Expression of palladin is associated with disease progression in metastatic high-grade serous carcinoma

Running title: Palladin in serous effusions

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Abstract

Objective: To analyze the expression and clinical role of the actin-associated molecule palladin in serous effusions.

Methods: PALLD mRNA expression by quantitative RT-PCR (qRT-PCR) was analyzed in 83 high-grade serous carcinoma (HGSC) effusions. Fifteen malignant mesothelioma (MM) effusions and 18 surgical HGSC specimens from the ovary were studied for comparative purposes. Palladin protein expression by immunohistochemistry was analyzed in another series consisting of 261 HGSC effusions.

Results: PALLD mRNA was significantly overexpressed in HGSC compared to MM effusions (p<0.001). Palladin expression by immunohistochemistry was found in HGSC cells in 106/261 (41%) effusions, most commonly focally (<5% of cells). PALLD expression was additionally higher in ovarian HGSC specimens compared to HGSC effusions (p<0.001). However, IHC showed only stromal expression of this protein in surgical specimens. PALLD mRNA expression in HGSC effusions was unrelated to clinicopathologic parameters, chemotherapy response or survival. Palladin protein expression was higher in post-chemotherapy, mainly disease recurrence, specimens compared to chemo-naïve effusions tapped at diagnosis (p=0.018), though it was unrelated to other clinicopathologic parameters or survival.

Conclusion: In conclusion, PALLD mRNA is overexpressed in HGSC compared to MM effusions, and its protein product is overexpressed in post-chemotherapy compared to pre-chemotherapy HGSC effusions, suggesting upregulation along tumor progression. The presence of this molecule in HGSC effusions, at the mRNA or the protein level, is unrelated to disease outcome.

Keywords: Palladin; quantitative RT-PCR; immunohistochemistry; high-grade serous carcinoma; effusion
1. Introduction

The cell cytoskeleton, consisting of microtubules, intermediate filaments and microfilaments, regulates cell structure, adhesion, motility and cellular trafficking under normal conditions. As in the case of other cellular functions, this system is deregulated in cancer cells, resulting in enhanced migration and invasion, proliferation and cell survival, and resistance to stress, including chemotherapy [1]. Deregulation of the cytoskeleton network is closely related to epithelial-mesenchymal transition (EMT), in which expression levels of cytoskeleton components, cell form and motility are affected [2].

Actin microfilaments are central regulators of motility, migration and invasion through regulation of phosphatidylinositol-3-kinase (PI3K) pathway signaling, secretion of enzymes modifying the tumor microenvironment and production of reactive oxygen species (ROS) [1]. Palladin is member of a family of actin-associated cytoplasmic proteins that additionally includes myopalladin and myotillin. All members contain immunoglobulin-like domains providing rigidity and correct spacing to the protein. Alternative splicing generates several palladin isoforms with a molecular size of 90, 140 and 200 kDa, which are present to a variable degree during embryogenesis, with a postulated role in organizing the cellular cytoskeleton in differentiating tissues, but are largely absent in adult organs. In addition to cross-linking actin filaments, palladin is able to bind several other proteins, including molecules that are deregulated in cancer, such as α-actinin, Ezrin and Src [3].

Ovarian cancer is the 7th most common cancer and the 8th most common cause of cancer death in women globally, with 239,000 new diagnoses and 152,000 deaths in 2018 [4]. The majority (>90%) of tumors designated as ovarian cancer are carcinomas, the most common type being high-grade serous carcinoma (HGSC), which in the majority of cases has its origin in the
fallopian tube rather than the ovary [5]. While patients with ovarian cancer currently live longer, due to optimized surgery and chemotherapy protocols, as well as targeted therapy, 5-years survival is only 45% and is considerably worse for patients with HGSC, a tumor often diagnosed at advanced stage and characterized by increasing chemoresistance along disease progression [6]. Data regarding the expression and role of palladin in ovarian carcinoma are limited to two studies. Palladin was reported to be upregulated in CI-1040 cells harboring BRAF mutation following inhibition of the mitogen-activated protein kinase (MAPK) pathway, based on serial analysis of gene expression (SAGE), though the most profound change observed was in CCND1, encoding cyclin D1 [7]. In another study, exposure of ovarian carcinoma cells to the morphogenetic protein epimorphin induced mesenchymal-to-epithelial transition (MET), with a 3-fold reduction in palladin level [8]. Data regarding the expression and clinical role of palladin in clinical ovarian carcinoma specimens are unavailable to date.

The objective of this retrospective study was to quantitatively analyze PALLD mRNA expression and its clinical relevance in HGSC effusions. Malignant mesothelioma (MM), a tumor with a metastatic pattern similar to HGSC, was studied for comparative purposes. Palladin protein expression was additionally analyzed in a large HGSC cohort.
2. Material and Methods

2.1. Patients and specimens

Quantitative RT-PCR (qRT-PCR) cohort: Effusions consisted of 83 HGSC specimens (63 peritoneal, 20 pleural) submitted to the Department of Pathology at the Norwegian Radium Hospital during the period of 1998 to 2004. A series of 18 surgical specimens of the ovary from patients with HGSC, operated at the Norwegian Radium Hospital, was additionally studied for comparative purposes, as was a series of 15 MM effusions (4 peritoneal, 11 pleural). The latter were submitted to the Departments of Pathology at the Norwegian Radium Hospital and Aalborg University Hospital, Aalborg, Denmark, during the period of 1998 to 2007. Effusions from Danish patients were studied with kind permission from Mr. Søren Nielsen (Department of Pathology, Aalborg University Hospital). MM effusions were from patients diagnosed with epithelioid or biphasic MM in biopsy specimens, and cells in the effusion specimens had epithelioid morphology. The difference in cohort size, except for the fact that MM is a relatively rare cancer, owed primarily due to the fact that the Norwegian Radium Hospital is a referral center for Gynecologic Cancer, whereas treatment of MM is divided between several hospitals in South-Eastern Norway.

Effusions were centrifuged immediately after tapping, and cell pellets were frozen at -70°C in equal amounts of RPMI 1640 medium (GIBCO-Invitrogen, Carlsbad, CA) containing 50% fetal calf serum (PAA Laboratories GmbH, Pasching, Austria) and 20% dimethylsulfoxide (Merck KGaA, Darmstadt, Germany). Cell blocks were prepared using the thrombin clot protocol.

Effusion specimens were diagnosed by an experienced cytopathologist (BD) based on smear and cell block morphology and IHC, based on established guidelines [8]. Frozen sections from all solid tumors were reviewed by an experienced gynecopathologist (BD), and only specimens with tumor cell population >50% and minimal or no necrosis were included.
IHC cohort: HGSC effusions (n=261; 216 peritoneal, 45 pleural) from 261 patients were submitted to the Department of Pathology at the Norwegian Radium Hospital during the period of 1998 to 2006. Effusions were centrifuged immediately after tapping, and cell pellets were used for preparation of cell blocks using the thrombin clot protocol. Clinicopathologic data for both HGSC cohorts are detailed in Table 1. A flow chart of the material analyzed is shown in Figure 1. Informed consent was obtained according to national and institutional guidelines. Study approval was given by the Regional Committee for Medical Research Ethics in Norway.

2.2. qRT-PCR
Effusions were centrifuged to obtain a cell pellet from which RNA was extracted using QIAsymphony (Qiagen, Hilden Germany). Details regarding reverse transcription, primer and probe design procedure and software, and efficiency testing were previously described [10]. The qRT-PCR reaction was run using the Perfecta qPCR ToughMix (Quanta Biosciences, Gaithersburg MD) and quantified on the Roche LightCycler 480 (Roche, Basel, Switzerland). Samples were analyzed in triplicate and average copy number was used for statistical analysis. PALLD primer and probe sequences were as follows:

**Forward**: AGCACTGACCGAGTGAGCAT

**Reverse**: CTTTTGTGGCTCCCTGAATGA

**Probe**: ACCAGGACAACCACGGCTACATCTGCCT

The beta-glucuronidase (GUS), TATA box binding protein (TBP) and mitochondrial ribosomal protein L19 (MRLP19) genes were used as reference genes following previous testing [10] applying established guidelines [11-13]. Primer and probe sequences were previously detailed [10].
2.3. IHC

Formalin-fixed, paraffin-embedded sections from 261 HGSC effusions were analyzed for palladin protein expression using the Dako EnVision Flex + System (K8012; Dako, Glostrup, Denmark). The palladin antibody was a mouse monoclonal antibody purchased from Novus Biologicals (cat # NBP1-25959, clone 1E6; Littleton, CO), applied at a 1:1000 dilution following antigen retrieval in HpH buffer (pH 9.0).

Following deparaffinization, sections were treated with EnVision™ Flex + mouse linker (15 min) and EnVision™ Flex/HRP enzyme (30 minutes) and stained for 10 minutes with 3’3'-diaminobenzidine tetrahydrochloride (DAB), counterstained with hematoxylin, dehydrated and mounted in Toluene-Free Mounting Medium (Dako). Positive control consisted of a tissue microarray of surgical ovarian carcinoma specimens. In negative controls, the primary antibody was replaced with isotype-specific mouse myeloma protein diluted to the same concentration as the primary antibody. Protocol optimization was based on in-house testing.

IHC scoring: Staining was scored by an experienced cytopathologist (BD), using a 0-4 scale as follows: 0=no staining, 1=1-5%, 2=6-25%, 3=26-75%, 4=76-100% of tumor cells.

2.4. Statistical analysis

Statistical analysis was performed applying the SPSS-PC package (Version 26). Probability of <0.05 was considered statistically significant. The Mann-Whitney U test or the Kruskal-Wallis H test was applied to analysis of the association between PALLD mRNA and palladin protein expression by IHC and clinicopathologic parameters (for 2-tier or 3-tier analyses, respectively). For this analysis, clinicopathologic parameters were grouped as follows: age: ≤60 vs. >60 years; effusion site: peritoneal vs. pleural; FIGO stage: III vs. IV; chemotherapy status: pre- vs. post-
chemotherapy specimens; residual disease (RD) volume: 0 cm vs. $\leq$1 cm vs. >1 cm; response to chemotherapy: complete response vs. partial response/stable disease/progressive disease.

Progression-free survival (PFS) and overall survival (OS) were calculated from the date of the last chemotherapy treatment/diagnosis to the date of recurrence/death or last follow-up, respectively. Univariate survival analyses of PFS and OS were executed using the Kaplan-Meier method and log-rank test. Platinum resistance was defined as PFS $\leq$ 6 months according to guidelines published by the Gynecologic Oncology Group (GOG) and progressive disease or recurrence was evaluated by the Response Evaluation Criteria In Solid Tumors (RECIST) criteria.
3. Results

qRT-PCR analysis of 83 HGSC and 15 MM effusions showed significantly higher *PALLD* expression in the former group (p<0.001). Based on this result, IHC analysis of a larger series of HGSC effusions was performed. The IHC cohort included the majority of HGSC effusions analyzed using qRT-PCR (Figure 1). This analysis showed palladin expression in HGSC cells in 106/261 (41%) effusions, with the following score: =1: 70 effusions; =2: 22 effusions; =3: 11 effusions; =4: 3 effusions. Staining was cytoplasmic, often with sub-membrane accentuation (Figure 2). Expression was limited to carcinoma cells. We additionally analyzed a series of 18 surgical specimens of the ovarian tumor from HGSC patients, in which *PALLD* expression was still higher than in the HGSC effusions (p<0.001). However, IHC analysis of a limited series of surgical specimens showed that expression of this protein was predominantly stromal (data not shown). Given the fact that the surgical specimens have not been microdissected, the qRT-PCR finding therefore needs to be viewed as probably affected by the presence of host cells. *PALLD* mRNA expression was unrelated to clinicopathologic parameters (p>0.05; data not shown). Palladin protein expression was significantly higher in post-chemotherapy effusions compared to pre-chemotherapy specimens tapped at diagnosis (p=0.018; Table 2), though its levels were not significantly related to other clinicopathologic parameters (p>0.05; data not shown).

In view of the reported role of palladin in PI3K signaling [1], the association between expression of this protein and the presence of p-AKT and p-mTOR, previously studied in 123 of the 261 HGSC effusions [14], was assessed. No significant association between the expression level of these proteins was found (p>0.05; data not shown).
Survival data were available for all HGSC patients. In the qRT-PCR cohort, the follow-up period ranged from 1 to 179 months (mean = 33 months, median = 24 months). PFS ranged from 0 to 148 months (mean = 9 months, median = 5 months). At the last follow-up, 81 patients were dead of disease, 1 was alive with disease and 1 was with no evidence of disease. PALLD mRNA levels were unrelated to OS (p=0.626) or PFS (p=0.778) in univariate survival analysis, with comparable data in separate analysis of patients with pre- and post-chemotherapy specimens (p>0.05; data not shown).

In the IHC cohort, the follow-up period ranged from 1 to 179 months (mean = 38 months, median = 27 months). PFS ranged from 0 to 148 months (mean = 12 months, median = 6 months). At the last follow-up, 248 patients were dead of disease, 4 were alive with disease and 6 were with no evidence of disease. Two patients died of complications and 1 patient was lost to follow-up. Palladin protein expression (any extent) was associated with a trend for longer OS (p=0.085; Figure 3-A), with no such trend for PFS (p=0.233) in univariate survival analysis.

Among clinical parameters, older age (p<0.001; Figure 3-B), FIGO IV stage (p<0.001; Figure 3-C) and higher RD volume (p=0.001; Figure 3-D) were significantly related to shorter OS. In Cox multivariate survival analysis, in which these 4 parameters were entered, only FIGO stage and RD volume emerged as independent prognosticators (Palladin: p=0.471; Age: p=0.373; FIGO stage: p=0.002; RD volume: p=0.006).

No significant association between palladin protein expression and survival was found in separate analysis of patients with pre- and post-chemotherapy specimens (p>0.05; data not shown).
4. Discussion

EMT is widely considered to have a tumor-promoting effect, and plays an important role in metastasis and chemoresistance, two of the main drivers of tumor progression. Our group has previously demonstrated in several studies that epithelial and mesenchymal/mesodermal markers, as well as their transcriptional regulators, are differentially expressed in ovarian carcinoma effusions compared to solid lesions [15-18]. It was therefore deemed relevant to assess whether palladin, an actin regulator often expressed in mesenchymal cells, is expressed in metastatic HGSC cells in effusions.

Unexpectedly, PALLD mRNA levels in HGSC specimens were higher than in MM effusions, despite the fact that the latter tumor has combined epithelial and mesenchymal histogenesis and expression profile. IHC performed on HGSC specimens confirmed that palladin expression in effusions is limited to carcinoma cells. This provides yet further evidence that HGSC, though to a lesser degree than MM, has the propensity to express mesenchymal markers, e.g. vimentin, as shown in our previous study [18].

IHC analysis of a tissue microarray containing a few surgical specimens of HGSC showed palladin expression only in stromal cells. This highlights the unique nature of tumor cells in effusions and concurs with data from other studies reporting the localization of palladin primarily to the stroma of other carcinomas, including those of pancreatic and renal origin [19-21], though expression in carcinoma cells was observed in 22/177 (12.4%) of tumors in the study by Salaria et al. [19]. Of note, PALLD mutation was additionally reported to be associated with familial pancreatic cancer [22]. Whether the absence of palladin from HGSC cells in solid lesions is a universal finding in HGSC, and whether stromal expression of palladin is informative of patient outcome in this cancer remains unanswered.
As cytoskeletal proteins participate in PI3K signaling, we analyzed whether the expression of palladin was related to expression of the activated forms of AKT and mTOR. No co-expression of these proteins was found, though this does not by any means rule out a functional association in HGSC cells.

Data regarding the predictive and prognostic relevance of palladin expression in carcinoma cells is lacking, though its expression in peritumoral stromal cells has been associated with poor survival in pancreatic and renal carcinoma [20,21]. In the present study, palladin protein was overexpressed in HGSC effusions tapped following exposure to chemotherapy compared to chemo-naïve effusions tapped at diagnosis. While these specimens were not patient-matched, this may suggest upregulation of this protein along disease progression. The presence of higher $PALLD$ mRNA levels or of its protein product was nevertheless unrelated to chemotherapy response or survival, as opposed to the performance of established clinical parameters, including age, FIGO stage and RD volume.

In conclusion, $PALLD$ mRNA is overexpressed in HGSC compared to MM effusions, and HGSC cells express its protein product, though most often focally. Palladin expression is higher following exposure to chemotherapy, but is not informative of disease outcome. Whether stromal palladin expression in surgical specimens has a prognostic role remains to be investigated.
References


Table 1: Clinicopathologic parameters of the HGSC qRT-PCR and IHC cohorts

<table>
<thead>
<tr>
<th>Parameter</th>
<th>qRT-PCR (n=83)</th>
<th>IHC (n=261)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (mean)</strong></td>
<td>35-87 years (62)</td>
<td>23-88 years (62)</td>
</tr>
<tr>
<td><strong>FIGO stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>II</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>III</td>
<td>44</td>
<td>154</td>
</tr>
<tr>
<td>IV</td>
<td>38</td>
<td>102</td>
</tr>
<tr>
<td><strong>Residual disease</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary debulking surgery (n=51 and n=140 for the qRT-PCR and IHC cohort, respectively)</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>≤1 cm</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>&gt;1 cm</td>
<td>29</td>
</tr>
<tr>
<td>Interval debulking surgery (n=18 and n=77 for the qRT-PCR and IHC cohort, respectively)</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>≤1 cm</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>&gt;1 cm</td>
<td>9</td>
</tr>
<tr>
<td>NA</td>
<td>14</td>
<td>44</td>
</tr>
<tr>
<td><strong>CA 125 at diagnosis (range; median)</strong></td>
<td>10-43800 (1474)</td>
<td>10-62400 (1238)</td>
</tr>
<tr>
<td><strong>Chemoresponse after primary treatment</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR</td>
<td>37</td>
<td>124</td>
</tr>
<tr>
<td>PR</td>
<td>13</td>
<td>48</td>
</tr>
<tr>
<td>SD</td>
<td>3</td>
<td>14</td>
</tr>
<tr>
<td>PD</td>
<td>17</td>
<td>41</td>
</tr>
<tr>
<td>NA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13</td>
<td>34</td>
</tr>
</tbody>
</table>
Abbreviations: NA = not available; CR = complete response; PR = partial response; SD = stable disease; PD = progressive disease

\( ^a \) Available for 55 and 202 patients in the qRT-PCR and IHC cohort, respectively

\( ^b \) Not available (missing data or disease response after chemotherapy could not be evaluated because of normalized CA 125 after primary surgery or missing CA 125 information and no residual tumor).
Table 2: The association between palladin expression in HGSC effusions and previous exposure to chemotherapy

<table>
<thead>
<tr>
<th>Effusion status</th>
<th>Staining extent (% of cells)</th>
<th>0%</th>
<th>1-5%</th>
<th>6-25%</th>
<th>26-75%</th>
<th>76-100%</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-chemotherapy (n=148)</td>
<td></td>
<td>98</td>
<td>32</td>
<td>12</td>
<td>4</td>
<td>2</td>
<td>p=0.018</td>
</tr>
<tr>
<td>Post-chemotherapy (n=107)</td>
<td></td>
<td>54</td>
<td>36</td>
<td>10</td>
<td>6</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
Figure legends

Figure 1: Flow chart of the material analyzed.

Figure 2: Immunohistochemistry
Palladin protein expression in 6 HGSC effusions. (A-B) Expression in the majority of tumor cells; (C-F) Focal expression.

Figure 3: Survival
A: Kaplan-Meier survival curve showing the association between palladin protein expression and overall survival (OS) for 261 HGSC patients. Patients with effusions with any (>0%) palladin expression (n=106; red line) had mean OS of 44.2 months compared to 36.6 months for patients with palladin-negative effusions (n=155, blue line; p=0.085).
B: Kaplan-Meier survival curve showing the association between patient age and OS for 261 HGSC patients. Older (>60 years) patients (n=142; red line) had mean OS of 31.4 months compared to 48.7 months for younger (≤60 years) patients (n=119, blue line; p<0.001).
C: Kaplan-Meier survival curve showing the association between FIGO stage and OS for 256 HGSC patients with advanced-stage disease. Patients diagnosed with stage IV disease (n=102; red line) had mean OS of 28.1 months compared to 45.5 months for patients with stage III disease (n=154, blue line; p<0.001).
D: Kaplan-Meier survival curve showing the association between residual disease (RD) volume and OS for 216 patients with debulking data. Patients debulked to no macroscopic disease (n=33; blue line) had mean OS of 66.7 months compared to 45.9 and 37.3 months for patients debulked to 1 cm (n=82, red line) and ≥2 cm (n=101, green line), respectively (p=0.001).