Osteopontin is a prognostic factor for survival of acute myeloid leukemia patients

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Running Head: Osteopontin in AML

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Scientific category: Hematopoiesis and Stem Cells; Myeloid Neoplasia
Abstract
Osteopontin (OPN) is a glycoprotein that is secreted by osteoblasts and hematopoietic cells. OPN suppresses the proliferation of hematopoietic stem-cells (HSC) in vitro and may regulate the HSC-pool. Increased serum OPN-concentrations occur in chronic myeloid leukemia, multiple myeloma and acute myeloid leukemia (AML). This investigation analyzed the prognostic impact of OPN in AML. We investigated expression and relevance of OPN in newly diagnosed AML-patients from two large study-groups (AMLCG,HOVON) by immunohistochemistry (IH;n=84), enzyme-linked immunoassays (ELISA) of blood/bone-marrow sera (n=41) and on the mRNA-level by analyzing microarray-data (n=261). Expression of OPN-protein was increased in AML-patients both in bone-marrow blasts (IH) and in bone-marrow serum (ELISA) compared to healthy controls. Patients expressing high levels of OPN within the bone-marrow (IH) experienced shortened overall survival (OS,p=0.025). Multivariate analysis identified karyotype, blast-clearance (day16) and the level of OPN-expression as independent prognostic factors for OS. This prompted us to analyse microarray data from 261 patients of a third patient cohort. The analysis confirmed OPN as a prognostic marker. In detail, high OPN mRNA-expression indicated decreased event-free survival (p=0.0002) and OS (p=0.001). The prognostic role of OPN was most prominent in intermediate-risk AML. These data provide evidence that OPN-expression is an independent prognostic factor in AML.

Keywords: osteopontin; acute myeloid leukemia; prognosis
INTRODUCTION

Treatment strategies for patients with acute myeloid leukemia (AML) are based on various prognostic factors including age and performance status of the patient, as well as cytogenetic and molecular characteristics of the leukemic clone. Key objectives in predicting the prognosis of AML are on the one hand, to define patients with high risk of relapse in order to apply more intensive therapy such as allogeneic stem cell transplantation, on the other hand to withhold aggressive therapy when final success seems highly unlikely. Hematological malignancies are developed and maintained not only by the molecular events inside the malignant cell clone but also by their interaction with the microenvironment. The crosstalk between stroma and malignant cells has a major impact on pathophysiological behaviour and progress of the disease.

Osteopontin (OPN) is a secreted glycoprotein of the SIBLING-family and is involved in physiologic and pathophysiologic processes. Its different effects are due to its multiple receptors and isoforms. Cleavage of osteopontin leads to molecular mass facilitating binding to various receptors like CD44, $\alpha_9\beta_1$, and $\alpha_4\beta_7$. In vitro, OPN has been shown to influence adhesion and migration of smooth muscle cells, endothelial cells and melanoma cells. OPN exerts dual functions as a chemoattractant cytokine and as an extracellular component.

Previous studies indicated a role for osteopontin in several malignancies. While most of the publications so far focused on solid cancers, there had been recent advances in deciphering the role of OPN in hematological malignancies. It has been shown that OPN contributes to bone-marrow adhesion and migration of hematopoietic stem cells (HSC). OPN may act as a negative regulator of the HSC through the inhibition of HSC division. HSC-adhesion to osteoblasts leads to a resting state of the HSC in the stem cell niche with protection from cytotoxic and apoptotic stimuli.

Here, we analyzed expression of OPN in bone-marrow, AML-blasts and serum of AML-patients. We demonstrate that the expression of OPN is increased on the mRNA and protein level in AML-patients compared to healthy controls. OPN emerged as an independent prognostic marker in AML.
PATIENTS AND METHODS

Patient characteristics

Immunohistochemical analysis (IH) cohort: Bone-marrow sections of 84 patients with newly diagnosed, untreated AML were randomly chosen. Three controls were taken from patients with various diseases but normal bone-marrow morphology. Bone-marrow core-biopsies were obtained at presentation. After core biopsy a bone-marrow aspiration was obtained through a separate puncture for cytological analysis. All patients were treated uniformly according to the German-AML-cooperative-group (AMLCG) trial as published in detail.\textsuperscript{13,14} All patients gave their informed consent in accordance with the Declaration of Helsinki and the study received approval from the local ethics committees of the participating institutions. Patient characteristics are summarized in Table 1.

Enzyme-linked immunosorbent assay (ELISA) cohort: Bone-marrow serum of 41 patients with newly diagnosed, untreated AML were randomly chosen. Bone-marrow serum from 4 healthy controls was obtained in addition. Serum was obtained by standard procedures. In addition, serum levels of OPN of the same patients were measured in peripheral blood-samples at the time of diagnosis before treatment. Blood samples were obtained at the time of diagnosis before the initiation of chemotherapy and stored at -80°C. All patients were treated according to the AMLCG-trial\textsuperscript{13,14}, patients gave their informed consent and the study received approval from the local ethics committees.

Microarray based analysis of mRNA-levels: Patients were treated as published according to the protocols of the Dutch–Belgian-Hematology–Oncology Cooperative group (available at www.hovon.nl).\textsuperscript{15-17} A total of 284 AML-patients provided bone-marrow aspirates or peripheral blood samples at the time of diagnosis. Patients had received a diagnosis of primary AML, which had been confirmed by means of a cytologic examination of blood and bone-marrow (Table 1). Blasts and mononuclear cells were purified by Ficoll–Hypaque (Nygaard) centrifugation and cryopreserved, as described.\textsuperscript{18} Patient-samples were analyzed with Affymetrix-U133A GeneChips. In 261 patients OPN-level could be measured. Further details have been published before.\textsuperscript{18}
**Immunohistochemistry**

*Immunohistochemical staining:* Bone-marrow specimens were fixed in paraformaldehyde, decalcified and embedded in paraffin. For staining we used the anti-human OPN-antibody (AF1433, R&D-Systems, MN, USA). Staining was performed by the alkaline phosphatase/anti-alkaline-phosphatase double-bridge-technique (Dako-APAAP-Kit; Dako, Glostrup, Denmark). Briefly, tissue-sections were deparaffinized and rehydrated. Samples were pretreated to promote antigen retrieval in a microwave oven in 10mmol/l sodium citrate. The primary antibodies were applied overnight. Subsequent steps were performed according to the manufacturer’s instructions. Sections were counterstained with erythrocin-solution.

*Immunohistochemical expression:* OPN-expression was semiquantitatively assessed by scoring the proportion and intensity of stained cells according to our published methods. The entire bone-marrow section was scanned at low magnification and the percentage of positive cells stained was estimated according to a 3-grade scale. The intensity was subsequently evaluated in 3 representative x500-fields. The mean of cellular staining intensity was subsequently multiplied with the number of positive cells according to the 3-grade scale. The results were expressed as arbitrary units (AU). Expression was evaluated in 2-3 sections processed in independent immunostainings and the mean value was calculated.

**Enzyme-linked immunosorbent assays (ELISA)**

ELISA were performed using available kits from R&D-Systems (Minneapolis, USA). Briefly, patient samples were collected using EDTA and stored at -80°C. Serum samples were added to separate microplates, each containing specific antibody. Mixtures were incubated at room temperature. Plates were washed to remove unbound antigen. Enzyme-linked antibodies specific for OPN were then added and the mixtures were incubated followed by washing steps. The substrate solution was added to the wells. Color
development was stopped and the intensity was measured. Optical density of each well was determined according to manufacturer’s instructions.

**Statistics**

Beyond descriptive statistical analyses, inferential analyses were performed using non-parametric methods. Differences in OPN expression between 2 groups were analyzed using the Mann-Whitney-U-test for independent groups. In the case of more than 2 groups the Kruskal-Wallis test was used. Correlation between metric variables was assessed by the Spearman-rank-correlation-coefficient ($r_s$). End points were defined as published. Patients who survived the entire follow-up period were censored at the date of last contact. Cut-off values of OPN-levels were determined by recursive partitioning. In addition, Bootstrap analysis and hazard ratio per unit were determined to identify the stability of the identified cut-off points. Survival curves were generated using the Kaplan-Meier method and the log-rank test was used to compare survival between groups. Univariate and multivariate Cox proportional hazards-regression analyses were performed to evaluate the impact of prognostic factors on survival. Factors found to be statistically significant at a 10%-level in the univariate analyses were included in the multivariate model. Two-sided $p$-values $\leq 0.05$ were considered to indicate significant differences. All calculations were performed using the SPSS-software (SPSSInc.,Chicago,IL,USA).

**RESULTS**

*Immunohistochemical analysis*

Expression of OPN was analyzed by immunohistochemical staining in bone-marrow core biopsies of 84 AML-patients. The patients’ characteristics are presented in Table 1. In AML and control patients, staining intensity varied from weak to moderate with some scattered cells showing strongly positive signals (Figure 1). OPN-positive leukemic blasts were widely and uniformly observed in a homogeneous pattern throughout the cellular regions of AML bone-marrow. Signals were associated with the cytoplasm as well as with the nucleus of leukemic blasts. In contrast to its expression in AML, OPN-expression in control bone-marrow samples was generally weak and limited to a few scattered positive
cells in the endostal region, with most of the cells being completely negative (Figure 1A). The staining scores for OPN-protein expression in AML-patients showed a median of 6.27AU([1–14]) and differed significantly from the OPN-expression found in bone-marrow sections of controls (1.33[1.3–2.0]AU; p=0.0059, Mann-Whitney-test). To verify the concordance of the 3 observers we performed a reliability analysis. The intraclass-correlation (ICC) analysis revealed good reliability (ICC: 0.654; 95% CI: 0.536-0.753). No significant association was found between the expression of OPN and age at diagnosis or patient gender.

Although OPN was consistently detected in leukemic blasts, staining scores were not associated with the percentage of leukemic blast infiltration. We then analyzed the correlation between OPN-expression and AML-subtypes according to FAB-classification, as defined by cytological analysis. Statistical analyses did not reveal differences in expression between subtypes M0 to M7 (Kruskal-Wallis-test, p=0.614). Also, no association was detected between the level of expression of OPN and the response to therapy at day 16 (p>0.5 for each variable).

We analyzed the association between pretherapeutic OPN-expression and prognosis in AML (Figure 1C,D). Optimized cut-off points were identified by recursive partitioning, and an AU=9.662 was defined as cut-off. 18.2% of the analyzed AML-patients had an OPN-expression level above that cut-off value. Univariate analysis revealed that patients expressing high levels of OPN (AU>9.662) in the bone-marrow had significantly shorter OS than those with low OPN-levels (p=0.025). The median survival time of high OPN-expressing AML-patients was more than 2-fold longer than survival time of patients with low OPN-expression (3.7 vs. 1.5 years). Figure 1 indicates the OS stratified according to OPN-expression. A multivariate Cox-regression analysis was performed that included the parameters: FAB-subtype, karyotype and OPN-expression. OPN emerged as independent prognostic factors for OS (p=0.03; hazard-ratio: 2.35, 95%CI:1.09–5.09). In addition, we observed a trend between high OPN-expression and relapse-free survival (RFS, Figure 1D), although this correlation failed to reach the level of significance (p=0.279). This is most likely explained by the lower number of patients available for this analysis.
**OPN-levels in patients’ serum**

Levels of OPN were analyzed in pretherapeutic bone-marrow and blood serum samples of patients with AML and healthy individuals (Table 1). The median level of OPN in AML-patients was 5.4ng/ml [0.3–20.0 ng/ml]. In blood serum of AML-patients levels of OPN were significantly increased as compared to controls. Blood OPN-protein levels in AML-patients were at a median of 7.4ng/ml [0.3–30.5 ng/ml]) and differed significantly from OPN-levels found in blood serum of controls (median:2.1ng/ml [0.83-3.22ng/ml]) (p=0.01, Mann-Whitney test). No statistical association between the levels of OPN in blood and bone-marrow and the variables gender or age at diagnosis of AML were observed. Levels of OPN did not significantly differ between different AML-subtypes as defined by the FAB-classification (p>0.1 for each variable). Furthermore, OPN-levels did not differ significantly among cytogenetic risk groups.

The bone-marrow of the AML-patients studied was highly infiltrated by leukemic blasts. The median (range) percentage of blasts was 70% (range,15–99%). The ELISA-levels of OPN were not positively correlated with the percentage of leukemic blast infiltration as determined by bone-marrow aspiration. Also OPN was not associated with white blood cell (WBC) count and lactate-dehydrogenase (LDH) levels. Furthermore, we tested if levels of OPN at first diagnosis could predict remission after induction therapy. From the 41 patients of our study population receiving intensive induction therapy, 78% achieved a CR. Levels of OPN in these patients did not significantly differ from those observed in patients not achieving a CR (p=0.280).

To determine the relationship of OPN-protein expression levels and prognosis in AML, we performed univariate Cox-proportional hazard analyses. As expected, the variables karyotype (favorable vs. intermediate/unfavourable), WBC and LDH displayed prognostic significance. Furthermore, using the dichotomous cut-off levels identified by recursive partitioning, OPN-protein expression in the bone-marrow was significantly associated with survival (Figure 2A,B). Blood OPN-levels showed no prognostic significance (data not shown). OS and EFS were significantly lower for patients with bone-marrow OPN-levels above 6.32ng/ml in the marrow serum.
**OPN is increased in AML-blasts at the mRNA-level**

To confirm the prognostic significance of OPN in AML we analyzed a published microarray analysis in AML-patients from the HOVON group using the database published by Valk et al.. OPN was expressed at a low signal to noise ratio but the specific queries concerning OPN-levels could be analyzed. The median OPN-mRNA expression level in these AML-patients was 4.9 (range[4.18–9.27]). Levels of OPN were significantly increased in the subgroup of AML M3, whereas it did not differ significantly between the other different AML subtypes as defined by the French–American–British (FAB) classification (p>0.1 for each variable; Kruskal–Wallis test). In the subgroup of the AML-M3, OPN-expression had no significant impact on EFS/OS. Also, OPN-levels did not differ significantly among cytogenetic risk groups. To determine the relationship of OPN and prognosis in AML, we initially performed univariate Cox-proportional-hazards analyses. An optimal cut-off value of the OPN-level was determined by recursive-partitioning. As expected, the variables karyotype and molecular genetics displayed prognostic significance. OPN was also significantly associated with survival (Figure 3). The hazard ratio (HR) of death was significantly higher for patients with high OPN-levels (HR 1.721, confidence-interval 1.24-2.40; p=0.001).

Subsequently, we performed multivariate Cox-regression-analyses incorporating all variables showing a significant association with survival in the univariate model at a 10%-level. The multivariate analysis of OPN-mRNA included the covariates FLT3, CEBPA, FAB-type and cytogenetics. OPN emerged as an independent predictor of survival (HR=1.708; 95%CI:1.141-2.557; p=0.009) (Suppl. Table:1). Figure 3 indicates the Kaplan-Meier curves of OS and EFS stratified for OPN-levels. An association was detected between high levels of OPN-expression and therapy resistance to induction therapy (p=0.009). We could only speculate that OPN may act already at that early timepoint as a negative regulator of leukemic cell proliferation and thus chemotherapy resistance. This prompted us to investigate whether OPN-expression levels at diagnosis would influence outcome even after an initial remission was reached. The analysis was restricted to patients that achieved a complete remission after induction chemotherapy. Nonetheless, OPN-expression levels at diagnosis showed a significant and independent impact on survival (Figure 3C; p=0.016).
Further, we analyzed OPN levels in subsequently relapsing patients versus patients with continuous CR and found significantly more OPN-high expressing blasts (12% vs 27%, p<0.012) in patients that subsequently developed a relapse. In the patient group who died in CR (n=36) the numbers were too low for statistical analysis. Only 9 patients were in the high OPN-group. In addition, we analysed whether OS and EFS were altered by exclusion of AML-M3 patients, who have a higher expression of OPN in contrast to the other FAB-types. The significance value for OS is not altered, whereas, as expected, the EFS was even more significant. The p-value changed from 0.0002 to 0.00003. For patients who reached a complete remission, high OPN-expression was still significant in terms of EFS (p=0.006). We subsequently investigated if OPN presents a prognostic factor in the different cytogenetic risk groups. Interestingly, OPN remained a significant prognostic factor in intermediate risk patients (Figure 3D; p=0.045).

**Validation of cut-off values and Hazard Ratio per unit increase in OPN**

In order to validate the determined optimal cut-off values of OPN-levels a bootstrap approach was applied. The 1000-bootstrap-samples results showed weak reproducibility. (Suppl.Table:2). This finding indicates that the cutpoints used in our analyses should not be regarded as the best that can be achieved in different experiments. Rather, the findings indicate that rising OPN-levels indicate increased risk for therapy failure. In all three analyses OPN-levels showed a unfavorable hazard ratio per unit increase in OPN that reaches significance in case of mRNA OPN (Suppl.Table:3).

**DISCUSSION**

In this retrospective study, we identified osteopontin expression as analyzed on the mRNA and protein level as a prognostic marker in AML. In three different patient cohorts and with various methods we demonstrate that increased expression of OPN was associated with shortened survival. While in control bone-marrow, OPN was only expressed by osteoblasts within the endosteal region, we could demonstrate OPN is readily expressed in AML blasts. The analyses (cut-off value defined by statistical optimization) revealed that OPN is
highly expressed in approximately 20% of AML (ELISA:23%; IH:18%, RNA:23%) and is neither associated with FAB-type, nor with cytogenetic or molecular alterations. In addition, in a recently published dataset from the Mile-study we also identified significantly increased levels of OPN in AML compared to normal bone-marrow (data not shown). Several studies indicated a role for OPN in different malignancies. While most of the publications had focused on solid cancers, there have been recent advances in deciphering the role of OPN in hematological malignancies. Increased serum OPN-concentrations have been reported in chronic myeloid leukemias, multiple myelomas and AML. Lee et al. demonstrated in a small cohort that marrow levels of higher OPN is associated with a significant shorter survival in AML-patients in a 1-year survival analysis, while 5-year analysis revealed no statistical difference. Powell et al. performed expression screen to identify targets in AML. These analyses identified OPN as a functionally relevant target. siRNA-mediated knockdown of OPN-expression induced cell death in both AML blasts and leukemic progenitors. These authors could demonstrate that in normal karyotype AML, increased expression of OPN at diagnosis is associated with poor prognosis (n=60). The important function of OPN in normal hematopoiesis appears to be the suppression of proliferation of primitive stem cells in the bone-marrow niche, as evidenced with OPN-/- mice and in-vitro colony formation assays. These data therefore provide strong evidence that OPN is an important component of the HSC-niche participating in HSC-location and as a physiologically negative regulator of HSC-proliferation. Furthermore, exogenous OPN suppresses the proliferation of primitive HSC in vitro. The relevance of this observation is demonstrated by the markedly enhanced cycling of HSC in OPN-/- mice and their hypersensitivity to exogenous stimuli. Considering these biologic effects of OPN and the significant influence of OPN-expression on prognosis as measured by EFS and OS in our study, we assume that OPN contributes to chemotherapy resistance in a paracrine/autocrine manner.

The self renewal of leukemic stem cells (LSC) may have an extrinsic component like OPN, as it is known for normal HSC. The importance of the leukemic stem cell miroenviroment has also been demonstrated. Their data indicated that mesenchymal stem cells are essential for the long-term survival and expansion of
leukemic lymphoblasts in childhood ALL.\textsuperscript{26} The microenvironment supports the repopulating potential and the ability to propagate and maintain the leukemic phenotype. Many studies reported that AML-LSC are quiescent or slowly dividing, whereas clonogenic progenitors are rapidly proliferating.\textsuperscript{27-29} However, there is emerging evidence that the complex interactions between LSC and their niche may also be targeted. Interestingly, targeting of CD44, a known ligand of OPN, eradicates human myeloid leukemic stem cells in mice.\textsuperscript{30} In summary, our data suggest that OPN, a molecule important for stem cell regulation, is closely associated with patient prognosis in AML. Further studies should test OPN as a biomarker and a possible target for AML-therapy.

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AUTHORS’ DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

All authors declared no conflicts of interest.

AUTHOR CONTRIBUTIONS

Conception and design: Ruediger Liersch, Torsten Kessler, Wolfgang E. Berdel, Rolf Mesters

Provision of study materials or patients: Ruediger Liersch, Christoph Schliemann, Iris Appelmann, Christoph Biermann, Torsten Kessler, Peter J. Valk, Ruud Delwel, Bob Löwenberg, Thomas Büchner, Wolfgang Hiddemann, Carsten Müller-Tidow, Wolfgang E. Berdel, Rolf Mesters

Collection and assembly of data: Ruediger Liersch, Christoph Schliemann, Torsten Kessler, Michael Bayer, Christian Schwöppe, Peter J. Valk, Ruud Delwel, Bob Löwenberg, Thomas Büchner, Wolfgang Hiddemann, Joachim Gerss, Carsten Müller-Tidow, Wolfgang E. Berdel, Rolf Mesters
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**Manuscript writing:** Ruediger Liersch, Carsten Müller-Tidow, Wolfgang E. Berdel, Rolf Mesters

**Final approval of manuscript:** All authors

**REFERENCES**


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**Table 1. Patients characteristics**
LEGENDS TO THE FIGURES

Figure 1. OPN protein expression by IH and survival.
OPN-expression in control bone-marrow (A) and acute myeloid leukemia (FAB AML M5; B) obtained at presentation. Immunohistochemical localization was performed using the respective specific antibodies and the alkaline phosphatase/anti-alkaline phosphatase technique (Dako-APAAP kit). Note the expression of OPN in the bone-marrow sections of AML patients (B; arrowhead) compared to the controls (A, arrow). Original magnification 200x; Kaplan-Meier curves of OS and Relapse-Free-Survival (RFS) of AML patients stratified for bone-marrow OPN expression are shown. OS of patients expressing high levels of OPN (≥9.662 AU) in the bone-marrow was significantly lower than of patients with low OPN expression (<9.662 AU) (C, p=0.025). Although with a visible trend, difference found for (RFS) was not significant (D, p=0.279).

Figure 2. Bone-marrow serum OPN expression (ELISA).
Kaplan-Meier curves for overall survival (OS, A) and event free survival (EFS, B) of AML patients stratified for bone-marrow serum OPN expression. OS of patients with low (<6.632 ng/ml) levels of OPN in the bone-marrow was significantly higher than in patients with high (≥6.632 ng/ml) OPN expression (A, p=0.018). Similar differences were found for EFS (B, p=0.008).

Figure 3: OPN mRNA expression and survival
High OPN expression on the mRNA level is associated with significantly (p=0.001) shorter overall survival (A) and event free survival (p=0.0002) (B). In the group of AML-patients, who reached remission, increased OPN expression still shows significantly shorter event free survival (C, p=0.006) and also in intermediate risk patients (as defined by cytogenetics) (D, p=0.045).
Figure 1

(A) Control

(B) AML

(C) Overall Survival

(D) Relapse Free Survival

Osteopontin

p = 0.025

p = 0.279
Figure 2

A: Osteopontin

- Overall Survival
- Low vs. High
- p = 0.018

B: Osteopontin

- Event Free Survival
- Low vs. High
- p = 0.008
Figure 3

(A) Overall Survival

(B) Event Free Survival

(C) Event Free Survival

(D) Event Free Survival

Osteopontin

Low

High

p=0.001

p=0.0002

p=0.006

p=0.045
Osteopontin is a prognostic factor for survival of acute myeloid leukemia patients

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