Circulating free DNA and p53 antibodies in plasma of patients with ovarian epithelial cancers

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Background: This study was conducted in order to evaluate the significance of circulating free DNA (CFDNA), blood plasma p53 antibodies (p53-Ab) and mutations of KRAS gene in the prognosis of ovarian epithelial cancers.

Patients and methods: A total of 126 patients were included in this study. KRAS mutations and CFDNA were detected by means of the PCR–restriction fragment length polymorphism (PCR-RFLP) and enriched by the PCR-RFLP method. Enzyme-linked immunosorbent assay was used to analyze plasma p53-Ab.

Results: KRAS mutations were detected in 27 (21.4%) of examined tumors. The frequency of KRAS mutations was especially high in mucinous cancers (P < 0.001). CFDNA and p53-Ab were frequently detected in patients with serous cancers in high grade (P < 0.001). The overall survival rate was significantly lower for patients with serous tumors and CFDNA and p53-Ab-positive than negative tumors (P = 0.022 and P < 0.001, respectively). In mucinous ovarian cancer, a worse overall survival was correlated with the KRAS mutations (P = 0.03).

Conclusions: The results of the present study suggested that a presence of KRAS mutations in mucinous ovarian cancer and CFDNA and p53-Ab in serous tumors was correlated with the highest risk of cancer progression.

Key words: CFDNA, epithelial ovarian cancer, KRAS codon 12 point mutation, p53-Ab

Introduction

Epithelial ovarian cancer comprises the majority of malignant ovarian tumors in adult women. About 190 000 new cases and 114 000 deaths from ovarian cancer are estimated to occur annually. The highest rates are reported in Scandinavia and Eastern Europe, the United States and Canada. The age-adjusted incidence rate in the United States is 12.48 per 100 000 women per year [1]. Low rates are found in Africa and Asia [2]. In Poland, the age-adjusted incidence is 10.8 per 100 000 women per year [3]. The overall 5-year survival rate for all stages combined range from 30% / INS> to 50%. Most women, however, present with late-stage disease, which is associated with a rate of about 20% [4].

Activation of protooncogenes is a feature of many malignancies and, not surprisingly, there have been numerous searches for oncogene mutations as well as for specific genes dysregulation involved in apoptotic, neoangiogenetic and transduction signal pathways [5]. In ovarian cancer, KRAS mutations are seen in 4%–30% of cases [6–10]. TP53 mutations have been found in 50% of cases [11–13]. The frequencies of p53 antibodies (p53-Ab) in serum vary from 8% to 46% [14–17]. Several methods have been used for circulating free DNA (CFDNA) quantification, but none have been evaluated in terms of reproducibility and therefore, results from different studies are not comparable [18–22].

This study was conducted in order to evaluate the significance of CFDNA, blood plasma p53-Ab and mutations of KRAS gene in the prognosis of ovarian epithelial cancers.

Materials and methods

Patient and clinical samples

A total of 126 patients with ovarian cancers (aged 18–79 years; median value = 58.3 years), all treated in the Department of Gynecology at District Hospital in Bialystok between 2002 and 2005, were included in this study. The protocol was previously approved by the Bioethical Committee of the Medical University of Bialystok. Patients were informed and gave their consent for the study. Follow-up data was completed until April 2010. At a median follow-up of 38 months (range 1–96), 85 patients had died as a consequence of cancer progression.
Primary treatment generally consisted of surgery, which entails total abdominal hysterectomy, omentectomy, multiple peritoneal and lymph node samplings as well as peritoneal washings for cytology. Adjuvant chemotherapy consisted of different platinum-based treatment regimens. Bilateral tumors were resected from 19 patients and remaining tumors were found unilaterally. Tumor stage and histological diagnosis of each case were determined according to the criteria of the International Federation of Gynecology and Obstetrics and the histological typing system of the World Health Organization, respectively. Patients were categorized as having limited disease (stage I or II) and advanced disease (stage III or IV). Tumors were graded as well (G1), moderately (G2), or poorly (G3) differentiated. Histologically, 64 (50.8%) of the patients had a serous cystadenocarcinoma, 18 (14.3%) a mucinous cystadenocarcinoma, 26 (20.6%) an endometrioid carcinoma and 18 (14.3%) was other (therein 10 cases of clear-cell carcinomas and 8 of different histological types). Two experienced pathologists evaluated the histological appearance of all tissue samples in a blinded fashion.

**KRAS mutation detection**

Tissue specimens of ovarian cancer were obtained during surgery and immediately frozen in liquid nitrogen and then stored at a temperature of −80°C until analyzed. Before DNA extraction, it was microscopically confirmed that the tumor specimens consisted mainly of carcinoma tissue (80%). Tumor samples (30–50 mg) were minced with a sterile scalpel and then digested overnight in 180 μl of tissue lysis buffer, containing 20 μl of proteinase K solution (10 mg/ml) at 37°C. DNA extraction from tissue lysates was carried out with the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, St Louis, MO), according to the manufacturer's instructions. Isolated DNA was stored at a temperature of −20°C before further assays.

The detection of KRAS mutations at codon 12 was carried out using PCR–restriction fragment length polymorphism (PCR-RFLP) method as described previously [7, 23]. PCR products were sequenced with the use of the sense primer K1, the ABI PRISM BigDye Terminator v3.0 Cycle Sequencing Ready Reaction (Applied Biosystems, Foster City, CA) and the automated ABI PRISM 377 DNA sequencer (Applied Biosystems). Mutations were confirmed by sequencing reaction with the use of the antisense primer DDSP. Antisense strain of PCR products was used for sequencing.

**circulating free DNA**

Three-milliliter sample of peripheral blood was collected in vials containing EDTA on the day before surgery. Plasma was immediately separated from the cellular fraction by centrifugation at 3000 g during the period of 20 min at a temperature of 4°C and the supernatant was stored at a temperature of −80°C until use.

The level of p53-Ab in plasma was measured with the use of anti-p53 ELISA II Kit (Pharmcell, Paris, France) according to the manufacturer's instruction. The value over 0.85 IU/ml indicates a probable presence of the antibody.

**statistical analysis**

Statistical analysis was carried out using Statistica software version 8.0 (StatSoft Inc., StatSoft Polska Sp. z o.o., Poland). A chi-square test was used to evaluate the relationship between categorical variables. Fisher's exact test was used to determine significance between the two groups. A P value of <0.05 was considered as statistically significant. In addition, survival time was calculated from the date of surgery to the date of death and survival analysis was carried out using the Kaplan–Meier method.

**results**

**KRAS gene mutations**

Our results show that mutations of the KRAS gene in codon 12 were present in 27 of 126 cases (21.4%) examined with epithelial ovarian cancer (Figure 1). We found this frequency to be lower in serous tumors (12.5%), with a slight increase to 23.1% in endometrioid tumors and an increase to 61.1% in mucinous carcinomas. These differences were statistically significant (P < 0.001) (Table 1). KRAS mutations were present in all analyzed types of the ovarian tumors, the most commonly, in grade 1 of histopathological differentiation. These differences are not statistically significant (Table 2).

Among patients with KRAS-mutated mucinous cystadenocarcinomas, the Kaplan–Meier survival estimates of the 1-year and 5-year survival rates were 97.4% and 90.8%, respectively, while the respective rates among patients with lack of KRAS mutations were 97.4% and 93.4%. Statistically significant difference was observed between survival rates over time (P = 0.03) (Figure 2). Presence of KRAS gene mutation was not associated with survival outcome in serous and endometrioid tumors (data not shown).

**circulating free DNA**

CDFNA was detectable in 55 of treated patients (43.7%). Among them, there were 39 patients with serous tumors (60.9%), 2 patients with mucinous tumors (11.1%) and 8 with endometrioid tumors (30.8%). The difference between the groups was statistically significant (P < 0.001). A positive correlation was found between the presence of CFDNA and the grade (mostly in G2 and G3; P = 0.001) at late tumor stage (III/IV; P < 0.001) and only in serous cancers (Tables 2 and 3 and Figure 3).

The Kaplan–Meier survival estimates of the 1-year and 5-year survival rates for patients with serous cystadenocarcinomas and the presence of CFDNA were...
97.4% and 90.8%, respectively, while the respective rates for patients with absence of CFDNA were 97.4% and 93.4%. The median overall survival was 21 months for patients with CFDNA presence and 52 months for patients with CFDNA absence. The differences were statistically significant (P = 0.022) (Figure 4). The survival rate was unaffected by presence of CFDNA in mucinous and endometrioid tumors (data not shown).

Table 1. Relationship between KRAS mutations, circulating free DNA (CFDNA) and p53 antibodies (p53-Ab) status in examined epithelial ovarian cancer

<table>
<thead>
<tr>
<th>Histology</th>
<th>No. of cases</th>
<th>KRAS mutations, n (%)</th>
<th>CFDNA, n (%)</th>
<th>p53-Ab, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>126</td>
<td>27 (21.4)</td>
<td>55 (43.7)</td>
<td>42 (33.3)</td>
</tr>
<tr>
<td>Serous</td>
<td>64</td>
<td>8 (12.5)</td>
<td>39 (60.9)</td>
<td>37 (57.8)</td>
</tr>
<tr>
<td>Mucinous</td>
<td>18</td>
<td>11 (61.1)</td>
<td>2 (11.1)</td>
<td>1 (5.6)</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>26</td>
<td>6 (23.1)</td>
<td>8 (30.8)</td>
<td>3 (11.5)</td>
</tr>
<tr>
<td>Othera</td>
<td>18</td>
<td>2 (11.1)</td>
<td>6 (33.3)</td>
<td>1 (5.6)</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

Chi-square test.

*aIncludes 10 cases of clear-cell epithelial ovarian cancer and 8 of epithelial ovarian cancer of other histological types.

97.4% and 90.8%, respectively, while the respective rates for patients with absence of CFDNA were 97.4% and 93.4%. The median overall survival was 21 months for patient with CFDNA presence and 52 months for patient with CFDNA absence. The differences were statistically significant (P = 0.022) (Figure 4). The survival rate was unaffected by presence of CFDNA in mucinous and endometrioid tumors (data not shown).

Table 2. Relationship between KRAS mutations, circulating free DNA (CFDNA) and p53 antibodies (p53-Ab) status in different grade of examined epithelial ovarian cancer

<table>
<thead>
<tr>
<th>Histology</th>
<th>Grade</th>
<th>No. of cases</th>
<th>KRAS mutations, n (%)</th>
<th>CFDNA, n (%)</th>
<th>p53-Ab, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serous</td>
<td>G1</td>
<td>29</td>
<td>6 (20.7)</td>
<td>5 (17.2)</td>
<td>4 (13.8)</td>
</tr>
<tr>
<td></td>
<td>G2</td>
<td>11</td>
<td>2 (18.2)</td>
<td>11 (100)</td>
<td>9 (81.8)</td>
</tr>
<tr>
<td></td>
<td>G3</td>
<td>24</td>
<td>0 (0)</td>
<td>23 (95.8)</td>
<td>24 (100)</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>0.06</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Mucinous</td>
<td>G1</td>
<td>9</td>
<td>7 (77.8)</td>
<td>1 (11.1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>G2</td>
<td>8</td>
<td>3 (37.5)</td>
<td>1 (12.5)</td>
<td>1 (12.5)</td>
</tr>
<tr>
<td></td>
<td>G3</td>
<td>1</td>
<td>1 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>0.17</td>
<td>0.51</td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td>Endometrioid</td>
<td>G1</td>
<td>12</td>
<td>5 (41.7)</td>
<td>1 (8.3)</td>
<td>1 (8.3)</td>
</tr>
<tr>
<td></td>
<td>G2</td>
<td>9</td>
<td>1 (11.1)</td>
<td>2 (22.2)</td>
<td>1 (11.1)</td>
</tr>
<tr>
<td></td>
<td>G3</td>
<td>5</td>
<td>0 (0)</td>
<td>5 (100)</td>
<td>1 (20)</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>0.10</td>
<td>&lt;0.001</td>
<td>0.79</td>
<td></td>
</tr>
</tbody>
</table>

Chi-square test.

positivity of plasma p53-Ab
Using the cutoff value, 42 (33.3%) of 126 patients were p53-Ab positive. The increased presence of Ab to p53 in plasma of women with serous ovarian cancer (57.8%)
was statistically significant as compared with other examined ovarian cancer ($P < 0.001$) (Table 1). Plasma of women with advanced stage (III/IV) of serous ovarian cancer was more likely to have detectable antibodies specific for p53 (96.4%) than samples taken from patients with early-stage (I/II) disease (27.8%) ($P < 0.001$) (Table 3). There was a tendency toward a higher presence of Ab to p53 in plasma of the serous cystadenocarcinomas than remaining histological subtypes of epithelial ovarian cancer. The p53-Ab was especially high in the G2 and G3 serous ovarian cancer (81.8% and 100%, respectively) ($P < 0.001$) (Table 2).

The Kaplan–Meier survival estimates of the 1-year and 5-year survival rates in p53-Ab-positive patients with serous cystadenocarcinomas were 86.4% and 72.7%, respectively, while the respective rates among p53-Ab-negative patients were 100% and 84.8%. The median overall survival was 11 months for p53-Ab-positive patient and 57 months for p53-Ab-negative patient. The differences were statistically significant ($P < 0.001$) (Figure 5). Presence of p53-Ab in plasma of patients with mucinous and endometrioid tumors did not influence survival significantly (data not shown).
contrast, type II tumors grow up rapidly, arising directly from the surface epithelium or inclusion cysts and metastasize early in the course of patients with high-grade advanced stage (III or IV) serous ovarian cancer. The fact that early-stage tumors of low-grade or in situ carcinomas could present DNA alterations in the plasma/serum/ascites circulation and this may explain the high level of CFDNA in blood. Invading cells have the ability of shedding DNA into the circulation and clearance including hydrolyzing enzymes. The mechanisms regulating appearance and distribution of CFDNA in blood are under pressure of factors that influence its circulation and clearance including hydrolyzing enzymes. The mechanisms regulating appearance and distribution of CFDNA in blood are under pressure of factors that influence its circulation and clearance including hydrolyzing enzymes. The mechanisms regulating appearance and distribution of CFDNA in blood are under pressure of factors that influence its circulation and clearance including hydrolyzing enzymes. The mechanisms regulating appearance and distribution of CFDNA in blood are under pressure of factors that influence its circulation and clearance including hydrolyzing enzymes.

Recent studies have reported an increase in the median levels of CFDNA in serum of patients with serous cancers in high grade compared with other histological subtypes. This result is in accordance with the finding of Kamat et al. [18, 21], who reported an increase in the median levels of CFDNA in serum patients with high-grade advanced stage (III or IV) serous ovarian cancer. Our study showed a significant difference between the presence of CFDNA in patients with serous cancers in high grade compared with other histological subtypes. This result is in accordance with the finding of Kamat et al. [18, 21], who reported an increase in the median levels of CFDNA in serum patients with high-grade advanced stage (III or IV) serous ovarian cancer. Our study showed a significant difference between the presence of CFDNA in patients with serous cancers in high grade compared with other histological subtypes. This result is in accordance with the finding of Kamat et al. 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cytoreduction. Probably, tumor biology and other mechanisms may also influence patient’s outcome. It is believed that a combined measurement of currently used tumor biomarkers will improve the sensitivity and specificity for ovarian cancer management. However, the values of longitudinal measurements of the used markers are yet to be determined.

### Table 4. Frequency of p53 antibodies (p53-Ab), circulating free DNA (CFDNA) and KRAS mutations in epithelial ovarian cancers

<table>
<thead>
<tr>
<th>Marker</th>
<th>Positive</th>
<th>Total</th>
<th>%</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53-Ab</td>
<td>143</td>
<td>652</td>
<td>22</td>
<td>Summary of 11 studies. Frequency of Abs 8.7%–92.3%. Few studies showed association with poor histological differentiation and poor survival.</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>113</td>
<td>19</td>
<td>Correlation with stage and grade. Association with worse survival.</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>193</td>
<td>13</td>
<td>No diagnostic value and no prognostic relevance for survival.</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>116</td>
<td>25</td>
<td>Detected exclusively in type II ovarian carcinoma. p53-Ab in ascites but not in serum was found to be a sign of unfavorable survival.</td>
</tr>
<tr>
<td></td>
<td>28 serum, 21 ascites</td>
<td>113</td>
<td>25</td>
<td>Tissue expression of p53 in ovarian tumors is associated with poor histological differentiation and the presence of detectable serum autoantibodies.</td>
</tr>
<tr>
<td></td>
<td>41</td>
<td>174</td>
<td>24</td>
<td>p53-Ab was associated with older patient, more aggressive tumors and reduced patient survival.</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>86</td>
<td>21</td>
<td>Preoperative serum p53-Ab had no prognostic relevance for survival.</td>
</tr>
<tr>
<td></td>
<td>104</td>
<td></td>
<td></td>
<td>Presence of p53-Ab to be an independent variable for prediction of survival in advanced-stage patients.</td>
</tr>
<tr>
<td>CFDNA</td>
<td>86</td>
<td>173</td>
<td>50</td>
<td>The relatively high percentage (50%) of cancer patients with apparently normal DNA levels would suggest that this test may have low diagnostic value.</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td></td>
<td></td>
<td>Free plasma DNA correlates with response to chemotherapy.</td>
</tr>
<tr>
<td></td>
<td>164</td>
<td></td>
<td></td>
<td>Elevated plasma CFDNA is an independent predictor for death.</td>
</tr>
<tr>
<td></td>
<td>104</td>
<td></td>
<td></td>
<td>Elevated CFDNA in epithelial ovarian cancer may have diagnostic value.</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>137</td>
<td>33</td>
<td>CFDNA can be detected in peritoneal fluid and is associated with a more aggressive tumor biology and reduced survival.</td>
</tr>
<tr>
<td>KRAS</td>
<td>46</td>
<td>73</td>
<td>63</td>
<td>Mutations of KRAS occurred in 68% invasive micropapillary serous carcinomas and in 61% serous borderline tumors.</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>37</td>
<td>27</td>
<td>KRAS mutations occurred significantly more frequently in mucinous than in serous carcinomas.</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>44</td>
<td>48</td>
<td>Mutation of KRAS was detected at a higher frequency in mucinous borderline tumor compared with serous tumor.</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>63</td>
<td>13</td>
<td>Presence of KRAS mutation was associated with advanced-stage disease.</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>74</td>
<td>22</td>
<td>Higher incidence of KRAS mutations was observed in mucinous tumors.</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>381</td>
<td>15</td>
<td>KRAS mutation is a common event in ovarian carcinomas of lower grade, lower stage and mucinous histotype.</td>
</tr>
</tbody>
</table>
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disclosure
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references


