Differential Associations of MMP-2 and MMP-14 with Stromal Amounts and T-lymphocyte Presence in Ovarian Cancer

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Abstract

Aim: Ovarian cancer prognosis is influenced by factors such as intratumoral T-lymphocyte presence and the amount of stroma formation relative to the epithelial tumor compartment. The regulation of these factors is unknown. Matrix metalloproteinases are involved in stromal remodeling and may be involved in T-cell trafficking as well. This study investigates the quantitative relationships between the matrix metalloproteinases MMP-2 and MMP-14 and both relative stroma amounts ('stroma percentage') and the presence of T lymphocytes in the stromal and epithelial compartments of ovarian cancer.

Patients and methods: In 86 patients with ovarian cancer, MMP-2 and MMP-14 expression and T-lymphocyte presence was determined using semi-quantitative immunohistochemistry. Stromal percentage was determined by area calculation in haematoxylin-eosin slides. Per slide, 3 random images at the tumor-stroma interface were investigated.

Results: Epithelial MMP-14 scoring correlated negatively with T-lymphocytes in tumor epithelium: correlation coefficient rho \( \approx -0.36 \) (p < 0.01) for CD3, \(-0.30 \) (p < 0.01) for CD8 and \(-0.24 \) (p < 0.05) for CD45Ro. Stromal MMP-2 was positively related to stroma percentage (rho 0.29; p < 0.01). No significant correlations were found for MMP-14 with stromal amounts, or MMP-2 with T-cell presence in stroma or tumor epithelium.

Conclusion: Opposite to our hypothesis that MMPs reduce stromal amounts and permit T cell trafficking into both stromal and epithelial tumor parts, MMP expression was associated with higher relative stromal amounts and lower presence of T lymphocytes. In particular, we found that epithelial MMP-14 expression was negatively associated with T-lymphocyte presence in the tumor suggesting a role for MMP-14 in negatively regulating T-cell trafficking by other means. Stromal MMP-2 expression was associated with an increase in stromal amounts suggesting an ineffective feedback on tumor stroma volume growth by the expression of this gelatinase. More research is necessary to determine the specific actions of MMPs in ovarian cancer.

Keywords: Ovarian cancer; MMP-2; MMP-14; T-lymphocytes; Stromal percentage; Immunohistochemistry

Introduction

Ovarian cancer is known for its bad prognosis due to the lack of screening possibilities and the late onset of symptoms leading to diagnosis mostly in advanced disease. Despite intensive treatment with debulking surgery and chemotherapy, 5-year survival rates remain low [1].

In the past few years, the investigation of the stromal compartments of tumors has increasingly gained importance [2-5]. Stromal alterations are commonly found around the invasive front of a malignant tumor. One such alteration can be described as a desmoplastic stroma reaction, characterized by a dense fibrotic network with deposition of various types of fibrillar collagen. The presence of desmosplastic stroma correlates with adverse prognosis in for example endometrial cancer as well as in a subset of ovarian cancers [5-7].

The maintenance of stroma often correlates with the upregulation of matrix metalloproteinases (MMPs), especially MMP-14, as a feedback loop to keep stroma at constant density [8]. In cancer however, this process is often deregulated and connective tissues develop into dense tumor stroma despite MMP upregulation and the possibility to break up tissue again [9-11].

MMPs are ubiquitous zinc-dependent proteases that are important in numerous physiological and pathophysiological processes, including ovulation and reproduction but also cancer progression and metastasis [12]. Specifically, MMP-14 and MMP-2 are involved in physiological growth of ovarian follicles [13] and due to their high expression rate, could also be involved in the growth of malignant ovarian cysts [14,15]. Some studies in ovarian cancer have found that MMP-14 and MMP-2 are independent prognosticators, whereas others have concluded that their predictive value is limited [16-18].

Other important processes in a tumor's stromal compartment include the body's own defense to tumor growth by immunomodulation by different immune cell types, and include, i.e., cytotoxic T-cell-mediated tumor cell killing. These T-cells arrive from neighboring...
Peripheral blood vessels and must travel through connective tissue compartments in order to reach and kill the tumor cells (Figure 1) [19].

The efficiency of T-cell trafficking is influenced by extracellular matrix (ECM) deposition, its geometry, which includes a guidance as well as a barrier function, and proteolytic remodeling which may open tracks of least resistance for moving cells. In malignant stroma the network densities, develops thickened matrix fibers and thus may hinder T-lymphocyte migration [3].

In ovarian cancer, both the amount of stroma deposition relative to the tumor mass ("stroma percentage") and T-lymphocyte presence influence prognosis [19-21]. Both survival-promoting presence of tumour infiltrating T-lymphocytes and survival-impairing high stroma percentage is reported for advanced ovarian cancer [19-21]. However, whether and how these survival-determining factors correlate with modulated MMP-expression typical in ovarian cancers remains unknown.

We here explored in a large regional cohort with long-term follow-up whether MMP-2 and MMP-14 expression was associated with these stromal and stroma-derived processes in ovarian cancer for the reduction of stroma mass by ECM turnover and attraction of T-cell influx. Our data indicate, opposite to our expectations, that MMP-2 expression positively correlates with relative stromal amounts and MMP-14 negatively associates with T-cell presence in tumor tissue.

**Patients and Methods**

**Clinical data**

Included in this retrospective cohort were all 116 patients diagnosed with ovarian cancer between January 1997 and December 2003 at St. Elisabeth Hospital and Tweesteden Hospital, both in Tilburg, and at Amphia Hospital, Breda all in, the Netherlands. Patients who had undergone neo-adjuvant chemotherapy with secondary cytoreductive surgery were excluded (n = 11), because previous chemotherapy may have influenced the immunohistochemical results of MMP's and T-lymphocytes of histopathological material obtained at the time of surgery [22]. All patients were followed until March 2013. Four patients emigrated and were thus lost to follow-up. For 15 patients, no residual tumor material was available for complete immunohistochemistry, leaving 86 patients for analysis. Included in this retrospective cohort were all 116 patients diagnosed with ovarian cancer between January 1997 and December 2003 at St. Elisabeth Hospital and Tweesteden Hospital, both in Tilburg, and at Amphia Hospital, Breda all in, the Netherlands. Patients who had undergone neo-adjuvant chemotherapy with secondary cytoreductive surgery were excluded (n = 11), because previous chemotherapy may have influenced the immunohistochemical results of MMP's and T-lymphocytes of histopathological material obtained at the time of surgery [22]. All patients were followed until March 2013. Four patients emigrated and were thus lost to follow-up. For 15 patients, no residual tumor material was available for complete immunohistochemistry, leaving 86 patients for which MMP-14 and MMP-2 expression and T-lymphocyte presence were determined. For a flowchart of the patients see Figure 2.

The patients underwent a laparotomy with staging or debulking surgery was found to be optimal if the maximum diameter (length, width or depth) of the individual residual tumor deposits were less than 1 cm. In patients with FIGO stage Ia or Ib ovarian cancer with differentiation grade I, no adjuvant therapy was given. All other patients received 6 to 9 courses of adjuvant platinum-based chemotherapy. The only treatment change during the study period was in adjuvant polychemotherapy. In 1999, the combination of cisplatin and cyclophosphamide was changed to carboplatin or cisplatin combined with paclitaxel. FIGO stage (I to IV) was categorized according to World Health Organization (WHO) criteria. Survival data were collected from the medical records, hospital-registered death certificates or registration by the general practitioner of the patient.

**Histopathological data**

All histopathological results and original slides were reviewed by an independent pathologist, specialized in gynecological pathology. Histology and differentiation grade were categorized according to WHO criteria, grading was based on the observer's impression of both architectural and cytological features [23]. In the original hematoxylin eosin slides, both the areas of tumor epithelium and stroma percentage were quantified from three random images taken adjacent to the epithelial tumor fields (Figure 3) and averaged according to Labiche et al. [21]. Necrotic or adenofibromatotic components of the tumor were excluded from the percentage calculation.

**Semi-quantitative immunohistochemistry**

Paraffin-embedded blocks were selected from the archives of the histopathology laboratory and immunohistochemistry was performed as described before [13]. In brief, sections of 3 μm thickness were deparaffinized in xylene and rehydrated with graded alcohol. Each slide included positive controls for MMP's consisting of placenta. Each run included negative controls without primary antibody. Endogenous peroxidase was blocked with 3% H2O2 and 5% normal goat serum. The primary antibodies used for staining are described in Table 1. After each incubation step, slides were washed two times with phosphate buffered saline (PBS).
Slides were incubated with secondary antibody, poly-HRP-GAM/R/R IgG Powervision (Immunologic, Duiven, the Netherlands) for 60 minutes at room temperature. Staining was done with diaminobenzidine (Immunologic, Duiven, the Netherlands) for 5 minutes. Slides were counterstained with hematoxylin.

**Scoring of immunostaining**

The scoring system according to Kamat et al. used for MMP-14 and MMP-2 incorporated intensity (0 = absent, 1 = weak, 2 = moderate, 3 = strong staining) and percentage of positive tumor cells (0 = 0%, 1 = 1-25%, 2 = 26-50%, 3 = 51-75%, 4 = 76-100% of cells) [16]. Two investigators scored all slides (MCCV, EB). If they disagreed, consensus was reached by consulting the gynaecopathologist (AAW).

T-lymphocyte count was recorded according to Zhang et al. T-lymphocytes were counted in three fields of the tumor compartment and three for the stromal compartment and averaged. CD3 was used as a pan-T-lymphocyte marker. CD8 was used as a cytotoxic T-lymphocyte marker as the presence of CD8 positive cytotoxic lymphocytes is most important for tumor cell killing [19]. The total amount of intratumoral T-cells consist of the CD3 positive cells. CD3 positive cells minus CD8 positive cells will make up the helper T-lymphocyte compartment. Of the helper T-lymphocyte compartment, the regulatory T-cells with CD45Ro as a regulatory T-lymphocyte marker seem to be the most interesting for prognosis [24]. For these markers, the same fields were counted as the fields used for CD3 determination. In order to make our result comparable to the results of Zhang et al. on clinical characteristics, we followed their methodology in the number and selection of fields and markers despite newer insights into the markers for regulatory T cells [19,25].

**Statistical analysis**

Statistical analysis was performed using the Statistical Package for Social Sciences 20.0 (SPSS Inc., Chicago, Il). Clinicopathological and survival data and immunohistochemistry scores of early-stage and advanced-stage patients were compared using independent samples t-tests for continuous variables if normally distributed, Mann-Whitney tests if continuous variables were not distributed normally and chi-square tests for categorical variables.

To determine the correlation coefficients between MMP-14 and MMP-2, T-lymphocytes and stromal volume, Spearman’s rho correlation coefficients were calculated; a value of 0.1 being considered a small correlation, 0.3 medium and 0.5 a high correlation.

**Results**

Of the 101 patients in this consecutive series from a regional laboratory, 33 were early-stage patients (FIGO Ia-IIa). The median follow-up time was 59 months (minimum 0- maximum 195). Patient characteristics including histopathological subtypes reflect the distribution pattern of diagnosis in the Netherlands 1989-2009 [1]. The patient characteristics are summarized in Table 1. Because the pathophysiology of early-stage ovarian cancer and advanced-stage ovarian cancer are different, we present the results for the two groups in the tables separately. To relate MMP staining to epithelial tumor or stromal areas and determine the number of T- cell infiltrates, firstly, MMP immunohistochemistry was performed and quantified (Figures 3 and 4) (Table 3 upper part). The MMP-14 and MMP-2 expression in the tumor epithelium was positive (Overall Score 1) in 53/94 and 84/94 of all patients (early and late stage together), respectively, and in the tumor stroma of 56/94 and 30/94 patients (with staining intensity scored weak or strong, respectively).

Second, Table 3 (lower part) shows the median T-cell count for the different markers. For the T-lymphocyte parameters, a large range of counts was observed for both the tumor epithelium and the stromal compartment. T-cells counts were recoded into < 5/HPF, 6-20/HPF and > 20/HPF according to Zhang [19]. Approximately half of the patients had five or less positive T-lymphocytes per HPF in the tumor epithelium (58.1% for CD45Ro, 58.1% for CD8 and 50.0% for CD3). In the stromal compartment, more CD45Ro, CD8 and CD3 positive T-lymphocytes were present. Only 27.9%, 32.6% respectively 19.8% of patients had five or less positive T-lymphocytes in the stromal compartment of the tumor. More T-cells were found –both epithelial and stromal- in advanced stage patients compared to early-stage patients, but only for cytotoxic T-cells (CD8) in stroma this difference was significant.

Third, in early-stage patients, the median percentage of stroma was 40% and in advanced-stage patients 30% (Table 3). Figure 3 illustrates different examples of a tumor with a low and a tumor with a high percentage of stroma. In the lower panels, the slide with CD3 immunostaining is presented.

In Figure 4 different examples of stroma are illustrated with A) necrotic and hemorrhagic stroma, B) more fibrillary and dense stroma and C) normal stroma with inflammatory cells.

Median overall survival for advanced-stage patients was 25

[38], [39]

Table 1: Antibodies for immunohistochemistry.
(minimum 0–maximum 191) months in the group of patients with five or less CD3 positive cells versus 47 (minimum 3–maximum 174) months in those with greater than five CD3 positive cells. For CD45Ro, median overall survival was 33 (minimum 0–maximum 191) and 40 (minimum 3–maximum 174) months, respectively, and for CD8 28 (minimum 0–maximum 191) and 46 (minimum 3–maximum 158) months, respectively. These differences were not statistically significant, but in line with the previous reported results on survival [19].

If the stroma percentage is dichotomized at the median of 30% for advanced-stage patients, median overall survival is 19 (minimum 0–maximum 174) months for patients with <30% stroma and 44 (minimum 6–maximum 191) months for patients with ≥30%. Also, this difference is not statistically different.

Correlation between MMP-14 and MMP-2 expression, T-lymphocytes and stromal volume

Contrary to our original hypothesis that MMP-14 expression in the tumor would positively correlate with T-cell influx, we found that epithelial MMP-14 expression negatively correlated with the presence of T-lymphocytes and yielded correlation coefficients rho = -0.36, p < 0.001 for CD3, rho = -0.30, p < 0.005 for CD8, and rho = -0.24, p < 0.03 for CD45Ro (Table 4). No significant correlations between stromal MMP-14 and MMP-2 and the presence of T-lymphocytes in the stromal compartment were found.

Neither significant correlations were found for MMP-2 expression in the tumor epithelium nor in the stroma on T-cell influx. Interestingly, high MMP-2 in stroma significantly correlated with high stroma percentage (rho 0.29, p < 0.008), which is also contrary to our original hypothesis.

In conclusion, MMP-14 expression in the tumor epithelium negatively correlates with T-cell influx and stromal MMP-2 expression correlates with the amount of stroma.

Discussion

In this study, we investigated the relationships between MMP-

Table 3: Results of immunostaining with p= values between early-stage and advanced stage using Mann-Whitney U tests.

![Figure 4: Panel of representative slides of A) normal stroma with inflammatory cells (arrowheads), B) fibrillar and dense stroma and C) dense and more heterogeneous stroma with series of 1 haematoxylin-eosin staining, II MMP-14 staining (brown) and III CD3 staining (dark brown) (10x10 magnification). Stromal compartments in the slides are indicated with stars and CD3 positive inflammatory cells with arrowheads.](image-url)
stroma are important, in particular, how the arrangement of collagen
MMP-14 expression on T lymphocyte presence is controversial. For
towards the tumor epithelium. Our result of the inverse effect of
14 expression and T-lymphocyte presence, indicating a role for
an inhibitory effect on vessel formation and invasion was found [28],
of stroma relative to tumor epithelium was not described in that study,
blockade and stroma formation in skin cancer, even though the amount
uncontrolled, stroma turnover. In line with this, in a study on MMP-2
result of less MMP-2 production which regulates part of the, though
in relation to the stroma mass. Also, perhaps is more stroma mass a
this longer survival goes along with overall less formed tumor mass
between stroma percentage and survival was also different than found
stroma percentage we found in early-stage patients, the relationship
center, resulting in a possible selection bias. In line with the larger
ours being a large regional cohort and theirs from a university referral
attributable to the different populations that the patients come from,
while early stage tumors were not included in their study. This may be
early-stage tumors, we found a median percentage of 40%,
with those in the study by Labiche et al. [21]. For advanced stage, the

tumor stroma followed by infiltration of the tumor mass, but clearly
MMP's on the ECM would facilitate T-lymphocyte infiltration of the
14 expression. It seems logical to assume that the degrading action of
lymphoid structures containing a reticular network are sometimes
aligned with a mesenchymal, and thus MMP-14-positive profile have less
lymphocyte infiltration than other tumor types such as high graded
serous and endometroid tumors [7]. From this perspective, high MMP-
14 expression in an ovarian tumor may reflect a mesenchymal profile
and thus contribute to less T-lymphocyte infiltration, for yet unknown
reasons. Unfortunately, analyzing different histopathological groups in
our sample will lead to a too small sample size to draw any conclusions.

Comparison of our results with those of earlier studies on
T-lymphocytes yields the following conclusions. Zhang et al, established
the influence of the presence of tumor-infiltrating lymphocytes on
prolonged survival in ovarian cancer [19]. This is in line with our
results. However, the underlying mechanisms for this finding are poorly
understood.

The roles that MMP-14 and MMP-2 play in malignant tumor
development are largely unknown. From the studies on normal ovaries
[8,30] and on auto-immune disease [31,32] MMP-14 expression
strength should positively correlate with T-cell infiltration, but
negatively correlate with stroma percentage. The findings in our
presented study show opposite results with the presence of MMP-14
in the tumor epithelium correlating negatively with T-lymphocytes
in the tumor epithelium. No relation between MMP-14 and stroma
percentage is found, only for stromal MMP-2 a positive correlation
is found. The results from the present study thus suggest that in the
ovarian environment, the roles of MMP-14 and MMP-2 in malignant
stroma are quite different from the roles that MMP-14 and MMP-2 may
play in healthy ovarian stroma with an expected positive correlation
between MMP-14 and T-cell infiltration and a negative correlation
between MMP-2 and stroma percentage [3].

In auto-immune disease, MMP-14 and MMP-2 are important
for T-lymphocyte influx via CD44, the integrin pathways and TGF-β
[31,33-35]. In ovarian cancer, we found in contrast a negative correlation
between the presence of the different types of T-lymphocytes and MMP-
14 expression. It seems logical to assume that the degrading action of
MMP's on the ECM would facilitate T-lymphocyte infiltration of the
tumor stroma followed by infiltration of the tumor mass, but clearly
other actions of MMP-14 on signaling pathways are more important.
It is possible that chemotactic signals in the micro-environment of
the tumor could be more important than the arrangement of the collagen
fibers [29,33]. The negative association of MMP-14 on T-lymphocyte
influx is in accordance with the negative effect of MMP-14 expression
and survival [16]. This negative correlation that we have found would
favor the use of MMP-14 inhibitors, which have been, however, already
been reported to be ineffective in advanced ovarian cancer [36]. Since
correlation coefficients of this MMP-14/T-cell presence relationship are
around 0.30, indicating a moderate effect, other factors must also play a
role, like maybe CD44 and TGF-β [33,37].

### Table 4: Spearman’s rho correlation coefficients for MMP-parameters and T-lymphocyte parameters and stroma percentage.

<table>
<thead>
<tr>
<th></th>
<th>CD45Ro tumor expression</th>
<th>CD8 tumor expression</th>
<th>CD3 tumor expression</th>
<th>percentage stroma</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-14 tumor epithelium</td>
<td>-0.24*</td>
<td>-0.30*</td>
<td>-0.36*</td>
<td>0.17</td>
</tr>
<tr>
<td>MMP-14 stroma</td>
<td>0.04</td>
<td>-0.17</td>
<td>-0.11</td>
<td>-0.05</td>
</tr>
<tr>
<td>MMP-2 tumor epithelium</td>
<td>-0.10</td>
<td>-0.15</td>
<td>-0.09</td>
<td>0.04</td>
</tr>
<tr>
<td>MMP-2 stroma</td>
<td>0.00</td>
<td>0.02</td>
<td>0.03</td>
<td>0.29*</td>
</tr>
</tbody>
</table>

*Correlation is significant at the 0.05 level (2-tailed)
**Correlation is significant at the 0.01 level (2-tailed)

Abbreviations: MMP: Matrix Metalloproteinase; CD: Cluster of Differentiation

example of lung cancer. When treated with collagenases, T-lymphocyte
movement towards the tumor increases. This is in favor of the concept
of partial degradation and thus widening of the collagen network
for increased T-cell migration [29]. More T-lymphocyte infiltration,
especially CD3+CD8+ lymphocytes will result in a better prognosis for
the patients [25]. In our advanced-stage patients, we found significantly
more CD8+ lymphocytes in stroma. In another example in lung cancer,
lymphoid structures containing a reticular network are sometimes
located far from the tumor at the boundary between tumor stroma
and healthy tissue [3]. In ovarian cancer, this boundary is often less
clear, because of the lack of an invasive front by microscopic detection.
However, also other adverse roles of MMP-14 are possible. Studies
on genetic profiling in ovarian cancer suggest that some tumor types
with a mesenchymal, and thus MMP-14-positive profile have less
lymphocyte infiltration than other tumor types such as high graded
serous and endometroid tumors [7]. It is challenging to interpret our results from a pathophysiological
viewpoint. The ovaries are a unique environment to study relationships
between MMP parameters and stromal factors. Most ovarian cancers
probably do not arise in the ovaries themselves, [26,27] and in terms of
disease characteristics, ovarian tumors are strikingly different to tumors
in other organs. The main distinguishing features of ovarian tumors are
their often cystic nature, their large size at detection and the prolonged
confinement of metastases to the peritoneal cavity.

Regarding stroma percentage, our results are not fully comparable
with those in the study by Labiche et al. [21]. For advanced stage, the
median stroma percentage was 30% in our group and 50% in their
group. In early-stage tumors, we found a median percentage of 40%,
while early stage tumors were not included in their study. This may be
attributable to the different populations that the patients come from,
ours being a large regional cohort and theirs from a university referral
center, resulting in a possible selection bias. In line with the larger
stroma percentage we found in early-stage patients, the relationship
between stroma percentage and survival was also different than found
in Labiche's study. In our group of advanced stage patients, those
with a stroma percentage larger than the median percentage of 30
had a longer survival, although not statistically significant. Perhaps
this longer survival goes along with overall less formed tumor mass
in relation to the stroma mass. Also, perhaps is more stroma mass a
result of less MMP-2 production which regulates part of the, though
uncontrolled, stroma turnover. In line with this, in a study on MMP-2
blockade and stroma formation in skin cancer, even though the amount
of stroma relative to tumor epithelium was not described in that study,
an inhibitory effect on vessel formation and invasion was found [28],
reasoning for a better overall survival.

We found a negative association between epithelial MMP-
14 expression and T-lymphocyte presence, indicating a role for
tumor-expressed MMP-14 in inhibiting T-lymphocyte trafficking
towards the tumor epithelium. Our result of the inverse effect of
MMP-14 expression on T lymphocyte presence is controversial. For
T-lymphocyte infiltration, structural characteristics of malignant
stroma are important, in particular, how the arrangement of collagen
fibers facilitates or hinders T-lymphocyte trafficking, as shown in an
The main strength of the present study is that it is based on a large regional cohort with long-term follow-up. We only excluded patients who had undergone neo-adjuvant chemotherapy, believing that including them might adversely influence the MMP staining results [22].

This study demonstrates the importance of characteristics of malignant stroma in ovarian cancer and the significant role for MMP-14 in T-lymphocyte influx. Clearly, further research is necessary to determine the specific actions of MMPs in ovarian tumor stroma, as well as their interactions with other molecules in this context.

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MCV designed the study, scored the immunohistology, performed statistical analysis and drafted the manuscript. EB carried out and scored the immunohistology under supervision of EdB. BJD supervised statistical analysis. AW conceived of the study, supervised scoring the immunohistologies and helped to draft the manuscript. TVK, KW and LM supervised the study and helped to draft the manuscript. All authors read and approved the final manuscript.

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