Soluble NKG2D Ligands in Hepatic Autoimmune Diseases and in Benign Diseases Involved in Marker Metabolism

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Abstract. *Background: Proteolytic shedding of the immunostimulatory NKG2D ligands MICA and MICB from cancer cells constitutes a novel immune escape strategy that diminishes antitumor reactivity by NKG2D-bearing cytotoxic lymphocytes. In consequence, serum levels of soluble MICA and MICB are frequently found to be elevated in cancer disease. Patients and Methods: As the diagnostic potential depends strongly on the organ-specific benign diseases and is affected by diseases involved in marker metabolism, both markers were analyzed by ELISA in sera of 141 patients with hepatic autoimmune diseases (34 autoimmune hepatitis, 35 primary sclerosing cholangitis, 72 primary biliary cirrhosis), 18 patients with acute bacterial infections, 21 patients with renal insufficiency, 13 patients with cholestasis and 62 healthy individuals. Results: Similarly to healthy controls (median sMICA <30 pg/mL; sMICB <30 pg/mL), low levels of both markers were generally found in sera of patients with hepatic autoimmune diseases. In contrast, significantly elevated concentrations of sMICA and sMICB were observed in sera of patients with acute infections (median sMICA 890 pg/mL; sMICB 111 pg/mL), in those with renal insufficiency (sMICA 195 pg/mL; sMICB 50 pg/mL), and in those with cholestasis (sMICA 1058 pg/mL; sMICB 146 pg/mL). Conclusion: While hepatic autoimmune diseases have no general impact on the amount of circulating sMICA and sMICB, acute bacterial infections, renal insufficiency and cholestasis can lead to notably elevated serum levels of the NKG2D ligands.*

The immunoreceptor NKG2D provides a costimulatory signal to CD8 T-cells and potently activates NK cells, even overcoming inhibitory signals by MHC class I molecules. Among the NKG2D ligands (NKG2DL) are the surface glycoproteins MICA and MICB which are broadly expressed by epithelial and haematopoietic tumors (1-4). In mice, NKG2DL expression by tumor cells strongly stimulates antitumor immunity leading to tumor rejection by NK cells and/or CD8 T-cells (3, 5). Because the strength of the antitumor response is critically dependent on NKG2DL surface levels (3), down-regulation of NKG2DL expression constitutes a novel tumor immune escape mechanism (2-7). We recently demonstrated that human tumor cells reduce NKG2DL surface levels by proteolytic shedding of MICA from the cell surface (7). Furthermore, soluble MICA (sMICA) was also reported to cause systemic down-regulation of NKG2D surface expression thereby impairing lysis of tumor cells by human CD8 T-cells (8, 9). As a consequence of NKG2D ligand-shedding by tumors, sMICA and sMICB are frequently found to be elevated in sera of patients with various malignancies (7, 9-13). In particular, they might play a role in late stages of tumor progression by overcoming the confining effect of NK cells and CD8 T-cells (11, 12). As the diagnostic potential of oncological biomarkers depends strongly on the extent of organ-specific benign diseases and is affected by diseases involved in marker metabolism, the levels of sMICA and sMICB were analyzed in sera of patients with hepatic autoimmune diseases, acute bacterial infections, renal insufficiency and cholestasis to test their influence on marker results.

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Patients and Methods

Patients. In total, levels of sMICA and sMICB were determined in sera of 141 patients with hepatic autoimmune diseases (34 autoimmune hepatitis, 35 primary sclerosing cholangitis, 72 primary biliary cirrhosis), 18 patients with acute bacterial infections and C-reactive protein levels >1.5 mg/dL, 21 patients with renal insufficiency and creatinin levels >2.5 mg/dL, 13 patients with cholestasis and bilirubin levels >2.5 mg/dL, and 62 healthy controls.

MICA-ELISA. SMICA and sMICB concentrations were determined by sandwich ELISA methods described elsewhere (7, 10). The monoclonal antibodies (mAb) and standards for both assays were generated by us as described in (10).

Plates were coated with the capture anti-MICA mAb AMO1 at 5 μg/mL in phosphate buffered saline (PBS), then blocked by the addition of 15% bovine serum albumin (BSA), washed and incubated with the standard (recombinant sMICA*04 produced in *E. coli*) or patient sera. Patient sera were diluted 1:3 in 7.5% BSA. Next, plates were washed, incubated with the detection mAb BAMO3 at 1 μg/mL, washed again, and then incubated with anti-mouse IgG2a-HRP (1:8000 dilution; Southern Biotechnologies, Birmingham, AL, USA). Finally, plates were washed and developed using the TMB Peroxidase Substrate System (KPL, Gaithersburg, MD, USA). Absorbance was measured at 450 nm.

MICB-ELISA. Plates were coated with the capture mAb BAMO1 at 5 μg/mL in PBS, then blocked by the addition of 15% BSA, washed and incubated with the standard (recombinant sMICB*02 produced in *E. coli*) or patient sera. Patient sera were diluted 1:3 in 7.5% BSA. Plates were washed, incubated with the detection mAb BMO2 at 1 μg/mL, washed again, and then incubated with anti-mouse IgG2a-HRP (1:8000 dilution; Southern Biotechnologies). Finally, plates were washed and developed using the TMB Peroxidase Substrate System (KPL). Absorbance was measured at 450 nm.

Statistics. Results were expressed as medians and dot plots, as well as percentages of values which were elevated with respect to the 95th percentile of healthy individuals. To compare different patient groups, the Wilcoxon test was used. A p-value <0.05 was considered statistically significant. Spearman's rank correlation testing was used to calculate correlations of the serum parameters. For all statistics, SAS software (version 8.2, SAS Institute Inc., Cary, NC, USA) was used.

Results

Similarly to healthy controls (median sMICA <30 pg/mL; sMICB <30 pg/mL), only low levels of both markers were found in sera of patients with hepatic autoimmune diseases (median sMICA <30 pg/mL; sMICB <30 pg/mL). In the various subgroups, sMICA and sMICB concentrations were very comparable. Elevations of marker levels above the 95th percentile of healthy controls (sMICA 330 pg/mL; sMICB 51 pg/mL) were observed in 0% (sMICA) and 3% (sMICB) of the patients with autoimmune hepatitis, in 4% (sMICA) and 4% (sMICB) of the patients with primary sclerosing cholangitis, and none of the patients with primary biliary cirrhosis (Figures 1 and 2).

In contrast, significantly elevated concentrations of sMICA and sMICB were observed in sera of patients with acute infections (median sMICA 890 pg/mL; sMICB 111 pg/mL). Of these patients, 83% (sMICA) and 61% (sMICB) had higher values than the 95th percentile of healthy controls (Figures 1 and 2). While there was no correlation between sMICA and C-reactive protein (median 21.0 mg/dL, range 1.5-39.9 mg/dL; R=0.18; p=0.476), sMICB correlated significantly with C-reactive protein (R=0.62; p=0.006) underlining the enhanced expression and shedding in situations of acute stimulations of the immune system (Figure 3 A, D).

In patients with renal insufficiency, serum levels of sMICA and sMICB were increased only moderately (median sMICA 195 pg/mL; sMICB 50 pg/mL); in consequence, only 29% (sMICA) and 48% (sMICB) of these patients had higher sMICA values than the 95th percentile of healthy controls (Figures 1 and 2). No correlation was observed between sMICA level and creatinine concentration (median 8.4 mg/dL, range 4.2-11.5 mg/dL; R=–0.29; p=0.191) or between sMICB level and creatinine concentration (R=0.15; p=0.526) (Figure 3 B, E).

However, highly elevated concentrations of sMICA and sMICB were found in patients with severe cholestasis (median: sMICA 1058 pg/mL; sMICB 146 pg/mL). Of these patients, 62% (sMICA) and 85% (sMICB) had higher values than the 95th percentile of healthy controls (Figure 1 and 2). There was neither a correlation between sMICA and serum bilirubin levels (median 13.4 mg/dL, range 2.9-51.8 mg/dL; R=–0.46; p=0.112) nor between sMICB and serum bilirubin levels (R=–0.14; p=0.648) (Figure 3 C, F).

When correlating sMICA and sMICB with each other in all individuals investigated, a significant correlation was obtained (R=0.68; p<0.001) (Figure 3 G). Whereas in patients with hepatic autoimmune diseases (R=0.11; p=0.187) (Figure 3 H) and those with renal insufficiencies (R=0.34; p=0.130) (Figure 3 I) no correlation was found, both markers showed a significant correlation in patients with acute infections (R=0.54; p=0.021) (Figure 3 J) and cholestasis (R=0.59; p=0.032) (Figure 3 K).

Discussion

The immunoreceptor NKG2D in conjunction with CD8 T-cells plays an essential role in NK cell activation and subsequent tumor rejection. Prominent ligands of NKG2D (NKG2DL) are the surface glycoproteins MICA and MICB, which are overexpressed in many tumors (1-4). To avoid the antitumor response stimulated by NK cells, down-regulation of NKG2DL expression, *e.g.* by proteolytic shedding of MICA and MICB from the tumor cell surface, has been

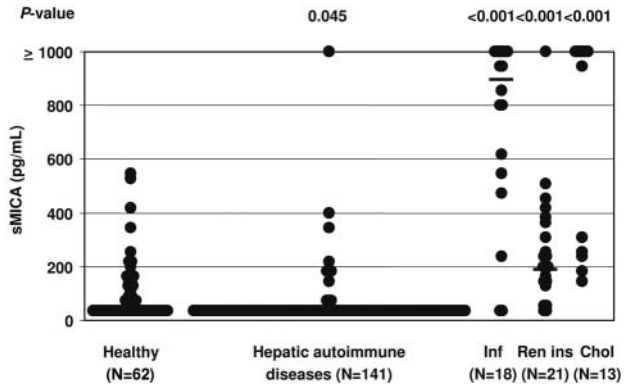

Figure 1. *Distribution of soluble MICA concentrations and medians (——) in sera of healthy controls, patients with hepatic autoimmune diseases, acute infections (Inf), renal insufficiency (Ren ins) and acute cholestatic liver diseases (Chol). P-values indicate the differences between healthy controls and the patient groups.*

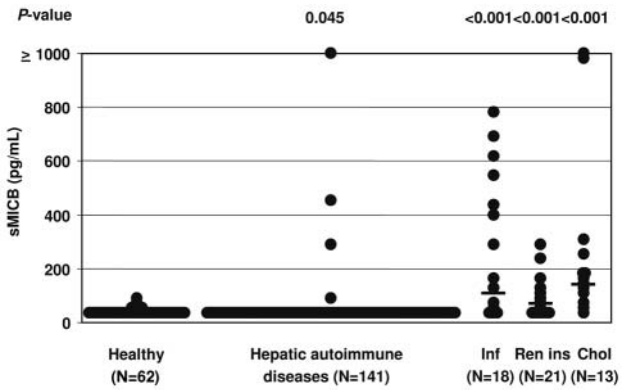

Figure 2. *Distribution of soluble MICB concentrations and medians (——) in sera of healthy controls, patients with hepatic autoimmune diseases, acute infections (Inf), renal insufficiency (Ren ins) and acute cholestatic liver diseases (Chol). P-values indicate the differences between healthy controls and the patient groups.*

found to be an effective tumor immune escape mechanism (2-7). As a consequence, soluble MICA and MICB are frequently observed to be elevated in sera of patients with various malignancies (7, 9-13). In particular, they seem to have a major function in late stages of tumor progression, impairing the antitumor effects of NK cells and CD8 T-cells (11, 12). However, it is known for several oncological biomarkers that the diagnostic potential is influenced by benign organ-specific diseases and diseases involved in marker metabolism. For example, CA 19-9 is frequently elevated during cholestasis, cytokeratin fragments and squamous cell cancer antigen during renal insufficiency, and CA 125 during peritoneal or pleural effusions (14). Therefore, the levels of the NKG2D ligands sMICA and sMICB were analyzed in sera of patients with various hepatic autoimmune diseases, acute bacterial infections, renal insufficiency and cholestasis to test the influence of these pathologies on the marker results.

Though an autoimmune reaction is supposed to be associated with the expression of immunostimulatory antigens on the surface of the autoantigen-presenting cells, it is not surprising that the concentrations of NKG2D ligands in serum were not elevated in patients with hepatic autoimmune diseases: First, NKG2D ligands are not known to be characteristic autoantigens in these pathologies; second, if they were present on autoantigen-presenting cells they are not necessarily expected to be shed from the surface; and third, most patients investigated were in a chronic state of disease.

However in sera of patients with acute infections, considerably elevated MICA and MICB levels were observed which showed no correlation with C-reactive protein concentrations. These results suggest an enhanced expression and shedding of NKG2D ligands in situations of acute stimulation of the immune system. However, this process seems not to correlate necessarily with the severity of disease, or even with the time-line of the production and secretion of acute phase proteins.

Because many biomarkers are eliminated by renal filtration or secretion and renal insufficiencies often impair marker metabolism, we expected considerably elevated concentrations of NKG2D ligands during impaired kidney function. But serum levels of sMICA and sMICB were increased only moderately in patients with renal insufficiencies and did not correlate with creatinine concentration.

However, the highly elevated concentrations of sMICA and sMICB in sera of patients with severe cholestasis indicate a potential role of the hepatic metabolization pathway for the elimination of soluble NKG2D ligands from the circulation even if there was no clear correlation with serum bilirubin levels. Further experiments may focus on degradation or modification processes at the cellular level.

Interestingly, we could not find a correlation of sMICA with sMICB in hepatic autoimmune diseases or renal insufficiency but did in patients with acute infections and cholestasis. These results may indicate that both ligands are expressed, shed and metabolized in a similar (at least partly) way in these patient groups.

Taken together, serum levels of the soluble NKG2D ligands sMICA and sMICB are elevated in only a few patients with hepatic autoimmune disease. They are increased moderately in patients with renal insufficiency and particularly strongly in those with acute infections and severe cholestasis. It is clear that the low patient number in the non-autoimmune disease group and the explorative character of the evaluation are limitations of this study. However, these findings open new directions for NKG2D

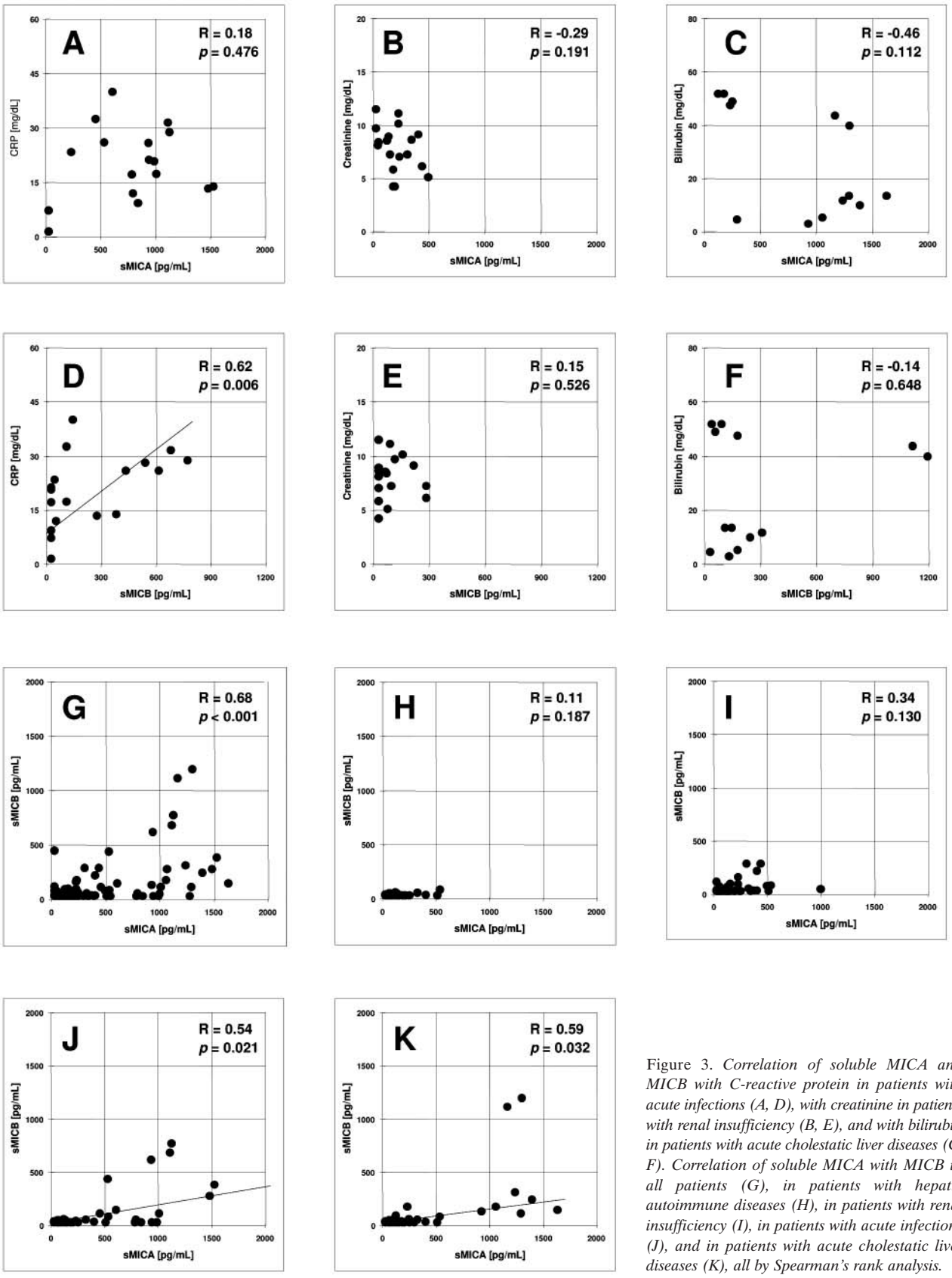

Figure 3. *Correlation of soluble MICA and MICB with C-reactive protein in patients with acute infections (A, D), with creatinine in patients with renal insufficiency (B, E), and with bilirubin in patients with acute cholestatic liver diseases (C, F). Correlation of soluble MICA with MICB in all patients (G), in patients with hepatic autoimmune diseases (H), in patients with renal insufficiency (I), in patients with acute infections (J), and in patients with acute cholestatic liver diseases (K), all by Spearman's rank analysis.*

ligand determinations and identify pathologies that may have to be taken into account if soluble NKG2D ligands are to be used for cancer diagnosis.

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