Macrophage Migration-inhibitory Factor Levels in Serum of Patients with Ovarian Cancer Correlates with Poor Prognosis

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Abstract. *Ovarian cancer is generally thought of as a cancer with poor prognosis. However, prognostic appraisal of the disease is based on tumor stages, surgical features or sensibility towards platinum-based chemotherapy. There are data that also grant immunological parameters such as CD8+ T-lymphocyte- (CD8 T-cell) infiltration in tumor tissue, a prognostic role. Macrophage migration-inhibitory factor (MIF) has been described as a tumor-derived protein which allows tumor cell immune escape from antitumoral host natural killer (NK) - and CD8 T-cells. This immune escape is functionally based on down-regulation of the receptor natural killer group 2D (NKG2D). We here report that the levels of the MIF protein which is known to be secreted in ascites and serum of patients with ovarian cancer, not only seems to correlate with common prognostic parameters such as tumor stage or platinum sensitivity, but also with CD8 T- and NK-cell infiltration in tumor tissue. We therefore believe that MIF may play a suppressive role in the host antitumor immune response, which may have a negative impact on the course of the disease. The fact that MIF levels in serum of patients at primary diagnosis correlate with platinum sensibility supports the hypothesis that serum MIF levels should be evaluated as a parameter reflecting tumor sensibility towards chemotherapy in early stages of the disease.*

Epithelial ovarian carcinoma is the most common cause of death from gynecological malignancies. Even with extended surgery and chemotherapy, overall 5-year survival rates do not

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exceed 20-40% (1). However it still remains unclear whether extensive surgical approaches such as radical lymphonodectomy (2) or other common parameters such as histological grading, age of patients or histological subtype really are of use in improving or appraising the prognosis of the disease. Especially in the early stages of the disease, it remains unclear whether extensive surgery or aggressive chemotherapy really is of prognostic value and of use for the individual patient (3). The strong correlation between long-term survival and favorable immunological parameters (4) suggest that the relief from tumor-induced immunosuppression may have considerable clinical impact in ovarian cancer. However, while antitumoral immune responses are strongly suppressed in ovarian cancer, malignant transformation is believed to occur in a pro-inflammatory environment (5), that is later maintained by tumor cells themselves (6). The formation of ascites is thought to occur when tumor cells metastasize into the peritoneal cavity which induces local inflammation, as well as the deregulation of the coagulation cascade and perhaps other, as yet still unknown, events (7). This means that the presence of ascitic fluid is a sign of local inflammation, but simultaneously cytolytic CD8+ T-lymphocyte (CD8 T-cell) or natural killer cell (NK cell) responses are suppressed (8). There are data that the presence, or even the amount, of ascitic fluid at primary diagnosis of ovarian cancer is of prognostic value (6).

Macrophage migration-inhibitory factor (MIF), a cytokine that has already been described in a variety of different cancer types (9), is secreted into malignant ascites of ovarian cancer and has been shown to suppress local antitumor immune responses (10). Originally, MIF was described as a product of T-cells after exposure to bacterial endotoxins that is responsible for lethal shock syndrome (11). However, MIF is produced by a variety of cells (12, 13). Cellular MIF production can be induced by glucocorticoids and MIF acts as a functional glucocorticoid antagonist, thus providing a negative feedback loop (14). Apart from its role in

endotoxemia, polymorphisms in the MIF promoter that lead to high serum MIF levels contribute to autoimmune diseases such as rheumatoid arthritis, inflammatory bowel disease, systemic lupus erythematosus and atopic dermatitis (15, 16). In tumor biology, MIF has been proposed as a biomarker for prostate cancer (17). Of note is a critical role for MIF in the angiogenesis of glioma and hepatocellular carcinoma where it also stimulates tumor cell migration (18). Tumor cell growth and survival may also be enhanced by MIF, since this cytokine suppresses p53 activity (19, 20), activates Cyclin D1 and E2F transcription factors (21) and has also impact on the extracellular signal-related kinase isoformes 1/2 (ERK 1/2) and protein kinase B pathway (AKT pathway) (22). The significant role of MIF in tumorigenesis is clearly demonstrated by the resistance of MIF-deficient fibroblasts towards malignant transformation induced by c-Myc, transforming protein p21 (H-Ras) or dominant-negative p53 (23). In antitumoral immunity MIF is attributed an important role since it affects the immune system by inhibiting cyotoxic T-lymphocyte (CTL) and NK cell responses (24, 25). Since antitumor immunity heavily depends on these cell types, MIF has also been implicated in the immune escape of tumor. The immunosuppressive effects of MIF may be caused by MIF-induced T-cell activation followed by activation-induced cell death (26) – a mechanism that might reconcile immune inhibitory with pro-inflammatory potential. Another mechanism seems to be the MIF-induced down-regulation of the receptor natural killer group 2D (NKG2D) on NK cells, mediated by MIF secretion of ovarian cancer cells into malignant ascites and serum of ovarian cancer patients (10). In the current study we provide data that suggest that the antitumoral MIF-effects in ovarian cancer may have impact on the clinical outcome of the disease since MIF levels in serum of patients with ovarian cancer correlate with markers that are used to describe poor prognosis of ovarian cancer.

Materials and Methods

Patients' characteristics. We examined sera of 68 patients that were diagnosed with ovarian cancer from 2002-2004. Blood samples were taken at the time of diagnosis which provided us with a retrospective period of five years. The file records of all patients were screened for the common prognostic factors in ovarian cancer: age of the patient, histological grading, tumor stage at primary diagnosis, R0 resection by surgery, and sensitivity to platinum-based chemotherapy for more than 12 months. These data were subsequently correlated with individual MIF levels in the serum of the patients at primary diagnosis. Furthermore, we sought for a correlation between MIF levels and NK and CD8 T-cell infiltration in tumor tissue at primary diagnosis, obtained by immunohistochemistry.

Enzyme-linked immunosorbent assay (ELISA). The concentration of soluble MIF in serum of patients with ovarian cancer and healthy controls was determined by ELISA using mouse anti-human MIF Mab289 as capture, and biotinylated goat anti-human MIF BAF289 as

detection antibody (both from R&D Systems, Wiesbaden, Germany) in a MaxiSorp 96-well plate (Nunc, Wiesbaden, Germany). Streptavidin-horse radish peroxidase and diaminobenzidine were used for color development. Absorbance was recorded at 450 nm in a Sunrise microplate reader (Tecan, Crailsheim, Germany) and quantified relative to a series of standards. The level of detection in this assay started at 0.029 ng/ml and reached up to a maximum level of 12 mg/ml.

Immunohistochemical staining. All tissue specimens were from the tumor bank of the University of Würzburg, School of Medicine, where they had been evaluated by at least two pathologists. Paraffin-embedded tissue samples of 37 ovarian adenocarcinomas were cut at 2 μm, placed on slides (Superfrost, Langenbrinck, Emmendingen, Germany), de-paraffinized with xylene and rehydrated in a descending alcohol sequence. Antigens were unmasked in 10 mM sodium citrate buffer in a microwave oven. Endogenous peroxidases were left to react with 3% H_2O_2 in methanol for 10 min. Slides were washed in phosphate buffered saline (PBS) and non-specific binding was blocked with 1% goat serum. Subsequently, slides were incubated for 1 h with a monoclonal mouse antibody to CD8 (Mab1509, R&D Systems), diluted in commercial antibody diluent (DakoCytomation, Hamburg, Germany) at 1:100, for the staining of CD8 T-cells. For staining of NK cells a monoclonal mouse antibody against CD56 (MAB 24081, R&D Systems) was used in analogy to the CD8 antibody. Biotinylated anti-mouse immunoglobulins and streptavidin-HRP (both from DakoCytomation) were used according to the manufacturer's protocol. Staining was developed for 5-10 min with diaminobenzidine. Nuclei were counterstained with hematoxylin. Stained sections were dehydrated by washing in graded ethanol and embedded in VitroClud (Langenbrinck, Emmendingen, Germany). For semi-quantitative evaluation, stained sections were rated by two independent individuals on a scale ranging from 0 to 3 where 0 signifies the absence of staining in 10 fields of view, 1 corresponds to single-positive cells in 10 fields, 2 denotes up to 10 positive cells in a diffuse pattern, 3 indicates more than 10 positive cells in a focused pattern. Statistical analysis was performed, using the Students *t*-test.

Results

MIF protein levels are elevated in a subgroup of patients with ovarian cancer. To evaluate adequate normal background levels of MIF in healthy individuals, the serum of 20 healthy donors was investigated additionally to that of patients with ovarian cancer by ELISA. The median MIF-level in this group was 0.699 ng/ml. The group of cancer patients interestingly could be divided into two groups based on secretion of MIF into serum. A so-called "low-MIF" group consisted of 31 patients with a mean serum level of MIF of 0.486 ng/ml. In this group, no patient had a MIF level higher than 1 ng/ml. The "high-MIF" group of 37 patients had a median MIF-level of 2.649 ng/ml. The difference in MIF between these two groups was statistically significant (*p*<0.05, Figure 1).

Correlation between MIF-levels and prognostic parameters of ovarian cancer. From the patient records common prognostic parameters of ovarian cancer were extracted. Of
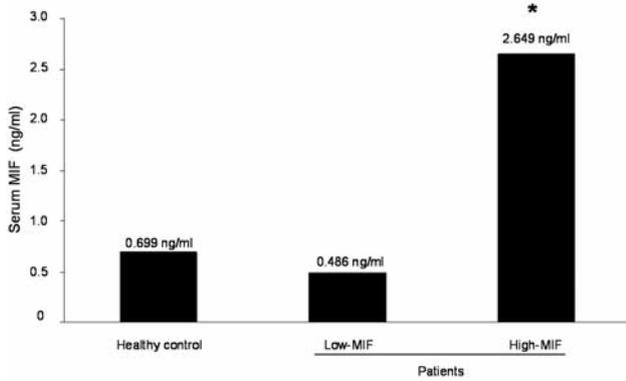
Figure 1. *Levels of the Macrophage migration-inhibitory factor protein (MIF) in serum of healthy individuals (mean 0.699 ng/ml, n=20) were similar to those of a subgroup of patients with ovarian cancer, the low-MIF group (mean 0.486 ng/ml, n=31). The high-MIF group of patients with ovarian cancer had significantly higher MIF levels in serum (mean 2.649 ng/ml, n=37) with no sample being under 1 ng/ml.*

Figure 2. *Macrophage migration-inhibitory factor (MIF) levels in serum of patients (n=68) correlate inversely with disease-free survival (DFS) after completed adjuvant or first-line therapy of ovarian cancer. The bold blue line indicates the regression line, the green lines indicate the 5-95% confidence interval.*

interest were initial stages of the disease as classified according to the Fédération Internationale de Gynécologie classification (FIGO, 27) at diagnosis, histological grading, age of patient at diagnosis, sensitivity to platinum-based chemotherapy from 6-12 months and R0 resection. Based on FIGO staging, FIGO I confers a favorable prognosis and FIGO II, III and IV unfavorable prognosis. Histological grading 1 also has favorable prognosis and grading 2 and 3 unfavorable prognosis. According to common literature (27) R0 resection and disease-free survival after platinum-based chemotherapy of 6-12 months were attributed as being indicative of favorable prognosis, as well as age over 60 years at diagnosis. Patients with high MIF levels were always more likely to have unfavorable prognostic parameters. However, the differences did not reach statistical significance.

Correlation between MIF-levels and disease-free survival (DFS). DFS was defined as years of survival after the completion of platinum-based chemotherapy in an adjuvant or a first line situation. Here it was evident that an inverse correlation between MIF level at the time of primary diagnosis or recurrence and DFS existed (Figure 2).

Unfortunately, this correlation also did not reach statistical significance. Furthermore, we sought for a difference in DFS between the low-MIF and the high-MIF groups. Of interest was the fact that there seemed to be a cut-off in DFS at 9 months according to MIF level. This means that a subgroup of patients with DFS of less than 9 months more likely had higher MIF levels than patients with a DFS greater than 9 months. In correlation, the MIF levels in the group with more than 9 months DFS were not less than 1 ng/ml (mean=2.2535 ng/ml MIF), whereas the ones of patients with DFS more than 9 months were
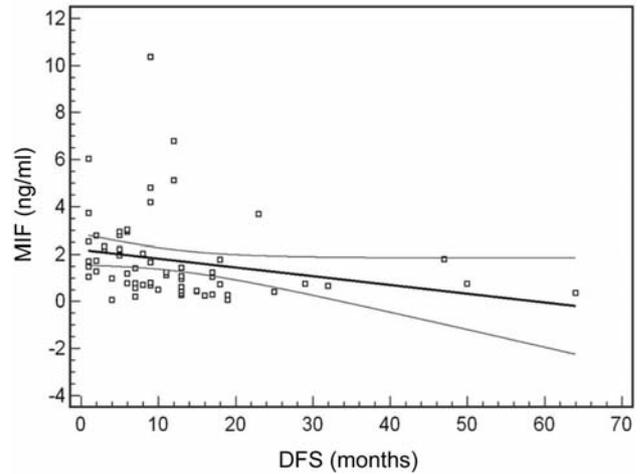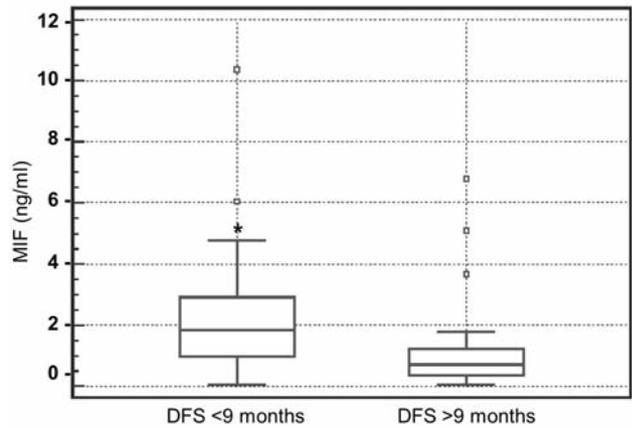

Figure 3. *Patients with disease-free survival of more than 9 months (n=31) had a significantly lower MIF serum level at the time of diagnosis (mean 1.1784 ng/ml) in comparison to patients with lower DFS (n=34, mean 2.254 ng/ml). This is in line with the findings for the high-MIF and low-MIF groups of patients. The box boundaries limit the interval of 25% to 75% of all data, the middle line indicates the mean of all data. The bars above and below the box indicate the 5% and 95% interval.*

very unlikely to exceed 1 ng/ml (mean=1.1784 ng/ml). This difference was statistically significant (*p*=0.0157). Therefore, patients of the high-MIF group had a shorter DFS in comparison to patients of the low-MIF group, with a cut off at 9 months DFS (Figure 3).

Correlation between MIF-levels and overall survival (OS). We also examined a difference in OS of patients of the high-
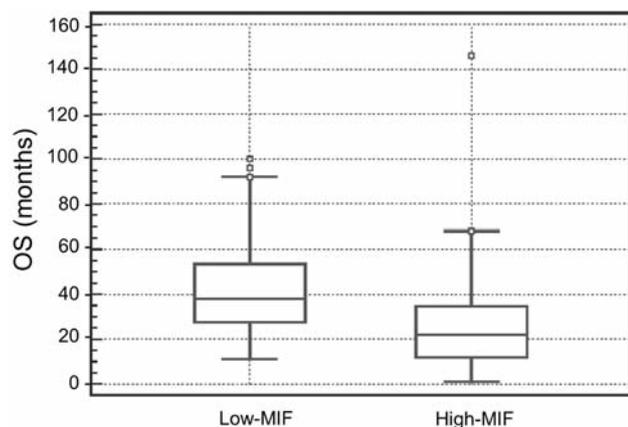
Figure 4. *Patients of the low-Macrophage migration-inhibitory factor (MIF) group (n=31) had longer duration of overall survival (OS, mean 39.8 months) in comparison to patients of the high-MIF group (n=37, mean 20.9 months). The box boundaries limit the interval of 25% to 75% of all data, the middle line indicates the mean of all data. The bars above and below the box indicate the 5% and 95% interval.*

Figure 5. *Tumor infiltration of antitumoral active CD8+ T-lymphocytes and natural killer cells was more frequent in patients of the low-Macrophage migration-inhibitory factor (MIF) group in comparison to patients of the high-MIF group. Data are obtained from 37 patients (low-MIF n=19 patients, high-MIF n=18 patients).*

and the low-MIF groups. It was evident that patients of the low-MIF group had a longer OS after 5 years (mean=39.8 months) in comparison to patients of the high-MIF group (mean=20.9 months). Unfortunately, this difference did not reach statistical significance (Figure 4).

Correlation between MIF levels and tumor stromal infiltration with CD8+ T-cells and NK-cells. Paraffin sections of 37 of the examined patients were evaluated immunohistochemically in order to determine the extent of tumor stroma infiltration with CD8+ T-cells and CD56+ NK cells. Stromal infiltration was quantified by a semiquantitative score, as described in the Materials and Methods. Patients of the low-MIF subgroup (n=19) had higher scores of CD8+ T-cell (mean=2.263) and CD56+ NK cell (mean=1) infiltration in the tumor stroma in comparison to patients of the high-MIF group (n=18), where mean scores for CD8+ T-cells were 1.56 and 0.33 for CD56+ NK cells. Unfortunately these differences also did not reach statistical significance (Figure 5).

Discussion

Ovarian cancer is still a disease with an unfavorable prognosis. To date, common prognostic parameters can be defined that may have an impact on OS and DFS. Such parameters are postoperative resection status, tumor stage, age and performance status of the patient, histological tumor type and histological tumor grading (27). Nevertheless, several authors have described coherence between immunological parameters and clinical outcome of the disease. In particular, infiltration of the tumor stroma by
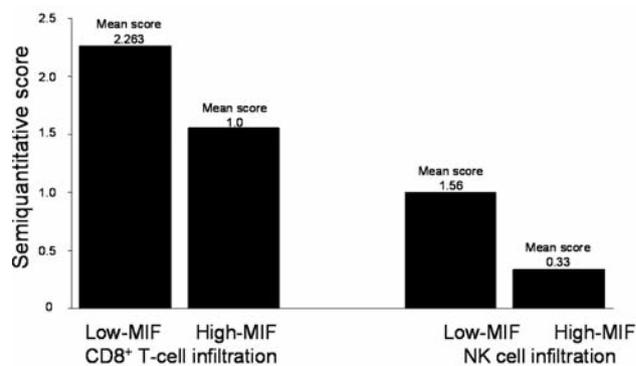
CD8+ T-cells seems to positively affect the course of the disease (28, 29, 30). Several authors have described a variety of tumor immune escape mechanisms promoted by tumor derived or other factors. These tumor immune escape mechanisms may therefore have strong impact on the prognosis of cancers (32). One such tumor-derived protein that provides a way for tumor immune escape is MIF, which leads *via* down-regulation of NKG2D receptors, to a reduction of the antitumoral abilities of CD8+ T-cells and NK cells toward ovarian cancer cells (10).

We therefore examined whether MIF levels in serum of patients with ovarian cancer correlate with prognostic parameters of this disease. To our great interest, it was possible to divide the examined patients into two groups according to the MIF level in serum at the time of diagnosis. This suggests that there may be a role for MIF in the course of ovarian cancer. Patients of the low-MIF group had MIF levels similar to those of healthy control individuals, MIF levels in the high-MIF group were significantly higher. Unfortunately, our collective only consisted of 68 patients. Nevertheless we found positive correlations between acknowledged clinical prognostic factors such as age of the patient at primary diagnosis, initial FIGO stage, histological grading, response to platinum-based chemotherapy and R0 resection after adjuvant surgery which did not reach statistical significance. However, it was evident that patients with ovarian cancer in our collective had differences in DFS at 9 months after completion of platinum-based chemotherapy. Literature does not clearly define whether platinum-sensitivity should be assumed after 6 or rather 12 months of DFS (33). However, patients with a DFS of less than 9 months had significantly higher MIF levels than those with DFS of more than 9 months, so that the differentiation into a high- and a

low-MIF group gains importance. The difference between these two groups also affects the duration of OS. Here, a clear but unfortunately not statistically significant difference between the low- and the high-MIF group was detected.

Nevertheless, the differences between the low- and the high-MIF group were consistent for all the examined parameters, which supports the theory that MIF-secretion in serum of patients with ovarian cancer may influence the prognosis of the disease. Literature describes a MIF-dependent down-regulation of NKG2D-receptors on CD8^{+} T-cells and NK-cells as being one possible mechanism for promotion of tumor immune escape (10, 34). This results in a reduction of the function of antitumoral immune cells. Assuming systemic effects of MIF secretion in the serum of patients with ovarian cancer, we also examined whether a difference in tumor stromal infiltration by CD8^{+} T-cells and NK cells between the high-MIF and the low-MIF group exists. Here it was evident that tumor tissue of patients of the low-MIF group exhibited clearly more CD8^{+} T-cell and NK-cell infiltration than tissue from patients of the high-MIF group. This underlines the suggestion that MIF may influence the prognosis of ovarian cancer by inhibiting the antitumor response of immune cells.

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