recently, Guerra et al. showed that the incidence of spontaneous tumors in NKG2D-deficient mice was increased compared with wild-type mice, suggesting that NKG2D is involved in early immune surveillance of spontaneous malignancy (9). Accordingly, Unni et al. reported an early induction of NKG2DL surface expression during spontaneous tumorigenesis in mice (10).

Thus far, eight ligands of NKG2D have been identified in humans, which are members of either the MIC (MICA and MICB) or the ULBP (ULBP1, ULBP2, ULBP3, ULBP4, RAET1G, and RAET1L) family (11). The biological significance of ligand diversity still remains to be elucidated, but it has been shown that their expression is induced by different stress signals such as heat shock, infection, or DNA damage (1, 12–14).

NKG2DL have been detected on a variety of *in vitro* cultured human tumor cells, including melanoma, and their engagement induces cell lysis by NK cells, $\gamma\delta$ T cells, and $\alpha\beta$ T cells (2–4, 15). Interestingly, tumor cells can escape NKG2D immunosurveillance by an enhanced shedding of ligands from the cell surface (16–19). Tumor-associated metalloproteases have been shown to mediate ligand release (17, 19–21), resulting in soluble NKG2DL (sNKG2DL) detectable in sera of cancer patients (16, 17, 19, 22–28). Several studies suggested that soluble MICA (sMICA) in patients' sera interferes with antitumor immunity by down-regulating NKG2D receptor expression on blood lymphocytes, leading to an impaired cytotoxic effector function (16, 25–28). However, whether NKG2DL shedding correlates with disease prognosis and whether individual NKG2DL differ in this regard still requires elucidation.

Within this work, we analyzed the expression of the MIC and ULBP2 molecules in tumor tissues and studied the presence of

sMICA and soluble ULBP2 (sULBP2) in sera from melanoma patients to determine their clinical significance. Melanoma was chosen as a tumor of particular interest, because we and others already showed the relevance of NKG2DL for an efficient killing of melanoma cells by CTLs and NK cells (3, 4, 15, 29), the latter being of specific importance for the elimination of MHC class I–negative tumor variants, as they arise during melanoma progression (29–32). Our analysis focused on MIC and ULBP2 molecules, because the surface expression of ULBP1 and ULBP3 has been shown to be low or even absent on melanoma cells (3, 15, 29). In melanoma tissues, we found MIC and ULBP2 to be heterogeneously expressed. To address the prognostic effect of NKG2DL, we measured the concentrations of sMICA and sULBP2 in sera from melanoma patients of different disease stages in comparison with the currently most widely established serologic marker S100B, a calcium-binding protein shed by melanoma cells (33). The serum concentrations of all three markers were subsequently correlated with the clinical stage and course of disease as well as with the survival of the corresponding patients.

Materials and Methods

Patient material. Serum samples from melanoma patients were selected from a deep-frozen serum bank hosted by the Clinical Cooperation Unit Dermato-Oncology at Mannheim. All samples were obtained and processed following a standardized protocol. Briefly, venous blood was drawn into gel-coated serum tubes (Sarstedt), clotted at room temperature for 30 to 60 min, and thereafter centrifuged at $2,500 \times g$ for 10 min. Serum was harvested and immediately frozen at -20°C . Thereafter, all samples underwent one additional freeze-thaw-cycle before the final thawing for analysis. Selection criteria were histologically confirmed melanoma; complete documentation of medical history, primary tumor characteristics, course of the disease, and follow-up; and no systemic treatment for at least 6 weeks before blood withdrawal to minimize confounding serum factors. Clinical data including S100B serum values at the time of blood withdrawal for the present study were extracted from patients' files. Serum samples of age-matched control volunteers were kindly provided by the Institute of Transfusion Medicine and Immunology. All controls were healthy blood donors undergoing regular physical and laboratory examinations. Peripheral blood mononuclear cells were isolated from freshly obtained heparinized blood samples by Ficoll-Hypaque density gradient centrifugation and cryopreserved before use. Tumor tissue was obtained from surgically excised cryopreserved melanoma metastases. Collection of sera, cells, and tissues as well as documentation of clinical data were done after informed consent with institutional review board approval. Disease staging was done according to the systematics of the American Joint Committee on Cancer (34).

Immunohistochemistry. Serial tissue sections derived from frozen tumor material were fixed with acetone for HMB-45 and MICA/B expression analysis or with paraformaldehyde for ULBP2 detection. After washing and blocking, the sections were incubated with either the anti-HMB-45 mouse monoclonal antibody (mAb; DAKOCytomation), the anti-MICA/B mAb 6D4 (eBioscience), or the polyclonal goat antibody specific for ULBP2 (BAMOMAB). To detect anti-HMB-45 and anti-MICA/B mAb binding, sections were subsequently incubated with secondary biotinylated goat anti-mouse IgG (Jackson ImmunoResearch, Dianova) followed by the addition of streptavidin-horseradish peroxidase (Jackson ImmunoResearch) and AEC substrate (Sigma). Detection of anti-ULBP2 antibody binding was done with the EnVision+Dual-Line System Horseradish Peroxidase kit from DAKO using DAB substrate.

Serum analyses. Frozen sera from patients and normal donors were thawed, diluted 1:3 in PBS, and analyzed for sNKG2DL by ELISA. For measurement of sMICA, the anti-MICA mAb AMO1 and the anti-MICA/B

capture mAb BAMO3 were used, both obtained from BAMOMAB. Determination of sULBP2 was carried out with the anti-ULBP2 capture mAb BUMO1 (BAMOMAB) and the anti-ULBP2 detection mAb 1298 (R&D Systems). MICA- and ULBP2-specific ELISA were done as described previously (19, 23). Results are indicated as mean of triplicates.

The serum concentration of S100B was measured using a sandwich immunoluminometric assay (LIA-mat Sangtec 100; Sangtec Medical). Briefly, serum samples were thawed, diluted (1:2), and thereafter subjected to polystyrene tubes precoated with a mAb recognizing the β -subunit of the S100 protein. After washing, captured S100B was incubated with a second mAb conjugated to an isoluminol derivative and thereafter detected by a light reaction quantified using a luminometer (Berthold ACL). The lowest measurable S100B concentration was determined as 0.02 $\mu\text{g/L}$; the intra- and inter-assay variations were <10%.

Flow cytometry. Cryopreserved peripheral blood mononuclear cells from melanoma patients and healthy donors were thawed and immediately used to determine the *ex vivo* NKG2D expression level on NK cells. Therefore, cells were stained with a combination of the following mouse mAbs: FITC-conjugated anti-CD3 mAb (Becton Dickinson), PE-labeled anti-CD56 mAb (Becton Dickinson), and APC-conjugated anti-NKG2D mAb (Miltenyi Biotec). Control staining was done with a combination of fluorescence-labeled isotype antibodies. Cells were incubated for 30 min on ice in the presence of the antibodies and, after washing, fixed in 4% formaldehyde. Cells were acquired by a FACSCanto II flow cytometer (Becton Dickinson) and data were analyzed with the FlowJo software.

Statistics. Statistical analyses of serum sNKG2DL concentrations were done using the Mann-Whitney test (patients versus controls; males versus females; tumor-bearing versus tumor-free) and the Kruskal-Wallis test (differences between disease stages). Overall survival of the patients was calculated from the date of blood withdrawal for serum sampling to melanoma-related death. Survival curves were estimated by the Kaplan-Meier method for censored failure time data. The log-rank test was used to compare survival probabilities between groups. The optimal cutoff points for sMICA and sULBP2 concentrations with regard to differentiation between prolonged and reduced overall survival were calculated using the method of exact distribution of maximally selected ranks (35). The proportional hazards model of Cox was used to identify independent predictors of survival in adjustment with relevant clinical covariates. All statistical tests were two-sided, and $P < 0.05$ was considered statistically significant. Statistical analyses were done using the statistical software package R.⁷

Results

Heterogeneous expression of MICA/B and ULBP2 in melanoma metastases. Our previous studies showed that melanoma cell lines established from tumor metastasis of different patients frequently coexpress MICA and ULBP2, whereas surface expression of ULBP1 and ULBP3 was low or even absent (29). Therefore, our immunohistochemical analyses on NKG2DL *in situ* expression by tissue samples from metastatic lesions ($n = 16$) focused on MIC and ULBP2 molecules (Fig. 1). In contrast to the melanoma cell lines, a very heterogeneous expression of MIC and ULBP2 between and even within individual tumor lesions was observed. Six of 16 tumors were positive for both ligands, 6 lesions expressed only MIC, and 2 samples were only positive for ULBP2 (Fig. 1A; Table 1). Moreover, none of the NKG2DL-positive tumors were characterized by a homogenous marker expression pattern; indeed, in some lesions, a minor fraction of the malignant cells expressed at least one of these ligands. Compared with ULBP2 (8 positive tumors), expression of MIC (12 positive tumors) was observed

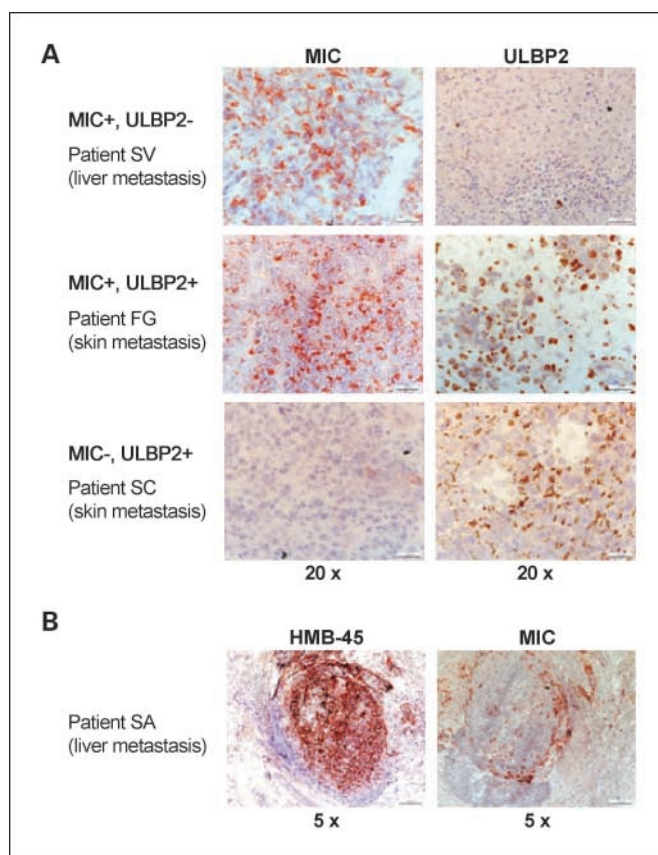


Fig. 1. NKG2DL expression in melanoma metastasis. Analysis of MIC and ULBP2 expression in serial sections of cryopreserved tissue samples from metastatic tumor lesions of different melanoma patients by immunohistochemistry. A, tumor cells in liver metastasis from patient SV express MIC only (MIC⁺, ULBP2⁻), malignant cells in skin metastasis of patient FG are positive for both ligands (MIC⁺, ULBP2⁺), and patient SC melanoma cells express ULBP2 only (MIC⁻, ULBP2⁺). B, MIC expressed in the periphery of a metastatic nodule. Tumor cells are marked by HMB-45 expression. Numbers indicate x-fold magnification.

at a higher frequency, indicating that both markers are not necessarily associated with each other in terms of expression strength and pattern. Interestingly, expression of NKG2DL seemed to be influenced also by the microenvironment; as in some metastases, a preferential staining at the invasive front was found (Fig. 1B).

sNKG2DL serum concentrations are elevated in melanoma patients. Based on the *in situ* expression of MIC and ULBP2 in melanoma, we next tested if these NKG2DL were detectable also as soluble molecules in sera of patients. For this purpose, we analyzed sera from 208 melanoma patients obtained at different stages of disease as well as 50 age-matched healthy controls for sMICA and sULBP2 levels by ELISA. In parallel, the serum concentration of the best-established and currently most widely used serologic marker S100B was measured. Patients had a mean age of 57.1 years; the median follow-up time was 38.3 months. Detailed patient characteristics are presented in Table 2. The mean serum concentrations of sMICA (257.4 pg/mL) and sULBP2 (45.6 pg/mL) in patients were significantly elevated compared with sMICA (90.3 pg/mL) and sULBP2 (2.6 pg/mL) in healthy controls (both $P < 0.0001$; Fig. 2; Table 2). Interestingly, sULBP2 was detectable less frequently in tumor

⁷ <http://www.r-project.org>

Table 1. *In situ* expression profile of MIC and ULBP2 in melanoma metastases

Melanoma metastases	NKG2DL expression profile			
	MIC ⁺ , ULBP2 ⁺	MIC ⁺ , ULBP2 ⁻	MIC ⁻ , ULBP2 ⁺	MIC ⁻ , ULBP2 ⁻
Brain (n = 1)	-	1	-	-
Liver (n = 4)	1	3	-	-
Lung (n = 4)	3	1	-	-
Skin (n = 7)	2	1	2	2
Total (n = 16)	6	6	2	2

patients than sMICA, corresponding to the *in situ* expression pattern of both NKG2DL. There was no significant correlation between gender or age of the patients and serum values of sMICA or sULBP2.

Serum levels of individual NKG2DL are differentially correlated with disease stage. Melanoma patients were grouped according to clinical stage: stage I/II primary tumors only, stage III regional metastases, and stage IV distant metastases. As depicted in Fig. 2 and Table 2, serum values of sULBP2 showed a strong, continuous, and highly significant increase with progressing disease stages ($P < 0.0001$; Fig. 2B); in contrast, for sMICA, there was no correlation with disease stage ($P = 0.36$; Fig. 2A), with only slightly increased serum values in stage IV patients. As expected, S100B serum concentrations were significantly correlated with stage of disease ($P < 0.0001$; Fig. 2C); however, the major increase was restricted to stage IV patients. Patients were further categorized according to tumor burden at the time when sera were obtained. Patients with a measurable tumor had significantly higher serum concentrations of sULBP2 ($P = 0.0003$; Fig. 2B) and S100B ($P < 0.0001$; Fig. 2C) than patients with clinically nonapparent tumor manifestations. Again, sMICA serum levels did not correlate with the patients' tumor load ($P = 0.75$; Fig. 2A).

Elevated serum levels of sNKG2DL are associated with poor prognosis. To analyze the prognostic effect of sNKG2DL serum concentrations compared with the established serologic marker S100B, the patients were grouped according to their serum levels of sMICA, sULBP2, and S100B, respectively. For this purpose, the cutoff value was determined as 400 pg/mL for sMICA and 50 pg/mL for sULBP2; for S100B, the cutoff value was 0.15 $\mu\text{g/L}$ as recommended by the manufacturer. Using the Kaplan-Meier method combined with the log-rank test, we observed a strong association of elevated serum levels of sULBP2 and S100B with a reduced overall survival ($P < 0.0001$; Fig. 3A). A similar but weaker association could be observed for sMICA ($P = 0.011$; Fig. 3A). Analyses of a subgroup of 79 patients with early stages of melanoma (stages I-III) revealed a strong association of elevated sULBP2 serum concentrations with a poor prognosis ($P < 0.0001$), whereas no significant correlation was observed for sMICA ($P = 0.55$) and S100B ($P = 0.07$; Fig. 3B). With regard to the subgroup of 129 patients with advanced metastatic disease, we found a strong correlation of sULBP2 and S100B (both $P < 0.0001$) and a weaker correlation of sMICA ($P = 0.032$) serum levels with overall survival (Fig. 3C).

sULBP2, but not sMICA, is an independent predictor of survival. A multivariate data analysis was done using the proportional hazards model of Cox including the well-known prognostic markers gender, age, histopathologic staging of the primary tumor (pT), tumor load, and serum S100B as well as our new markers of interest, serum sMICA and sULBP2, using the previously established cutoff values. This analysis revealed three markers as independent predictors of prognosis with the following ranking: whereas sMICA did not turn out as an independent prognostic factor ($P = 0.27$), sULBP2 is a strong independent predictor of prognosis ($P = 0.0015$) ranking after tumor load ($P < 0.0001$) but before S100B ($P = 0.013$).

Unaltered NKG2D surface expression on peripheral NK cells of melanoma patients with high sNKG2DL serum levels. Previous studies showed that elevated levels of sMICA in sera of tumor

Table 2. sNKG2DL and S100B in sera from melanoma patients

	n	sMICA (pg/mL), mean (25%/75%)	sULBP2 (pg/mL), mean (25%/75%)	S100B ($\mu\text{g/L}$), mean (SE)
Controls	50	90.3 (6.0/109.5)	2.6 (0.0/0.0)	NA
Gender				
Male	34	79.4 (9.0/109.5)	1.9 (0.0/0.0)	NA
Female	16	113.6 (2.0/97.5)	4.2 (0.0/0.0)	NA
Patients	208	257.4 (185.0/344.0)*	45.6 (0.0/53.5) [†]	0.71 (2.08)
Gender				
Male	106	230.6 (172.0/347.5)	45.0 (0.0/52.3)	0.75 (0.24)
Female	102	285.3 (190.0/340.8)	46.2 (3.0/53.8)	0.68 (0.17)
Stage (American Joint Committee on Cancer)				
I/II	25	223.4 (114.0/325.0)	0.0 (0.0/0.0)*	0.07 (0.01)*
III	54	190.9 (111.0/279.5)	29.4 (0.0/42.8)*	0.09 (0.02)*
IV	129	291.8 (215.0/404.0)	61.2 (13.0/69.0)*	1.13 (0.24)*
Tumor load				
Tumor-bearing	117	253.0 (195.0/350.0)	63.4 (13.0/70.0)*	1.20 (0.28)*
Tumor-free	91	263.1 (153.0/327.0)	22.7 (0.0/24.5)*	0.24 (0.09)*

NOTE: Data for sNKG2DL are represented as mean (25% quartile; 75% quartile); data for S100B are mean (SE). Statistical analyses were done using the Mann-Whitney test (patients versus controls; males versus females; tumor-bearing versus tumor-free) and the Kruskal-Wallis test (differences between disease stages).

Abbreviation: NA, not analyzed.

* $P < 0.0005$.

[†] $P < 0.005$.

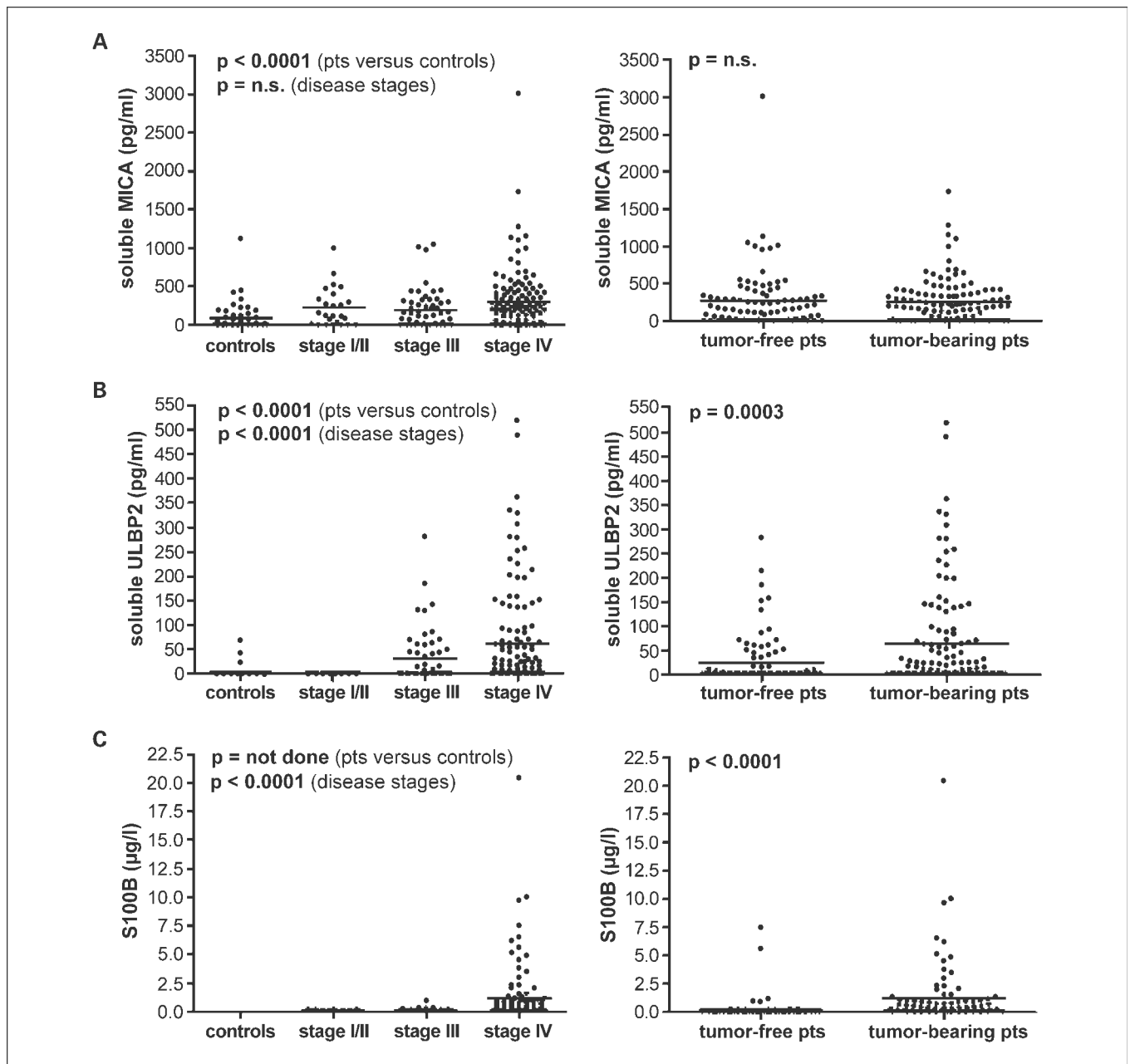


Fig. 2. Detection of sMICA and s100B in sera from melanoma patients. Sera from 208 melanoma patients and 50 healthy controls were analyzed for the presence of sMICA (A) and sULBP2 (B) by ELISA. Sera from melanoma patients were additionally examined for s100B (C). Patients are grouped by disease stage (left) and tumor load (right), respectively. Patient numbers of each group are given in Table 1. Dots, values for individual sera; lines, mean values.

patients were associated with a down-regulation of NKG2D receptor expression on peripheral blood NK cells. Thus, we selected cryopreserved peripheral blood mononuclear cells from seven patients with elevated sMICA and/or sULBP2 serum levels and determined the *ex vivo* expression of NKG2D on CD3⁺CD56⁺ NK cells. For control, NK cells from cryopreserved peripheral blood mononuclear cells of five age-matched healthy donors were analyzed. As depicted in Fig. 4A, NK cells from patients with relatively high (patient RR) and low (patient EM) sMICA serum levels, respectively, exhibited comparable NKG2D surface expression. Only marginal differences in NKG2D expression levels were observed among these

patients. Notably, similar variations in NKG2D expression were measured for NK cells obtained from healthy donors (Fig. 4B). Thus, in our patient cohort, the elevated levels of sNKG2DL in sera were not associated with a significant down-regulation of NKG2D expression on peripheral NK cells.

Discussion

Our study, for the first time, shows that individual NKG2DL are of differential clinical significance in melanoma. Comprehensive analysis of >200 sera from melanoma patients for sNKG2DL revealed that elevated sULBP2, in contrast to sMICA,

is a strong indicator of poor prognosis. Moreover, comparison of sULBP2 as a prognostic marker with the currently most widely used serum marker S100B confirmed sULBP2 as an independent prognostic factor, which is actually superior to S100B. Notably, the superiority of sULBP2 mainly results from the strong correlation of elevated sULBP2 serum levels with poor clinical outcome in early-stage patients (stage I-III); in fact, S100B had no significant association with prognosis in this patient group. In patients with advanced metastatic disease (stage IV), however, sULBP2 and S100B turned out as equally strong prognostic indicators. This finding is of major importance, because numerous serologic molecules have been described as prognostic biomarkers of melanoma, but only very few have been shown to be superior to S100B and LDH (33, 36). The latter was not tested in our study, because LDH is known to be elevated in stage IV patients only and therefore is not a useful marker for early stage (stage I-III) patients like those tested in the present study (37).

We reported previously that *in vitro* cultured melanoma cells frequently coexpress MICA and ULBP2. This observation prompted us to establish the *in situ* expression pattern of both

ligands. Immunohistochemistry analysis on cryopreserved tissue samples from melanoma metastases revealed a heterogeneous expression of MIC and ULBP2 between and also within tumors. In some lesions, only a few malignant cells expressed these molecules. Our observation on ligand heterogeneity is in line with studies by Vetter et al., comparing expression of MIC in primary and metastatic melanoma *in situ*. They found MIC to be expressed less frequently in metastases (65%) compared with primary lesions (78%) of cutaneous melanoma; this difference was even more pronounced in cases of uveal melanoma, with MIC being detectable in 50% of primary tumors but not in any of the metastases (38, 39). We extended these findings by showing that besides MIC melanomas also express ULBP2 molecules *in situ* and that both ligands are not necessarily coexpressed. Notably in some melanoma lesions, NKG2DL-positive cells were accentuated in the periphery of the tumor, suggesting that the microenvironment influences ligand expression. It is tempting to speculate that tumor cell-intrinsic genetic and epigenetic alterations, accumulating during disease progression as well as cell-cell and cell-matrix contacts, account for NKG2DL heterogeneity in addition to soluble factors. Indeed,

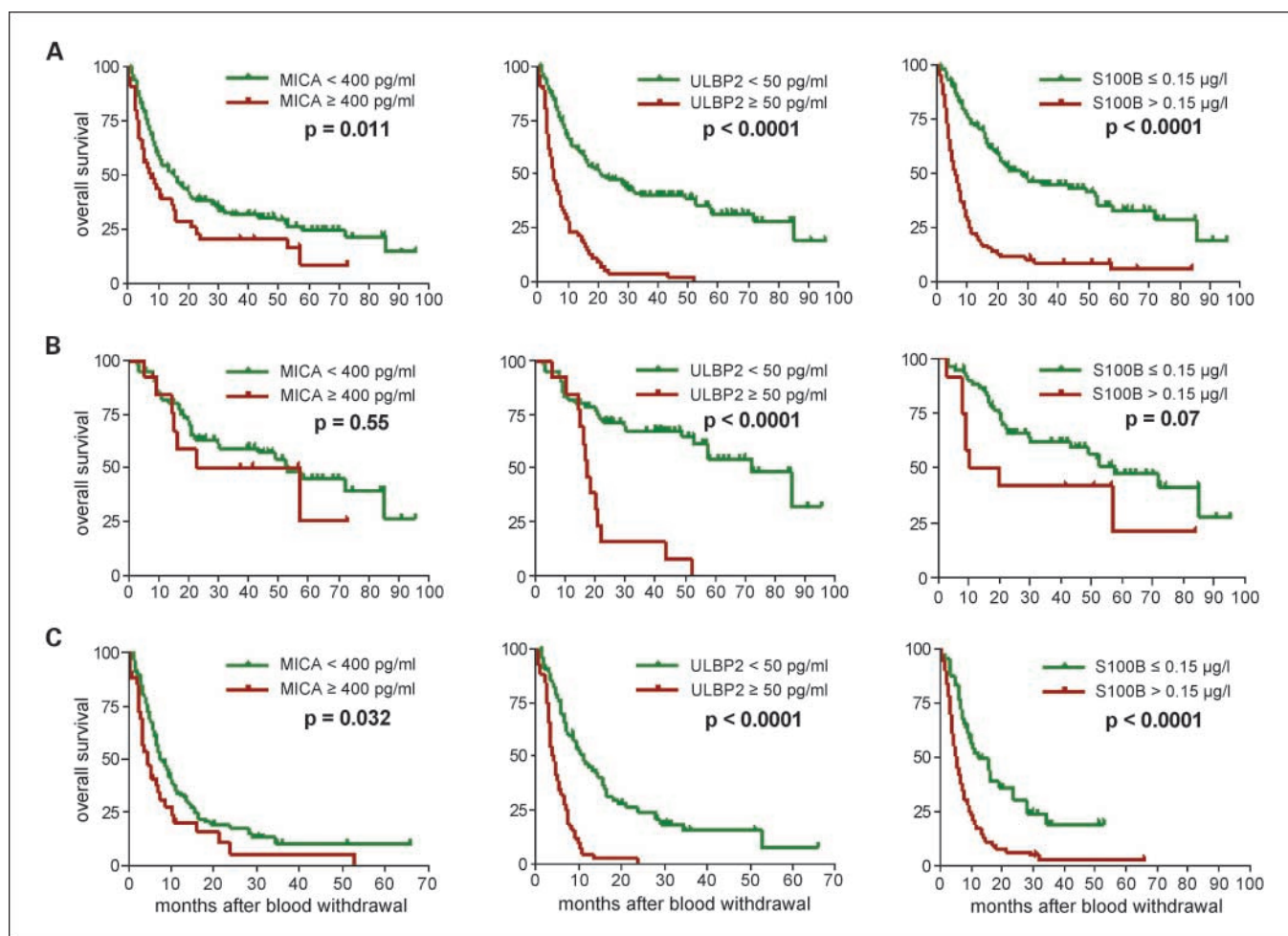


Fig. 3. sNKG2DL and S100B are associated with poor prognosis. Kaplan-Meier curves represent the overall survival of (A) 208 stage I to IV, (B) 79 stage I to III, and (C) 129 stage IV melanoma patients, calculated from the time of blood withdrawal for serum sampling until melanoma-related death. Patients are grouped by their serum values of sNKG2DL and S100B, respectively. Short vertical lines, censored observations. Statistical differences were calculated by log-rank test.

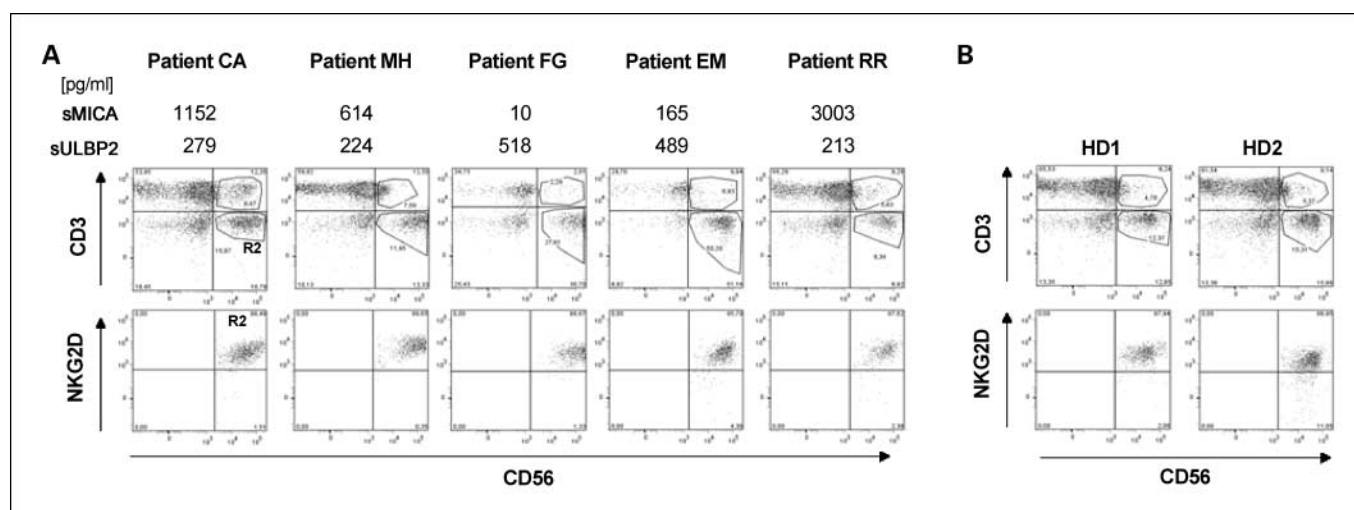


Fig. 4. NKG2D receptor expression on peripheral blood NK cells. Intensity of NKG2D surface expression on NK cells was determined by flow cytometry. Peripheral blood NK cells from melanoma patients show *ex vivo* NKG2D expression levels similar to age-matched healthy donor NK cells. **A**, concentrations of sMICA and sULBP2 in sera of five melanoma patients are depicted. Peripheral blood NK cells from these patients were analyzed for NKG2D surface expression. *Bottom*, expression levels of NKG2D on gated CD3⁺CD56⁺ NK cells; *top*, corresponding R2 gate. Representative staining from 5 of 7 patients. **B**, peripheral blood NK cells from healthy donors (HD) were analyzed for NKG2D expression as described above. Representative staining from 2 of 5 donors.

the immunosuppressive cytokine transforming growth factor- β , secreted by tumor cells and various regulatory immune cells, has been shown to decrease NKG2DL expression (40). Furthermore, we and others reported a negative effect of IFN- γ on the surface expression of MICA and ULBP2 (29, 41, 42).

Besides, surface expression of MIC and ULBP2 molecules can also be diminished by an enhanced proteolytic shedding, which renders sNKG2DL detectable in serum of patients with different cancers (16, 17, 21–27, 43). It was shown that sMICA levels increase with tumor progression for prostate cancer, hepatocellular carcinoma, and others (23, 25, 26, 28). In case of multiple myeloma, this increase was reported to be associated with poor prognosis (44). However, for some tumors, such as lung cancer, high sMICA levels are detectable already in early stages of the disease with no increase during progression (23).

Thus far, one study reported elevated concentrations of sULBP2 in leukemia patients (4 of 23), whereas sULBP2 was not detectable in sera of 19 patients with gastrointestinal tumors (19). Our comparative analysis showed that both sMICA and sULBP2 are present in sera from melanoma patients. Interestingly, sULBP2 is of strong clinical significance for melanoma patients' prognosis, whereas this is not the case for sMICA. In accordance with our data, Li et al. recently reported a correlation of ULBP2 expression in tissue samples from ovarian cancer patients with poor prognosis ($P < 0.05$); in contrast, MIC expression was not correlated with prognosis (45).

The mechanisms underlying the strong association of sULBP2 with poor prognosis remain elusive. Previous studies suggested that elevated levels of sMICA in sera from cancer patients cause a down-regulation of NKG2D on peripheral blood NK cells and T lymphocytes (16, 25–28), thereby promoting tumor immune escape. However, by comparing NKG2D expression on NK cells obtained from peripheral blood of stage IV melanoma patients versus age-matched healthy donors, we did not observe significant differences. Even NK cells from patients with relatively high sMICA levels (up to 3 ng/mL) did not show diminished receptor expression. Thus, it is tempting to speculate

that additional soluble factors might interfere with NKG2D down-regulation. For example, Groh et al. detected high concentrations of sMICA (up to 30 ng/mL) in sera from patients with rheumatoid arthritis, the peripheral blood T cells of which did not exhibit a down-regulation of NKG2D. Signals elicited by interleukin-15 and tumor necrosis factor- α , cytokines that are abundant in sera of rheumatoid arthritis patients, prevented NKG2D down-regulation by sMICA (46). Other studies showed that a strong reduction in NKG2D expression was dependent on sustained receptor triggering by cell-bound ligands (47, 48).

In conclusion, we observed a heterogeneous *in situ* expression of MIC and ULBP2 molecules in malignant melanoma and detected elevated levels of sMICA and sULBP2 in patients' sera. Most importantly, these sNKG2DL differentially relate to the prognosis of melanoma patients with sULBP2 as an independent prognostic marker, which is superior to the established serologic marker S100B. Notably, sULBP2 is a strong prognostic marker even in early disease stages (stage I–III), a subgroup in which S100B is only of weak prognostic value (33). Our results also suggest that a potential negative effect of ULBP2 expression and shedding by tumor cells on patients' prognosis may not be attributed to a systemic NKG2D down-regulation. This raises questions regarding the expression of the various NKG2DL by tumor cells. Although signals and pathways controlling expression of the diverse NKG2DL are far from being elucidated, one could speculate that, for example, ULBP2 might be under control of signals associated with a highly malignant tumor phenotype *in vivo*. Indeed, signaling induced by the BCR/ABL oncogene has already been shown to influence NKG2DL expression in chronic myelogenous leukemia (49). Better understanding of NKG2DL regulation in malignancies is important for therapeutic intervention and thus should be addressed by future investigations.

Disclosure of Potential Conflicts of Interest

A. Steinle has an ownership interest in BAMOMAB.

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