The Value of Sequential Serum Measurements of Gelatinases and Tissue Inhibitors During Chemotherapy in Ovarian Cancer

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Abstract. Background: Gelatinases and tissue inhibitors of metalloproteinases (TIMPs) are involved in tumour invasion and metastasis. High pre-operative TIMP-1 serum values have been previously found to correlate to aggressive features in ovarian cancer patients. This study has examined the clinical value of sequential serum measurements of gelatinases and TIMPs during ovarian cancer treatment. Patients and Methods: Serum gelatinase and TIMP values were measured in 48 patients receiving cytotoxic chemotherapy for ovarian cancer. The serum values were analysed by enzyme-linked immunosorbent assay (ELISA) in the third and sixth chemotherapy cycles, and compared to the treatment response. Results: Increased MMP-9 (matrix metalloproteinase-9), TIMP-2 and MMP-2 mean serum values were observed in optimally operated patients with a complete response to treatment, compared to those with a partial response. No significant differences were found in the change of circulating TIMP-1 values in the different chemotherapy response groups. Conclusion: Measuring the serum values of gelatinases or TIMP-2 sequentially during chemotherapy might be beneficial for response evaluation in optimally operated patients, whereas the serum TIMP-1 values might not contribute to an evaluation of the treatment response.

Although most ovarian malignancies respond well to primary chemotherapy, there is a high incidence of relapse during or after the treatment.

Matrix metalloproteinases (MMPs) are a group of proteolytic enzymes, which are capable of degrading most of the components of the ECM (3). Gelatinases A and B (MMP-2 and MMP-9, respectively) play an important role in the cancer invasion process, being involved in the degradation of type IV collagen, as well as other essential extracellular matrix components (3-5). Gelatinases are also involved in angiogenesis (6-8).

Tissue inhibitors of metalloproteinases (TIMPs) are the major endogenous regulators of MMP activity. Four members of the TIMP family have been characterized to date, namely TIMP-1, TIMP-2, TIMP-3 and TIMP-4. Both TIMP-1 and TIMP-2 are capable of inhibiting the activities of most known matrix metalloproteinases (9), although TIMP-1 binds MMP-9 in particular (10), and TIMP-2 has a higher affinity for MMP-2 (11). Recent research suggests that TIMP-1 and TIMP-2 are multifunctional proteins with diverse actions, for example TIMP-2 seems to have a role in the activation of MMP-2 (12), and TIMP-1 in promoting cancer cell proliferation (13). Additionally, both inhibitors also inhibit angiogenesis (14, 15).

MMP-2 and MMP-9 and the tissue inhibitors of metalloproteinase-1 and -2 have been previously analysed in pre-operative serum samples of ovarian cancer patients, and compared the results with different clinicopathological factors (16). High TIMP-1 values were found to correlate to aggressive features of ovarian cancer. Also a poor survival rate was evident in the patients with high TIMP-1 levels. In the present study the serum values of gelatinases and TIMPs were analysed sequentially in the third and sixth chemotherapy cycles of these same patients to assess their correlation to treatment response. Additionally, to evaluate the reliability of serum as a source of gelatinases and TIMP analyses, the pre-operative serum and plasma measurements of individual patients were compared. The study was approved by the Local Ethical Committee of the University of Oulu.

Key Words: Ovarian cancer, follow-up, gelatinases, TIMP-1, TIMP-2.
Patients and Methods

Patient selection. This study was conducted as a continuum to our previous analysis of gelatinases and their tissue inhibitors in pre-operative serum samples of ovarian cancer patients. The primary study group consisted of 59 patients with epithelial ovarian cancer treated in the University Hospital of Oulu between 1995-2001. Of these women, 57 received platinum based post-operative chemotherapy. Those patients who received their adjuvant treatment in the University Hospital of Oulu had follow up serum samples taken in their third and sixth chemotherapy cycles. These samples were analysed by the enzyme-linked immunosorbent assay (ELISA) for MMP-2 and MMP-9, and their tissue inhibitors TIMP-1 and -2. The final study group consisted of 48 women with samples from the pre-treatment stage and the third cycle, 35 of them also had a sample from the sixth cycle. The patient characteristics are presented in Table I.

All the patients underwent surgery. All stage I and II cancers were operated optimally with no residual tumour remaining. In stages III and IV cancer, the limit for optimal operation result was set at a residual tumour of less than 2 centimetres. This was achieved in 41% of the patients. Twenty-eight patients treated before 1999 received cytotoxic chemotherapy post-operatively with a combination of platinum and cyclophosphamide. The subsequent 18 patients received a taxane-platinum combination, and 2 had platinum monotherapy.

Sixteen patients in this study population had both pre-operative serum and plasma (EDTA) samples available. These 16 pre-operative plasma samples were analysed by ELISA contemporaneously with the serum samples, and compared the values on an individual basis.

Determining TIMP-1, MMP-9, TIMP-2 and MMP-2 by ELISA. The ELISA procedure was performed according to standard protocols with standard samples included in each microtiter plate, and measurements were performed in duplicate (17). Similar standard curves for each of the proteins were required in every lot; otherwise the procedure was repeated. Specific monoclonal antibodies (SBA Sciences, Oulu, Finland) that recognise both the free protein and that bound to gelatinase/ tissue inhibitor for TIMP-1, MMP-9 and TIMP-2 were used for coating the wells. Both the latent and active forms of MMP-9 were recognised. For MMP-2, a method which recognises only the MMP-2/TIMP-2 complex was used, in which the primary serum value was calculated. The pre-operative samples were analysed for TIMP-1, MMP-9, TIMP-2 and MMP-2 in plasma samples and standards for TIMP-1, TIMP-2 and MMP-2/TIMP-2 were incubated for 60 minutes, and for MMP-9 overnight. The wells were thoroughly washed between each stage of the procedure, in the first phase with PBS, and in the later stages with PBST (0.05% Tween 20 in PBS). The bound proteins were detected with polyclonal antibodies produced in chickens against each of the analyses (anti-TIMP-1 ab, anti-MMP-9 ab, anti-TIMP-2 ab or anti-MMP-2 ab; SBA Sciences, Oulu, Finland). A peroxidase-labelled anti-chicken antibody (Chemicon International, CA, USA) was used to detect the bound polyclonal antibody, and an OPD-solution (o-phenylenediamine dihydrochloride) (Sigma Chemical Co., USA) was used to visualise the peroxidase label. The reaction was stopped with 1.8 M H2SO4. The optical density of the solution was measured on 492 nm (Anthos 2000 microplate reader), and the results were calculated from the linear parts of the standard curves.

Statistical analysis. The patients were first divided into three groups according to treatment response: complete response (no sign of a tumour in clinical, radiological or laboratory examinations), partial response (residual tumour left, 50% decreased from the primary situation in clinical, radiological or laboratory examinations) and progressive disease (increased tumour load in clinical, radiological or laboratory examinations) (18, 19). The results were analysed graphically and by analysis of variance to compare the changes in TIMP-1, MMP-9, TIMP-2 or MMP-2 mean serum concentrations of different patient groups in the third and sixth chemotherapy cycles. The patients were then divided into two groups according to the change in their serum values, namely, those with an increased serum value and those with a decreased value in the follow-up. The patient distribution according to the treatment response in these groups was analysed by the Chi square test or Fisher’s exact test. The t-test was used to compare the changes in the means and the absolute mean values of the two different treatment response groups in the further analysis with homogeneous patient cohorts. Pearson’s correlation test for continuous variables was used to compare the pre-operative serum and plasma values of individual patients. In the statistical analysis, a p-value less than 0.05 was considered significant.

Results

The changes in TIMP-1, MMP-9, TIMP-2 and MMP-2 serum values during chemotherapy. The follow-up serum values were analysed for TIMP-1, MMP-9, TIMP-2 and MMP-2 in each patient during the chemotherapy, and the ratio to the primary serum value was calculated. The pre-operative sample was marked as 1.0. Thus, the higher follow-up values were above 1.0, and lower ones less than 1.0. The patients were grouped into those with complete response, those with partial response and those with progressive disease.
There were no statistically significant differences between the changes of the values in the different response groups, in the third or sixth chemotherapy cycles, in any of the proteins. However, TIMP-1 and MMP-9 serum values stayed at about the pre-operative level for complete respondents, whereas in partial respondents or patients with progressive disease the values decreased to between 43-85% from the pre-operative level during chemotherapy. In TIMP-2 and MMP-2 measurements, serum values increased to approximately 150% of the pre-operative level by the sixth chemotherapy cycle in all patient groups. Thus, the changes in TIMP-1 and MMP-9 serum values were in line with each other, as were the changes in TIMP-2 and MMP-2 serum values during chemotherapy.

The preceding data was analysed also by dividing the patients into those with an increasing serum level and into those with a decreasing serum value for each of the proteins. There was no statistically significant difference in the patient distribution according to the treatment response in these patient groups. However, a tendency for higher serum values was seen in patients with a favourable response to chemotherapy within all the operability groups. In the MMP-9 measurements patients with poor chemotherapy responses tended to have decreasing serum MMP-9 values during the treatment compared to almost non-changing or slightly increasing values in the patients with favourable chemotherapy responses within all the operable groups. This difference was statistically significant in patients with optimally operated tumours ($p=0.043$). In optimally debulked patients also the absolute serum values of MMP-9 were higher in good respondents compared to poor respondents during chemotherapy.

In TIMP-2 and MMP-2 measurements there was a clear tendency towards increasing serum values during chemotherapy in patients with a favourable response to cytotoxic treatment within all the operable groups. For TIMP-2 this was statistically significant in optimally debulked patients with stage III-IV disease ($p=0.027$), and for MMP-2 in the group of all optimally operated patients ($p=0.014$). Also the absolute serum values were higher in patients with good responses to treatment compared to the ones with poor responses.
significant correlation between the serum and plasma values for TIMP-1 \((p=0.0004)\), MMP-9 \((p=0.024)\) and MMP-2 \((p=0.005)\) (Figure 1). There was no statistically significant correlation between the plasma and serum TIMP-2 values \((p=0.139)\). The mean plasma values of the analysed proteins were lower than the mean serum values. For TIMP-1 the average plasma value was 62% of the average serum value, for MMP-9 39%, for TIMP-2 93% and for MMP-2 65%.

**Discussion**

There are no previous reports of sequential serum analyses of gelatinases and TIMPs during chemotherapy in ovarian cancer. In general, there are only a few studies with repeated MMP and/or TIMP measurements during adjuvant treatment or the post-operative follow-up of any malignancies. Ranuncolo et al. have analysed repeated MMP-9 activity by zymography in the plasma of patients with breast cancer (20). In contrast to the present study, they found decreasing MMP-9 activity in the patients achieving a complete response, whereas the patients showing progression had increasing MMP-9 activity. Additionally, plasma MMP-9 activity increased prior to clinical detection of recurrence. In the work of Waas et al., on the other hand, neither proMMP-2 nor -9 activity, determined by gelatin zymography in consecutive plasma samples, appeared to be of value for monitoring the disease status in patients operated for colorectal cancer (21).

Pre-operative serum values of MMP-9, TIMP-2 and MMP-2 were not found to be prognostic factors in epithelial ovarian cancer in our preceding study (16). However, in sequential serum measurements during chemotherapy the changes in their serum values seemed to reflect the treatment response in optimally operated ovarian cancer patients. An increase in the serum value of these proteins was associated with a good response to the chemotherapy. The mechanism of this increase is unclear. It is possible that the cytotoxic effect on the remaining residual tumour cells released these proteins into circulation. The increase of MMP-2, TIMP-2 and MMP-9 could also be mediated by cytokines, such as interleukins. It is known that interleukins modify MMP production (22, 23), and that they are affected by platinum agents (24, 25).
In our previous work, a high pre-operative TIMP-1 serum value was found to be correlated to the majority of the aggressive features of ovarian cancer, including poor prognosis (16). In the current work complete respondents to the chemotherapy retained their pre-operative serum TIMP-1 value during the chemotherapy, whereas poor respondents seemed to have falling values when analysing the study population as a whole. Further analysis revealed that the decreasing serum values occurred mostly in the non-optimally operated patients with the highest pre-operative values, regardless of the treatment response. The changes were not, however, statistically significant. In conclusion, sequential measurements of serum TIMP-1 during cytotoxic chemotherapy were not found to contribute to the evaluation of treatment response.

Jung et al. have criticised the use of serum as a source of measurement especially in the MMP-9 analyses (26, 27). They have found up to 20-fold higher MMP-9 values in serum compared to contemporaneous plasma samples of the same patient (26, 27). MMP-9 has been suspected to be released from blood cells during serum collection procedures. In the present study, a statistically significant correlation was found between TIMP-1, MMP-9 and MMP-2 plasma and serum values in individual patients. However, the mean plasma values of the analysed proteins were lower than the mean serum values, with the biggest difference seen in MMP-9, where the average plasma value was only 39% of that in the serum samples. Furthermore, similar correlations to the clinicopathological factors of ovarian cancer were found in serum and plasma analyses for TIMP-1 and TIMP-2, and only slight differences were seen in MMP-9 and MMP-2 measurements (data not shown). Our data is too limited to draw conclusions on the use of serum as a sample source. However, it is important to identify the sample source used in the different studies, and a direct comparison of the results derived from analyses using different sample sources is not possible.

In conclusion, our preliminary results indicate that the measurement of serum levels of gelatinases and tissue inhibitors-1 and -2 during adjuvant chemotherapy has only limited value in predicting the responsiveness of ovarian cancer to treatment. This pertains especially to TIMP-1. Further studies with larger patient cohorts would be necessary to confirm these results.

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