Down-regulation of proteasomal subunit MB1 is an independent predictor of improved survival in ovarian cancer

Ninke Leffers
Marloes J.M. Gooden
Anna A. Mokhova
W. Martin Kast
H. Marike Boezen
Klaske A. ten Hoor
Harry Hollema
Toos Daemen
Ate G.J. van der Zee
Hans W. Nijman

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Abstract

Objective: To investigate the expression and to determine the prognostic impact of components of the antigen processing and presentation pathway (APPP) in ovarian cancer.

Methods: Expression of MB1, LMP7, TAP1, TAP2, ERp57, ERAP1, β2-microglobulin and the α-chains, HLA-B/ C and HLA-A, of the MHC class I molecules was evaluated on tissue microarrays containing primary tumor samples from 232 FIGO stages I–IV ovarian cancer patients. Expression levels were correlated to clinicopathological data and disease specific (DSS) survival.

Results: Patients with expression of all components of the MHC class I complex, i.e. HLA-A+–β2-m+ and HLA-B/C+–β2-m+ patients, more often had expression of LMP7, a component of the immunoproteasome than patients with other phenotypes (p < 0.001). These patients were also more prone to loss of MB1, part of the constitutive multicatalytic proteasome (p < 0.05). Nuclear MB1 expression was an independent predictor of worse DSS (HR 1.94, 95% CI 1.16–3.26, p = 0.012). The HLA-B/C+–β2-m+ phenotype was an independent predictor of a better prognosis (HR 0.63, 95% CI 0.40–0.99, p = 0.047). Median DSS was longer for patients with normal nuclear expression of LMP7 (57.4 vs. 31.0 months, p = 0.029).

Conclusions: The prognostic influence of the proteasomal subunit MB1 and the MHC class I complex in ovarian cancer provides a rationale for targeting these specific APPP components in ovarian cancer.
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Introduction

Ovarian cancer is the second most common gynecological cancer and the leading cause of death from gynecological malignancies in the Western world (1). The exploration of immunotherapy as a new treatment modality for this disease relies on evidence of improved clinical outcome when intratumoral T-lymphocytes are present in ovarian cancer patients, which is most likely a reflection of an anti-tumor immune response (2). Immunotherapy can potentially overcome the poor survival rate seen with standard treatment, i.e. surgical debulking and adjuvant platinum based chemotherapy (3;4), by inducing or augmenting anti-tumor immune responses. Different strategies have been employed, including adoptive transfer of anti-tumor T cells or natural killer (NK) cells, as well as active immunization with tumor antigens, such as p53. Contrary to some promising murine studies (5-7), immunotherapeutic trials in human subjects have only reached minimal clinical benefit (8;9). This may partly be due to the development and exploitation of immune escape mechanism by the tumor. Down-regulation or absence of components of the antigen processing and presentation pathway (APPP) is believed to be such mechanism as presentation of antigens either processed within the MHC class I or the MHC class II pathway is a prerequisite for recognition by CD8+ cytotoxic and CD4+ helper T-lymphocytes, respectively (10;11).

The mainstay of the anti-tumor immune response is via MHC class I. In the MHC class I pathway, cytoplasmic and nuclear proteins are degraded by the interferon-γ inducible subunits LMP7, LMP2 and LMP10 (components of the immunoproteasome), which can replace subunits MB1(X), delta(Y) and zeta(Z) of the multicatalytic constitutive proteasome (10-14). Immunoproteasomes are generally thought to be more efficient at the production of antigenic peptides than the constitutive proteasome (13) and they tend to produce more and on average slightly longer peptides (15). Immunoproteasomes are preferentially located near the endoplasmic reticulum (ER), whereas the constitutive proteasome can be found throughout the cytoplasm (16). Proteasomes may also be present in the nucleus, especially in case of cell stress (17). Next peptides of a suitable length are transported from the cytoplasm into the endoplasmic reticulum by the transporter associated with antigen processing (TAP), consisting of subunits TAP1 and TAP2 (10;11;13;14;18). Once in the ER, aminopeptidases such as endoplasmic reticulum aminopeptidase 1 (ERAP1) can further trim peptides to a correct size for adequate MHC class I binding. Chaperones (e.g. ERP57) facilitate loading of peptides into the MHC class I molecule (10;11;19-21). Finally, a fully assembled heterotrimeric MHC class I/peptide complex (α-chain, β2-microglobulin and a bound antigenic peptide) is transported to the cell surface, where it may be recognized by CD8+ cytotoxic T lymphocytes (11;13;14;20). A schematic overview of this pathway is given in figure 1.

Although defects and down-regulation in all of the aforementioned components have been identified in several types of cancer, most APPP defects have some, little or no significant relation with clinical parameters and disease outcome (11;22-32). In ovarian cancer, a study of five APPP components recently showed that the number of down-regulated components is an independent prognostic factor (33). We investigated the expression and clinical relevance of these and essential
additional components of the APPP in paraffin embedded tissues obtained from a large series of well-documented ovarian cancer patients.

Materials and methods

Patients
Patients were identified from a database containing clinicopathological and follow-up data of all epithelial ovarian cancer treated with primary debulking surgery according to standard treatment protocols by gynecological oncologists of the University Medical Center Groningen (Groningen, The Netherlands) between May 1985 and April 2003. Patients were selected if sufficient paraffin embedded tissue was available. All patients were staged according to the FIGO classification, and resected tumors were graded and classified by a gynecological pathologist based on the World Health Organization criteria. Adjuvant chemotherapy was given if indicated. Follow-up of all patients was performed regularly up to ten years with gradually increasing intervals.

Figure 1 Schematic overview of the MHC class I dependent antigen processing and presentation pathway
**Institutional review board approval**

All clinicopathologic and follow-up data of patients referred to the Department of Gynecologic Oncology of the UMCG are prospectively collected and stored in a computerized database. For the present study, all relevant data were retrieved from our computerized database and transferred into a separate password-protected anonymous database. Patient identity was protected by study-specific unique patient codes. Patients’ true identity was only known to two dedicated data managers, who have daily responsibility for the larger database. In case of uncertainties with respect to clinicopathologic and follow-up data, the larger databases could only be checked through the data managers. Based on this information, according to Dutch law no further approval from our Institutional Review Board was needed.

**Tissue samples and construction of tissue microarrays**

Paraffin-embedded tissue blocks and corresponding hematoxylin & eosin stained slides constructed from tumor tissue obtained at primary debulking surgery were retrieved from the pathology archives. Tissue microarrays (TMA) were constructed as described previously (34-36). Briefly, primary tumor samples were arrayed using a tissue microarrayer (Beecher Instruments, Silver Spring, Maryland) by taking four representative cores with a diameter of 0.6 mm from marked tumor sites from the individual paraffin embedded tumor block onto a recipient paraffin block at pre-defined array locations. For staining, 4μm sections were cut from each TMA block and applied to APES-coated slides. H&E staining was performed to verify the presence of tumor in the arrayed samples.

**Immunohistochemical staining**

TMA sections were dewaxed and rehydrated, whereupon antigen retrieval was performed in a citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by a 3% H2O2 solution, after which sections were incubated overnight at 4 ºC with the primary antibodies in the following dilutions: anti-MB1 (clone SJJ-3) 1:10, anti-LMP7 1: 100 (clone 1B3, Novus Biologicals, Heerhugowaard, The Netherlands), anti-TAP1 1:20 (clone H-300, Santa Cruz Biothechnology Inc., Heidelberg, Germany), anti-TAP2 1:20 (clone H-210, Santa Cruz Biothechnology Inc., Heidelberg, Germany), anti-ERp57 (clone TO-2) 1: 20, polyclonal anti-ERAP1 1:100 , polyclonal anti-β2-microglobulin 1:400 ( DAKO, Glostrup, Denmark) and anti-MHC class I heavy chain antibodies HCA2 1: 500 (HLA-A) and HC-10 1:100 (HLA-B/C). The anti-ERp57 and anti-MB1 antibodies were a gift from Dr S. Ferrone, University of Pittsburgh, Pittsburgh, PA. The HCA2 and HC-10 antibodies were a gift from Dr. J.J. Neefjes, Netherlands Cancer Institute, Amsterdam, The Netherlands. The anti-ERAP1 antibody was a gift from Dr. M. Tsujimoto, Riken, Wako, Saitama, Japan. Sections were subsequently incubated with DAKO Envision+ (Dako, Heverlee, Belgium). Antigen-antibody reactions were visualized with 3,3-diaminobenzidine, the chromogenic substrate for peroxidase, and hematoxylin was used to counterstain the tissue.
Down-regulation of proteasomal subunit MB1 is an independent predictor of improved survival in ovarian cancer

Table 1 - Clinicopathological characteristics and survival data

<table>
<thead>
<tr>
<th></th>
<th>Total (n=232)</th>
<th>Early stage (n=64)</th>
<th>Late stage (n=166)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>57.7 (14.2)</td>
<td>53.1 (14.5)</td>
<td>59.7 (13.6)</td>
</tr>
<tr>
<td>5 year DSS</td>
<td>39%</td>
<td>79%</td>
<td>25%</td>
</tr>
<tr>
<td><strong>FIGO Stage</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I</td>
<td>45 (19.4%)</td>
<td>45 (70.3%)</td>
<td></td>
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<tr>
<td>Stage II</td>
<td>19 (8.2%)</td>
<td>19 (29.7%)</td>
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<tr>
<td>Stage III</td>
<td>133 (57.3%)</td>
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<td>133 (80.1%)</td>
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<tr>
<td>Stage IV</td>
<td>33 (14.2%)</td>
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<td>33 (19.9%)</td>
</tr>
<tr>
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<td>2 (0.9%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Tumor type</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serous</td>
<td>128 (55.2%)</td>
<td>13 (20.3%)</td>
<td>115 (69.3%)</td>
</tr>
<tr>
<td>Mucinous</td>
<td>27 (11.6%)</td>
<td>18 (28.1%)</td>
<td>8 (4.8%)</td>
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<tr>
<td>Endometroid</td>
<td>33 (14.2%)</td>
<td>19 (29.7%)</td>
<td>14 (8.4%)</td>
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<tr>
<td>Clear Cell</td>
<td>17 (7.3%)</td>
<td>6 (9.4%)</td>
<td>10 (6.0%)</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>9 (3.9%)</td>
<td>2 (3.1%)</td>
<td>7 (4.2%)</td>
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<tr>
<td>Mixed Tumours</td>
<td>10 (4.3%)</td>
<td>5 (7.8%)</td>
<td>5 (3.0%)</td>
</tr>
<tr>
<td>Other</td>
<td>8 (3.4%)</td>
<td>1 (1.6%)</td>
<td>7 (4.2%)</td>
</tr>
<tr>
<td><strong>Tumor Grade</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade I</td>
<td>39 (16.8%)</td>
<td>29 (45.3%)</td>
<td>9 (5.4%)</td>
</tr>
<tr>
<td>Grade II</td>
<td>52 (22.4%)</td>
<td>22 (34.4%)</td>
<td>29 (17.5%)</td>
</tr>
<tr>
<td>Grade III</td>
<td>104 (44.8%)</td>
<td>7 (10.9%)</td>
<td>97 (58.4%)</td>
</tr>
<tr>
<td>Undifferentiated</td>
<td>14 (6.0%)</td>
<td>2 (3.1%)</td>
<td>12 (7.2%)</td>
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<td>4 (6.3%)</td>
<td>19 (11.4%)</td>
</tr>
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<td><strong>Residual disease</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 2 cm</td>
<td>119 (51.3%)</td>
<td>62 (96.9%)</td>
<td>55 (33.1%)</td>
</tr>
<tr>
<td>&gt;= 2 cm</td>
<td>109 (47.0%)</td>
<td>2 (3.1%)</td>
<td>107 (64.5%)</td>
</tr>
<tr>
<td>Missing</td>
<td>4 (1.7%)</td>
<td>0 (0.0%)</td>
<td>4 (2.4%)</td>
</tr>
<tr>
<td><strong>Type of chemotherapy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No chemotherapy</td>
<td>32 (13.8%)</td>
<td>21 (32.8%)</td>
<td>11 (6.6%)</td>
</tr>
<tr>
<td>Platinum containing</td>
<td>122 (44.0%)</td>
<td>22 (34.4%)</td>
<td>79 (47.6%)</td>
</tr>
<tr>
<td>Platinum &amp; taxane containing</td>
<td>72 (31.0%)</td>
<td>13 (20.3%)</td>
<td>59 (35.5%)</td>
</tr>
<tr>
<td>Other</td>
<td>24 (10.3%)</td>
<td>7 (10.9%)</td>
<td>17 (10.2%)</td>
</tr>
<tr>
<td>Missing</td>
<td>2 (0.9%)</td>
<td>1 (1.6%)</td>
<td></td>
</tr>
</tbody>
</table>

DSS = disease specific survival; FIGO = International Federation of Gynaecology and Obstetrics.

**Evaluation of immunostaining**

Scoring was done independently by two investigators, without prior knowledge of the clinicopathological parameters. The semi-quantitative quality control system proposed by Ruiter et al. (37) was used, in which both intensity of staining and percentage of positive tumor cells are determined. The intensity of staining was scored as 0, 1, 2, or 3, indicating absent, weak, positive, or strong positive expression, respectively. The percentage of positive cells was scored as 0 for 0%; 1 for 1–5%; 2 for 5–25%; 3 for 25–50%; 4 for 50–75% and 5 for 75–100%. The sum of both scores was used to identify three categories of expression: normal expression (total score 7–8), partial loss (3–6) and total loss (0–2), as previously described by Mehta et al. (28). Immunohistochemical staining demonstrated strong positive expression of all examined markers in stromal tissue and
Down-regulation of proteasomal subunit MB1 is an independent predictor of improved survival in ovarian cancer.

Figure 1 Representative example of positive and negative / weakly positive staining pattern of several APPP components. a, b) MB1; c, d) LPM7; e, f) HLA-B/C; and g, h) β2-m expression by ovarian tumor cells (400x).
Down-regulation of proteasomal subunit MB1 is an independent predictor of improved survival in ovarian cancer

Table 2 Expression levels of APM components

<table>
<thead>
<tr>
<th>Component</th>
<th>Normal</th>
<th>Loss</th>
<th>Missing</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB1 nucleus</td>
<td>156 (67.2%)</td>
<td>65 (28.0%)</td>
<td>11 (4.7%)</td>
</tr>
<tr>
<td>MB1 cytoplasm</td>
<td>127 (54.7%)</td>
<td>94 (40.5%)</td>
<td>11 (4.7%)</td>
</tr>
<tr>
<td>LMP7 nucleus</td>
<td>88 (37.9%)</td>
<td>134 (57.8%)</td>
<td>10 (4.3%)</td>
</tr>
<tr>
<td>LMP7 cytoplasm</td>
<td>56 (24.1%)</td>
<td>166 (71.6%)</td>
<td>10 (4.3%)</td>
</tr>
<tr>
<td>TAP1</td>
<td>189 (81.5%)</td>
<td>24 (10.3%)</td>
<td>19 (8.2%)</td>
</tr>
<tr>
<td>TAP2</td>
<td>185 (79.7%)</td>
<td>28 (12.1%)</td>
<td>19 (8.2%)</td>
</tr>
<tr>
<td>ERp57</td>
<td>147 (63.4%)</td>
<td>77 (33.2%)</td>
<td>8 (3.4%)</td>
</tr>
<tr>
<td>ERAP1</td>
<td>183 (78.9%)</td>
<td>39 (16.8%)</td>
<td>10 (4.3%)</td>
</tr>
</tbody>
</table>

Both components | All other phenotypes | Missing |
<table>
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</tr>
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<tbody>
<tr>
<td>HLA-A and β2-m</td>
<td>114 (49.1%)</td>
<td>102 (44.0%)</td>
</tr>
<tr>
<td>HLA-B/C and β2-m</td>
<td>162 (69.8%)</td>
<td>56 (24.1%)</td>
</tr>
</tbody>
</table>

Table 3 Associations of MHC class I components with other APPP components

<table>
<thead>
<tr>
<th>Component</th>
<th>Co-expression HLA-A/β2-m</th>
<th>Co-expression HLA-B/C/β2-m</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HLA-A/β2-m</td>
<td>All other phenotypes</td>
</tr>
<tr>
<td>MB1 nucleus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal</td>
<td></td>
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</tr>
<tr>
<td>73 (48.7%)</td>
<td>77 (51.3%)</td>
<td>0.048</td>
</tr>
<tr>
<td>40 (63.5%)</td>
<td>23 (36.5%)</td>
<td></td>
</tr>
<tr>
<td>loss</td>
<td></td>
<td></td>
</tr>
<tr>
<td>56 (45.9%)</td>
<td>66 (54.1%)</td>
<td>0.015</td>
</tr>
<tr>
<td>57 (62.6%)</td>
<td>34 (37.4%)</td>
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<tr>
<td>LMP7 nucleus</td>
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<td></td>
</tr>
<tr>
<td>normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>65 (76.5%)</td>
<td>20 (23.5%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>48 (36.9%)</td>
<td>82 (63.1%)</td>
<td></td>
</tr>
<tr>
<td>loss</td>
<td></td>
<td></td>
</tr>
<tr>
<td>39 (72.2%)</td>
<td>15 (27.8%)</td>
<td>0.001</td>
</tr>
<tr>
<td>74 (46.0%)</td>
<td>87 (54.0%)</td>
<td></td>
</tr>
<tr>
<td>TAP1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>109 (59.9%)</td>
<td>73 (40.1%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2 (8.3%)</td>
<td>22 (91.7%)</td>
<td></td>
</tr>
<tr>
<td>loss</td>
<td></td>
<td></td>
</tr>
<tr>
<td>97 (56.1%)</td>
<td>76 (43.9%)</td>
<td>0.065</td>
</tr>
<tr>
<td>10 (37.0%)</td>
<td>17 (63.0%)</td>
<td></td>
</tr>
<tr>
<td>ERp57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>88 (62.0%)</td>
<td>54 (38.0%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>26 (35.1%)</td>
<td>48 (64.9%)</td>
<td></td>
</tr>
<tr>
<td>loss</td>
<td></td>
<td></td>
</tr>
<tr>
<td>107 (60.1%)</td>
<td>71 (39.9%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>7 (18.4%)</td>
<td>31 (81.6%)</td>
<td></td>
</tr>
</tbody>
</table>

Associations between APPP components and fully assembled MHC class I complexes were evaluated using the Chi square-test. Bold signifies p<0.05.
tumor-infiltrating inflammatory cells, thereby providing an internal positive control. As the proteasome containing the MB1 or LMP7 subunit may be present in both the nucleus and cytoplasm (17) and a difference in intensity was apparent between these organelles, both were scored separately. Staining of ERp57, ERAP1, TAP1, and TAP2 was evaluated in the cytoplasm, while for β2-microglobulin, MHC class I α (heavy) chain (HC-10 and HC-A2) cell membrane staining was graded.

**Statistical Analysis**

Patients were entered into analyses only when at least two cores with viable tumor cells were available. To perform statistical analyses, expression levels were dichotomized. Thus, for MB1, LMP7, ERp57, and ERAP1, patients with total and partial loss were taken together. TAP1 and TAP2 expression were naturally dichotomous, since no total loss was observed. Therefore, partial loss was set off against normal expression. Finally, components of MHC class I complex were dichotomized by comparing patients with a heavy chain+ - β2-m+ phenotype to patients with all other phenotypes. Associations between expression levels of the APPP components and clinicopathological parameters were tested using the χ2 test. Differences in disease-specific survival (DSS) based on expression levels were plotted using Kaplan-Meier survival curves and evaluated by log-rank tests. DSS was defined as the time from primary debulking surgery until death due to ovarian cancer or the date of last follow-up. Subsequently, multivariate analysis was performed with Cox proportion hazards model and was stratified for type of chemotherapy. Only those variables that were significantly associated with DSS in univariate analyses, were entered into the Cox proportion hazards model.

All analyses were performed using SPSS version 14.0 software package for windows (SPSS Inc., Chicago, USA). P values <0.05 were considered significant (tested 2-sided).

**Results**

**Patient characteristics**

Paraffin embedded tumor tissue was available for 259 ovarian cancer patients. Twenty-seven patients were excluded because no samples were available from primary debulking surgery. Patient characteristics are summarized in table 1. The majority of patients presented with serous histology, late stage, and/or high grade disease. Thirty-two patients did not receive chemotherapy, 21 of whom were diagnosed with early stage disease. The remaining 11 patients were either unfit or unwilling to receive chemotherapy. Of the patients treated with chemotherapy 87.9% received a platinum-based regimen. Estimated five-year DSS rate was 39%. Median DSS was 33.8 months (95% C.I.: 22.2-45.4).
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Figure 2 Disease-specific survival (in months) of FIGO stage I-IV ovarian cancer. Cumulative survival time was estimated by the Kaplan-Meier method. Log Rank test was used to evaluate survival differences between groups. A) Nuclear and B) cytoplasmic expression of MB1 is associated with worse disease specific survival. C) Nuclear expression of LMP7 is associated with better disease-specific survival. D) Better disease-specific survival was observed in patients with the HLA-B/C – β2-m phenotype.

Expression levels of APPP components
Expression levels of all APPP components are shown in table 2. Representative staining patterns of several components are depicted in figure 2. Percentages of missing data vary from 3.4-8.2%. Partial and/or total loss of expression was witnessed for all evaluated components.

The presence of a heavy chain and β2-m are prerequisites for the formation of a stable MHC class I complex; co-expression of HLA-A and β2-m and HLA-B/C and β2-m was observed in 49.1%, respectively 69.8% of patients. Relationships between HLA-A - β2-m or HLA-B/C - β2-m and other APPP components are shown in table 3. For both combinations, there was a striking paradox between MB1 and LMP7 expression, i.e. the majority of HLA-A+ - β2-m+ and HLA-B/C+ - β2-m+ patients had normal expression of nuclear and cytoplasmic LMP7, a component of the immunoproteasome, whereas these patients were more often prone to loss of nuclear and/or
Down-regulation of proteasomal subunit MB1 is an independent predictor of improved survival in ovarian cancer.

### Table 4

Multivariate Cox regression analysis of disease specific survival survival in ovarian cancer patients*

<table>
<thead>
<tr>
<th></th>
<th>HR</th>
<th>95% CI</th>
<th>p-value</th>
<th>HR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age ≥ 58 years</td>
<td>1.19</td>
<td>0.76-1.87</td>
<td>0.440</td>
<td>1.16</td>
<td>0.74-1.82</td>
<td>0.510</td>
</tr>
<tr>
<td>Grade III/undiff</td>
<td>2.09</td>
<td>1.26-3.47</td>
<td>0.005</td>
<td>1.94</td>
<td>1.16-3.22</td>
<td>0.011</td>
</tr>
<tr>
<td>Non-serous tumor</td>
<td>0.48</td>
<td>0.28-0.82</td>
<td>0.008</td>
<td>0.50</td>
<td>0.29-0.87</td>
<td>0.014</td>
</tr>
<tr>
<td>FIGO stage III/IV</td>
<td>2.99</td>
<td>1.15-7.74</td>
<td>0.024</td>
<td>3.11</td>
<td>1.19-8.15</td>
<td>0.021</td>
</tr>
<tr>
<td>Residual tumor</td>
<td>2.45</td>
<td>1.53-3.93</td>
<td>&lt;0.001</td>
<td>2.46</td>
<td>1.54-3.93</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MB1 nucleus</td>
<td>1.94</td>
<td>1.16-3.26</td>
<td>0.012</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MB1 cytoplasm</td>
<td></td>
<td></td>
<td></td>
<td>1.40</td>
<td>0.89-2.22</td>
<td>0.150</td>
</tr>
<tr>
<td>Age ≥ 58 years</td>
<td>1.04</td>
<td>0.67-1.63</td>
<td>0.859</td>
<td>1.05</td>
<td>0.40-0.99</td>
<td>0.047</td>
</tr>
<tr>
<td>Grade III/undiff</td>
<td>1.72</td>
<td>1.04-2.82</td>
<td>0.033</td>
<td>1.92</td>
<td>1.16-3.20</td>
<td>0.012</td>
</tr>
<tr>
<td>Non-serous tumor</td>
<td>0.51</td>
<td>0.30-0.86</td>
<td>0.012</td>
<td>0.52</td>
<td>0.30-0.89</td>
<td>0.016</td>
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<tr>
<td>FIGO stage III/IV</td>
<td>2.96</td>
<td>1.18-7.39</td>
<td>0.020</td>
<td>2.93</td>
<td>1.17-7.34</td>
<td>0.022</td>
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<tr>
<td>Residual tumor</td>
<td>2.60</td>
<td>1.61-4.20</td>
<td>0.000</td>
<td>2.48</td>
<td>1.55-3.98</td>
<td>&lt;0.001</td>
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<tr>
<td>LMP7 nucleus</td>
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<td>0.47-1.13</td>
<td>0.163</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA-B/C - β2-m+</td>
<td></td>
<td></td>
<td></td>
<td>0.63</td>
<td>0.40-0.99</td>
<td>0.047</td>
</tr>
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</table>

* Analyses performed stratified for type of chemotherapy; FIGO = International Federation of Gynecology and Obstetrics; HR = hazard ratio; CI = confidence interval; bold signifies p<0.05.

We found that HLA-A+ - β2-m+ patients were also more likely to show normal expression of TAP1, ERp57, and ERAP1 than patients with loss of MHC class I components. HLA-B/C+ - β2-m+ patients often expressed TAP1 and ERAP1 while these components tended to be down-regulated in patients who lacked one or both components of the MHC class I complex (table 3).

Association with clinicopathological factors

Loss of nuclear LMP7 was more frequently observed in patients ≥58 years old (67.8 vs. 51.5%, p=0.014; data not shown), as was the case for loss of cytoplasmic LMP7 (82.6 vs. 65.3%, p=0.003). The HLA-A+ - β2-m+ phenotype was more frequently observed in patients with FIGO stage III/IV disease (70.5 vs. 55.9%, p=0.043; data not shown).

Association with disease-specific survival

Median DSS was 51.5 months shorter for patients with normal nuclear expression of MB1 as opposed to patients with loss of nuclear MB1 (figure 3a; median DSS 27.0 vs. 78.5 months, p=0.005). Likewise, DSS was shorter for patients with normal cytoplasmic expression of MB1 (figure 3b; median DSS 30.5 vs. 60.2 months, p=0.024). On the contrary, nuclear expression of LMP7 was associated with improved DSS (figure 3c; median DSS 57.4 vs. 31.0 months, p=0.029). DSS was more than twice as long in patients with the HLA-B/C+ - β2-m+ phenotype as opposed to patients with any another phenotype (figure 3d; median 45.2 vs. 21.1 months, p=0.015).

We repeated the univariate survival analyses in a subgroup containing 166 patients with advanced stage disease only (data not shown). Improved DSS was demonstrated based on down-regulation...
Down-regulation of proteasomal subunit MB1 is an independent predictor of improved survival in ovarian cancer

of nuclear MB1 expression (median 45.2 vs. 17.8 months, p=0.008) and the HLA-B/C+ - β2-m + phenotype (median 25.7 vs. 13.4, p=0.028).

Multivariate analysis
Only the above-mentioned variables that were significantly associated with DSS in the univariate analyses were entered into the Cox proportion hazards model, which was additionally adjusted for well-known prognostic factors (table 4).
After stratification for type of chemotherapy, normal nuclear MB1 expression was found to be an independent prognostic factor for shortened DSS (HR 1.94, 95% CI 1.16-3.26, p=0.012). Furthermore, the HLA-B/C+ - β2-m+ phenotype was demonstrated to be an independent predictor of longer DSS (HR 0.63, 95% CI 0.40-0.99, p=0.047). Likewise, MB1 expression and the HLA-B/C+ - β2-m+ phenotype were found to be independent predictors of DSS in the advanced stage subgroup (HR 1.78, 95% CI 1.06-2.98, p=0.028, resp. HR 0.55, 95% CI 0.35-0.87, p=0.010).

Discussion
The antigen processing and presentation pathway bridges many cellular organelles, ultimately resulting in the MHC restricted presentation of a small peptide sequence on the cell surface. Recognition of this peptide-MHC complex by the immune system can lead to a potentially life-prolonging anti-tumour immune response (2). Individual components of the APPP may have a prognostic value and/or a therapeutic potential. At the level of the proteasome, our study indicates that the presence of MB1 is an independent predictor of shorter DSS in ovarian cancer. In contrast, expression of LMP7 is associated with longer DSS. At the cell membrane level, the HLA-B/C+ - β2-m+ phenotype is a predictor of longer DSS. Furthermore, patients with heavy chain and β2-m expression are more likely to express LMP7, while patients lacking one of the MHC class I components more often express MB1.
Whereas previous studies reported mainly on the immunoproteasome (22;27;28;38;39), we observed an important role for the constitutive proteasome. To our knowledge, the negative influence of MB1 expression on prognosis of (ovarian) cancer patients has not previously been described, and opposes the results of a study in renal cell carcinoma, where MB1 expression did not influence survival (39). It has been suggested that there is a reciprocal relationship between MB1 and LMP7, which may explain the observed negative prognostic influence of MB1 expression (39;40). We were, however, unable to find such an association between expression levels of LMP7 and MB1.
An alternative explanation for the survival benefit associated with MB1 loss might be a mechanism similar to that observed with proteasome inhibitors, e.g. Bortezomib (41). These agents inhibit the ubiquitin-proteasome pathway in tumour cells, resulting in accumulation of proteins involved in cell cycle regulation, proliferation, differentiation, and apoptosis. Ultimately, proteasome inhibitors increase apoptosis and decrease chemoresistance (42). Patients in whom
Down-regulation of proteasomal subunit MB1 is an independent predictor of improved survival in ovarian cancer

the constitutive proteasome is intrinsically down-regulated might enjoy a similar benefit.

Nuclear expression of the immunoproteasome component LMP7 was associated with longer DSS. Similarly, differences in DSS based on LMP7 expression have been reported for several types of cancer (28;38;39). These survival benefits can be interpreted as the result of immunologic mechanisms by which the generation of more immunogenic peptides results in more efficient activation of the immune system. Since LMP7 expression conveys a positive effect on DSS, proteasome inhibition might not be beneficial for patients expressing LMP7, which suggests that more selective proteasome inhibitors may be needed for optimal clinical benefit. Alternatively, one could envision treating patients with agents that enhance LMP7 and/or MHC class I expression. Histone deacetylase inhibitors, e.g. trichostatin A and valproic acid, have been shown to have these properties, but more specific compounds would be desirable (43).

The final step of the antigen processing and presentation pathway is cell surface expression of a fully assembled heterotrimeric MHC class I complex consisting of a heavy chain molecule, β2-microglobulin, and a bound antigenic peptide. In this study, expression of the HLA-A and HLA-B/C heavy chain molecules was analyzed in combination with β2-microglobulin. We observed that the HLA-B/C+ - β2-m+ phenotype is an independent positive prognostic factor for DSS, confirming the recently published findings of Rolland and colleagues in ovarian cancer (44).

Strikinglly, we found that MB1 expression correlates with loss of the heavy chain+/ β2-m+ phenotype, while patients with LMP7 are likely to express the fully assembled MHC class I complex. This implies that in cases where the immunoproteasome dominates over the constitutive proteasome, effective antigen presentation is more likely. Moreover, the presence of upstream APPP components could be necessary for formation of a stable MHC class I complex (45). Thus, the generation of suboptimal peptide sequences by the constitutive proteasome is exacerbated by impaired presentation of the peptide on the cell surface, resulting in escape from immune detection, thereby promoting ‘immunoediting’, i.e. the selective outgrowth of tumour components with low immunogenicity. To further substantiate this, the association of expression of the (immuno)proteasome to tumor-infiltrating lymphocytes was determined using the results from a recently published study of tumour-infiltrating lymphocytes in the same population (46). Higher numbers of tumour-infiltrating CD8+ T-lymphocytes are more frequently observed in patients with loss of MB1 expression as well as in patients with positive LMP7 expression (data not shown). Whether down-regulation of the constitutive and up-regulation of the immunoproteasome result in an increase in lymphocyte infiltration, or whether the presence of interferon-y producing lymphocytes facilitates the switch from constitutive to immunoproteasome needs to be elucidated.

While we found prognostic significance associated with components at either the beginning or the very end of the APPP, we did not detect any survival differences based on expression levels of proteins active at the level of the endoplasmatic reticulum. Summation of APPP components, however, similar to the approach of Han et al. (33) indeed correlated with worse survival in patients with down-regulation of one or more components as opposed to TAP1+ TAP2+ HLA-B/ C+ β2-microglobulin+ patients (data not shown). Furthermore, combination of these results with
Down-regulation of proteasomal subunit MB1 is an independent predictor of improved survival in ovarian cancer

A tumour-infiltrating lymphocytes revealed that patients with expression of all four components more frequently have an abundance of tumour-infiltrating CD8+ T-lymphocytes. Moreover, we also find that both the absolute number of APPP components and CD8+ T-lymphocytes are independent prognostic predictors of longer disease specific survival, next to some well-acknowledged prognostic factors (33;46).

In conclusion, our results underscore a pivotal role for the (immuno)proteasome and MHC class I complex in determining prognosis in ovarian cancer patients. In view of the complexity of the APPP, these results provide a rationale for targeting these specific components in targeted therapy in cancer.

Acknowledgements

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References

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