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

Annette Paschen,1 Antje Sucker,1 Bettina Hill,1 Iris Moll,1 Marc Zapatka,3 Xuan Duc Nguyen,2 Geok Choo Sim,1 Isabelle Gutmann,1 Jessica Hassel,1 Jürgen C. Becker,4 Alexander Steinle,5 Dirk Schadendorf,1,6 and Selma Ugurel4

**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)
Translatable Relevance

NKG2D is an activating immunoreceptor of cyto-
toxic lymphocytes, the ligands of which, MIC and
ULBP molecules, are expressed on tumor cells. Tu-
mors 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 el-
levated 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 inde-
pendent prognostic factor, which is superior to
S100B. This mainly results from the strong correla-
tion of elevated sULBP2 serum levels with poor
clinical outcome in early-stage patients. Thus, our
study reveals marked differences in the clinical sig-
ificance 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, tu-
mor 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 spontane-
ous 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 tumorogene-
sis 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 di-
versity 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 melanomas, and their engage-
ment induces cell lysis by NK cells, γδ T cells, and αβ T cells
(2–4, 15). Interestingly, tumor cells can escape NKG2D immu-
osurveillance 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 pa-
patients (16, 17, 19, 22–28). Several studies suggested that solu-
able 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 NKG2D shedding
 correlates with disease prognosis and whether individual
NKG2D 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 NKG2D 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 me-
lanoma 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 prog-
nostic 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 Coopera-
tion 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 tem-
perature for 30 to 60 min, and thereafter centrifuged at 2,500 × g for
10 min. Serum was harvested and immediately frozen at −20°C. There-
after, all samples underwent one additional freeze-thaw cycle before the
final thawing for analysis. Selection criteria were histologically con-
firmed melanoma; complete documentation of medical history, prima-
tary tumor characteristics, course of the disease, and follow-up; and no
systemic treatment for at least 6 weeks before blood withdrawal to min-
imize confounding serum factors. Clinical data including S100B serum
values at the time of blood withdrawal for the present study were ex-
tracted 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 mono-
nuclear cells were isolated from freshly obtained heparinized blood
samples by Ficoll-Hypaque density gradient centrifugation and cryopre-
served before use. Tumor tissue was obtained from surgically excised
cryo-preserved melanoma metastases. Collection of sera, cells, and tis-
sues 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 ex-
pression 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 bio-
tinylated goat anti-mouse IgG (Jackson Immunoresearch, Dianova) fol-
lowed 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 Horse-
adish 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 mea-
urement of sMICA, the anti-MICA mAb AMO1 and the anti-MICA/B
suluble NKG2D Ligands in Sera of Melanoma Patients
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 MICA and ULBP2 molecules (Fig. 1). In contrast to the melanoma cell lines, a very heterogeneous expression of MICA and ULBP2 between and even within individual tumor lesions was observed. Six of 16 tumors were positive for both ligands, 6 lesions expressed only MICA, 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 MICA (12 positive tumors) was observed 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).

### 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 MICA and ULBP2 molecules (Fig. 1). In contrast to the melanoma cell lines, a very heterogeneous expression of MICA and ULBP2 between and even within individual tumor lesions was observed. Six of 16 tumors were positive for both ligands, 6 lesions expressed only MICA, 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 MICA (12 positive tumors) was observed 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 MICA 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
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 sNKG2D 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 μg/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 = 0.53) 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 sNKG2D serum levels.** Previous studies showed that elevated levels of sMICA in sera of tumor

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

<table>
<thead>
<tr>
<th>Melanoma metastases</th>
<th>MIC&lt;sup&gt;+&lt;/sup&gt;, ULBP2&lt;sup&gt;+&lt;/sup&gt;</th>
<th>MIC&lt;sup&gt;-&lt;/sup&gt;, ULBP2&lt;sup&gt;+&lt;/sup&gt;</th>
<th>MIC&lt;sup&gt;+&lt;/sup&gt;, ULBP2&lt;sup&gt;-&lt;/sup&gt;</th>
<th>MIC&lt;sup&gt;-&lt;/sup&gt;, ULBP2&lt;sup&gt;-&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain (n = 1)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Liver (n = 4)</td>
<td>1</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lung (n = 4)</td>
<td>3</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Skin (n = 7)</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>Total (n = 16)</td>
<td>6</td>
<td>6</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

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**Table 2. sNKG2DL and S100B in sera from melanoma patients**

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

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.
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 MICA 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 MICA in primary and metastatic melanoma in situ. They found MICA 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 MICA being detectable in 50% of primary tumors but not in any of the metastases (38, 39). We extended these findings by showing that besides MICA 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,
Peripheral blood NK cells from healthy donors (up to 3 ng/mL) did not show significant differences. Even NK cells from patients with melanoma 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.

Besides, surface expression of MIC and ULBP 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, 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.


Clinical Cancer Research

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