The Platelet-activating Factor Receptor’s Association with the Outcome of Ovarian Cancer Patients and its Experimental Inhibition by Rupatadine

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The platelet-activating factor receptor's association with the outcome of ovarian cancer patients and its experimental inhibition by Rupatadine

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Abstract

The platelet-activating factor receptor (PAFR) and its ligand (PAF) are important inflammatory mediators that are overexpressed in ovarian cancer. The receptor is an important player in ovarian cancer development. In this study, we aimed to evaluate the prognostic value of PAFR in epithelial ovarian cancer (EOC) and the potential use of its antagonist, Rupatadine as an experimental treatment.

Tissue microarrays of ovarian cancer patients, most markedly those with a non-mucinous subtype, immunohistochemically overexpressed PAFR. Elevated cytoplasmic PAFR expression was found to significantly and independently impair patients' overall and recurrence-free survival (OS: median 83.48 vs. 155.03 months; p=0.022; RFS: median 164.46 vs. 78.03 months; p=0.015). In vitro, especially the serous ovarian cancer subtypes displayed an elevated PAFR gene and protein expression. siRNA knockdown of PAFR decreased cell proliferation significantly, thus confirming the receptor's protumorigenic effect on ovarian cancer cells. The clinically approved PAFR antagonist Rupatadine effectively inhibited in vitro cell proliferation and migration of ovarian cancer cells. PAFR is a prognostic marker in ovarian cancer patients and its inhibition through Rupatadine may have important therapeutic implications in the therapy of ovarian cancer patients.

Keywords

Ovarian cancer, platelet-activating factor receptor (PAFR), Rupatadine, platelet-activating factor (PAF)
**Introduction**

Ovarian cancer is one of the five leading types of cancer death among women of all ages. Over recent years, the outcome of ovarian cancer patients has remained poor, with a five-year survival rate of less than 45%. The high case fatality rate of ovarian cancer is mostly attributed to a late-stage diagnosis due to an occult growth of the tumor within the peritoneal cavity and a lack of screening methods. Therapeutic options are still limited. Debulking surgery is the most effective curative treatment choice. Additionally, adjuvant platinum-based chemotherapy combined with anti-angiogenic agents or followed by poly-ADP-ribose-polymerase inhibitors are established treatment options. However, most of ovarian cancer patients are diagnosed when the metastasis has already occurred. Hence, it is vital to develop new and effective prognostic and therapeutic options.

Chronic inflammation is a key factor in the pathogenesis of ovarian cancer and other malignancies. In ovarian cancer, chronic inflammation in the tumor environment can be linked to tumor formation, progress, and metastasis. Important inflammatory lipid mediators are the platelet-activating factor (PAF) and its receptor (PAFR). PAFR is a G-protein coupled receptor that signals through G-proteins and associated phosphorylation pathways. The receptors’ sole ligand, PAF, which was first described as an inducer of platelet degranulation and aggregation, is an essential proinflammatory activator of neutrophils, macrophages, platelets, lymphocytes, and endothelial cells.

The role of PAF and PAFR in various cancers, including ovarian cancer, has been investigated in recent years. In ovarian cancer, PAFR is overexpressed and has been identified as an important player in tumor development, metastasis, antiapoptosis, and angiogenesis. However, the receptor’s significance on long-
term survival of ovarian cancer patients is not yet known. The inhibition of PAFR with specific antagonists (Web 2086 and Ginkgolide B) showed promising antiproliferative effects with reduced tumor growth in ovarian cancer models
\textsuperscript{14,15}. Another, not yet analyzed, antagonist of PAFR is Rupatadine \textsuperscript{16}. It is a clinically approved and used antihistaminic drug for allergic diseases \textsuperscript{17}. Due to its inhibition of PAFR and good safety profile, we considered it as a potential drug candidate in ovarian cancer \textsuperscript{16}.

The study aimed to first assess the clinical importance of PAFR on long-term patient’s outcomes. On a molecular level, we examined PAFR gene and protein expression in different subtypes of ovarian cancer cells. To investigate the role of PAFR in epithelial ovarian cancer (EOC), we performed PAFR gene knockdown and evaluated its effect on EOC proliferation. In a drug repurposing approach, we antagonized PAFR with the clinically approved Rupatadine. To evaluate the antagonists’ influence on EOC development, we conducted proliferation and migration assays.

\textbf{Materials and Methods}

\textbf{Patients}

Ovarian cancer samples from 156 patients who underwent surgery for EOC from 1990-2002 at the Department of Obstetrics and Gynecology, Ludwig-Maximilians-University in Munich, Germany were included (Table 1). Written informed consent was obtained from all patients. We only included patients with the definite diagnosis of ovarian cancer in this study; borderline tumors or benign tumors were excluded. Clinical data were retrieved from the patients’ chart, and the Munich Cancer Registry (MCR) provided the follow-up data. The histological classification (serous (n=110),
endometrioid (n=21), mucinous (n=13), clear cell (n=12)) and tumor grading according to the WHO criteria were performed by a gynecological pathologist. Unfortunately, the BRCA status of the patient collective was not accessible. Most patients (67.9 %) presented with advanced stage disease (FIGO III and FIGO IV). All patients that were staged as FIGO II–IV received carboplatin and paclitaxel as adjuvant chemotherapy.

### Table 1: Patient characteristics

<table>
<thead>
<tr>
<th>Parameters</th>
<th>N</th>
<th>Percentage</th>
</tr>
</thead>
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<tr>
<td><strong>Histology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>serous</td>
<td>110</td>
<td>70.5%</td>
</tr>
<tr>
<td>clear cell</td>
<td>12</td>
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</tr>
<tr>
<td>endometrioid</td>
<td>21</td>
<td>13.5%</td>
</tr>
<tr>
<td>mucinous</td>
<td>13</td>
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</tr>
<tr>
<td><strong>Lymph node</strong></td>
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</tr>
<tr>
<td>pNX</td>
<td>61</td>
<td>39.1%</td>
</tr>
<tr>
<td>pN0</td>
<td>43</td>
<td>27.6%</td>
</tr>
<tr>
<td>pN1</td>
<td>52</td>
<td>33.3%</td>
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<tr>
<td><strong>Distant Metastasis</strong></td>
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<tr>
<td>pM0/X</td>
<td>150</td>
<td>96.2%</td>
</tr>
<tr>
<td>pM1</td>
<td>6</td>
<td>3.8%</td>
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<tr>
<td><strong>Grading</strong></td>
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<td></td>
</tr>
<tr>
<td><strong>serous</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>low</td>
<td>24</td>
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</tr>
<tr>
<td>high</td>
<td>80</td>
<td>72.7%</td>
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<tr>
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<tr>
<td>G1</td>
<td>6</td>
<td>28.6%</td>
</tr>
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<tr>
<td>G3</td>
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<tr>
<td>G1</td>
<td>6</td>
<td>46.2%</td>
</tr>
<tr>
<td>G2</td>
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<td>46.2%</td>
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<tr>
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**clear cell**

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**FIGO**

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</tr>
<tr>
<td>II</td>
<td>10</td>
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<tr>
<td>III</td>
<td>103</td>
<td>66.0%</td>
</tr>
<tr>
<td>IV</td>
<td>3</td>
<td>1.9%</td>
</tr>
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</table>

**Age**

| (Median) years | 62 ± 12 |

**Deaths**

| Deaths | 100 |

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**Ethics approval**

The Ethics Committee of the Ludwig-Maximilians-University, Munich, Germany, approved this study (approval numbers 227-09 and 18-392). All tissue samples utilized for this investigation were collected from material from the archives of the Department of Obstetrics and Gynecology, University Hospital, LMU Munich, Munich, Germany, having initially been used for pathological diagnostics. The diagnostic procedures were concluded before the current study was conducted. During the analysis, the observers were fully blinded for patients’ data.

**Immunohistochemistry**

Tissue microarrays of formalin-fixed paraffin-embedded ovarian cancer tissue samples were prepared and stained as previously described. As primary antibody we used the polyclonal rabbit IgG anti-PAFR antibody (Abcam, Cambridge, UK). Kidney tissues served as a positive and negative control.

The intensity of the PAFR expression was assessed by the immunoreactive score (IRS). This is a semi-quantitative scoring system which is well established. The IRS is obtained by multiplying the staining intensity (negative=0, weak=1, moderate=2,
strong=3) with the percentage of positive cells (negative=0, ≤10% =1, ≥11%, ≤50%=2, ≥51%, ≤80%=3, ≥81%=4) resulting in a IR score between 0-12. In the cytoplasm, a score between 0-3 was marked as low and 4-12 as high.

In our study, two independent scorers analyzed the intensity and distribution pattern of PAFR in the cytoplasm. The light microscope “Immunohistochemistry Type 307–148.001 512 686” by Leitz (Wetzlar, Germany) was used. The camera was produced by Fissler (IH- Camera 3CCD Colour Video Camera). For image acquisition, the software “Discuss Version 4,602,017-#233 (Carl C. Hilgers Technical Office) was used. Image bit depth: 24 mm; time and space resolution data: 760 + 574 pixel.

**Cells, culture conditions and reagents**

The OVCAR-3 (serous, BRCA WT), UWB1.289 (serous, BRCA1 negative), ES-2 (clear cell) and TOV 112D (endometrioid) cell lines were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD, USA). All cell lines were cultured in RPMI 1640 medium (ThermoFisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS) without antimycotics/ antibiotics in a humidified atmosphere (37°C, 5% CO2). All cell lines were tested for mycoplasma before the experiments.

**qPCR**

RNA isolation was performed according to the manufacturer’s protocol by using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands). RNA concentrations were adjusted, and cDNA synthesis was carried out with the MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (Epicentre, Madison, WI, USA). qPCR was carried out with FastStart Essential DNA Probes Master and gene-specific primers (Roche, Basel, Switzerland). The primer sequences are available in Table S1. The relative expression
was calculated by the 2-ΔΔCt method applying β-Actin and GAPDH as housekeeping genes.

**siRNA knockdown**

For siRNA knockdown experiments, UWB1.289 cells were transfected with small interfering RNA (siRNA; 3 different sequences for PAFR) using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA). A scrambled negative control siRNA (Qiagen, Hilden, Germany) was used as a reference. UWB1.289 cells were cultured in six-well plates and transfected when cell density reached 60-70 %. Opti-MEM Reduced Serum Medium (Thermo Fisher Scientific, Waltham, MA, USA) containing siRNA-PAFR and Lipofectamine RNAiMAX was used for the treatment of the cells. After 36 h cells were harvested and used for further analysis.

**Western blotting**

Wells were washed twice in ice-cold PBS and lysed for 15 min at 4°C in 200 µl RIPA buffer containing protease inhibitor (Sigma-Aldrich Co., St. Louis, MO, USA). The protein concentration of the lysates was determined by Bradford assay. Then, cellular extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). After blocking the membrane for 1 h with casein (Vector Laboratories, Burlingame, CA, USA), blots were incubated with diluted primary antibodies gently shaking overnight at 4°C. As primary antibodies a rabbit polyclonal antibody against PAFR (1:200 dilution; Cayman, Ann Arbor, MI, USA) and a mouse monoclonal antibody against β-actin (1:1000 dilution; Sigma, St. Louis, USA) were employed. β-actin western blots served as controls. Subsequently, blots were washed with 1:10 casein three times and then subjected to biotinylated anti-mouse/anti-rabbit IgG secondary antibodies and ABC-
AmP reagent (VECTASTAIN ABC-AmP Kit for rabbit IgG; Vector Laboratories, Burlingame, CA, USA). The antibody complexes were visualized with 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium chromogenic substrate (Vectastain ABC-AmP Kit; Vector Laboratories, Burlingame, CA, USA). Western blotting detection and analysis was performed with Bio-Rad Universal Hood II and the corresponding software Quantity One (Bio-Rad Laboratories Inc., Hercules, CA, USA).

**Cell proliferation**

Cell proliferation of OVCAR-3, UWB1.289, ES-2, TOV 112D was assessed with a dimethyl-thiazolyl-diphenyl-tetrazolium bromide (MTT) assay (Roche, Mannheim, Germany). Cells were seeded at a density of 5x10^3 cells per well into 96-well plates and incubated in RPMI 1640 medium with 10% FBS for 24 h, before being treated. The siRNA transfection (PAFR gene knockdown) was conducted as described above. The cells were inhibited with the PAFR antagonist Rupatadine (Sigma-Aldrich Co., St. Louis, MO, USA) or the respective carrier solution (ethanol, DMSO) at the indicated concentrations. After treatment for 72 h, MTT-labelling (5 mg/ml in PBS dye solution) was performed according to the manufacturer's manual. A spectrophotometric reading analyzed absorbance with the Microplate Reader (BioTek, Winooski, VT, USA) at 595nm.

**Wound healing**

The cells were seeded in 12-well plates (4x10^5) for 24 h. To create an artificial wound, the central fields of confluent monolayers were scratched manually with 200 μl pipette tips through the entire center of the wells. Digital images were taken after treatment with Rupatadine at 0h and 24 h. Cell migration was monitored with an inverse phase-contrast microscope (Leica Dmi1, Germany). Photos of cell migration area were
analyzed with the software Image J (https://image.j.nih.gov/ij/), and the cell migration area was calculated as the difference of the scratch areas between 24 h and 0 h.

**Statistical analysis**

Data were analyzed employing the SPSS (v21, IBM, Armonk, New York) and GraphPad Prism Version 8.00 softwares (CA, USA). Spearman coefficient was employed to correlate data, while the Kaplan-Meier curves were drawn to compare survival times between groups.

The chi-square statistic of the log-rank (Mantel-Cox) test was applied to test differences in overall and recurrence-free survival for significance. Cut-off points were obtained through the receiver operator curve (ROC) \(^{21-23}\). Multivariate cox-regression was performed to analyze covariates. For unpaired data, if D'Agostino-Pearson omnibus normality test indicated Gaussian distribution, an unpaired t-test for side-by-side comparisons or one-way ANOVA with Tukey’s posttest for multiple comparisons was performed. Statistical significance for all tests was set as p=0.05 and data was expressed as means ± SD.

**Results**

**Elevated cytoplasmic PAFR expression in serous, clear cell and endometrioid ovarian cancer patients**

Out of 148 successfully stained ovarian cancer specimens, 147 (99%) samples showed a positive cytoplasmic PAFR expression. In the cytoplasm, the median immunoreactive score (IRS) was 6 (0-12). When analyzing the staining distribution between the different histological subtypes, the serous (median IRS= 6), clear cell (median IRS= 6), and endometrioid (median IRS= 6) samples demonstrate a
significant elevated expression compared to the mucinous subtype (median IRS= 2; p=0.000, Fig.1).

**Figure 1**

PAFR expression correlates with clinical and pathological data

The correlation between cytoplasmic PAFR expression and clinical and pathological data such as histology, FIGO and grading was analyzed. A significant negative correlation was found between cytoplasmic PAFR expression and histology (p=0.011, Rho= -0.222). High grading in serous cancer patients correlated significantly with cytoplasmic PAFR expression (p=0.031, Rho= 0.189, Table 2).
Table 2: Correlation analysis between positive cytoplasmic PAFR expression and clinicopathological data

<table>
<thead>
<tr>
<th>Variables</th>
<th>p</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histology</td>
<td>0.011</td>
<td>-0.222</td>
</tr>
<tr>
<td>FIGO</td>
<td>0.749</td>
<td>0.029</td>
</tr>
</tbody>
</table>

Grading

<table>
<thead>
<tr>
<th>Grading</th>
<th>p</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>serous- low grading</td>
<td>0.647</td>
<td>0.040</td>
</tr>
<tr>
<td>serous- high grading</td>
<td><strong>0.031</strong></td>
<td>0.189</td>
</tr>
<tr>
<td>clear cell, endometrioid and mucinous-G1 to G3</td>
<td>0.051</td>
<td>-0.174</td>
</tr>
</tbody>
</table>

**PAFR as a negative independent prognostic factor in ovarian cancer patients**

In this study, patients' median survival time was 51.2 ± 57.6 months and median months free of recurrence were 56.4 ± 57.6. The median age of the women were 62 ± 12 years with range of 31- 88 years. Ovarian cancer patients with an increased cytoplasmic PAFR expression (IRS >3) had a significantly worse outcome compared to the other subgroup. The mean survival time of patients with an elevated PAFR expression (IRS >3) was 83.48 months vs. 155.03 months for patients with low expression (IRS <3); p=0.022, Fig.2a. Similarly, patients with raised PAFR expression had a significantly higher chance of cancer recurrence (p=0.015 164.46 vs. 78.03 months, Fig.2b).
In a multivariate cox-regression analysis, we analyzed various clinicopathological parameters, as well as cytoplasmic PAFR expression for recurrence free survival. Patients with elevated cytoplasmic PAFR expression demonstrated a higher hazard ratio (HR= 4.069) than those with lower PAFR expression (p=0.045). Out of the other coefficients, FIGO status proved to significantly increase the hazard of patients (HR= 4.316; p= 0.000, Table 3).

Table 3: Multivariate analysis of clinicopathological parameters and cytoplasmic PAFR expression

<table>
<thead>
<tr>
<th>Covariate</th>
<th>Coefficient ((b_i))</th>
<th>([HR = \exp(b_i)])</th>
<th>95% CI</th>
<th>p-value</th>
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<tbody>
<tr>
<td></td>
<td>Lower</td>
<td>Upper</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FIGO (I,II vs. III, IV)</td>
<td>1.462</td>
<td>4.316</td>
<td>2.222</td>
<td>8.384</td>
</tr>
<tr>
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<td>-0.526</td>
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</tr>
<tr>
<td></td>
<td>serous high</td>
<td>0.692</td>
<td>1.999</td>
<td>0.742</td>
</tr>
</tbody>
</table>
PAFR knockdown reduced ovarian cancer cell proliferation

We first detected PAFR gene and protein expression in different ovarian cancer cell lines. In the PCR, the expression of PAFR was significantly higher in the serous, BRCA-1 negative ovarian cancer cell lines (UWB1.289) compared to the other groups (p= <0.0001, Supplementary information). We further analyzed PAFR protein expression by Western blot. The PAFR protein band was highly expressed in the UWB1.289 cell line. TOV 112D, OVCAR-3 and ES-2 also showed protein expression. This data is consistent with previous findings that PAFR is overexpressed in malignant ovarian cancer.24

To prove that PAFR upregulation significantly contributes to cancer cell proliferation, we reduced PAFR expression through siRNA interference in UWB1.289 cells. Three specific siRNA sequences significantly reduced PAFR expression compared to the negative control (p=<0.0001; Fig.3a). Cell proliferation was confirmed to be significantly lower in the PAFR knock-down cancer cells than in the cells used as control group (p=0.0003; Fig.3b).

<table>
<thead>
<tr>
<th>clear cell, endometrioid and mucinous-G1 to G3</th>
<th>0.043</th>
<th>1.044</th>
<th>0.685</th>
<th>1.591</th>
<th>0.841</th>
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</thead>
<tbody>
<tr>
<td>Patients’ age (≤45 vs. &gt;45 years)</td>
<td>-0.196</td>
<td>0.822</td>
<td>0.416</td>
<td>1.624</td>
<td>0.572</td>
</tr>
<tr>
<td>PAFR cytoplasmic IRS &gt;3</td>
<td>1.403</td>
<td>4.069</td>
<td>1.031</td>
<td>16.059</td>
<td><strong>0.045</strong></td>
</tr>
</tbody>
</table>


Rupatadine has an inhibitory effect on proliferation and migration of ovarian cancer cells

To investigate the effect of the PAFR antagonist, Rupatadine, on ovarian cancer we performed functional assays in different ovarian cancer cells. All cell lines demonstrated a significantly reduced cell viability after being treated with Rupatadine. This effect was highlighted by the response of OVCAR-3, UWB1.289, ES-2, and TOV112D cells to 30 μM of Rupatadine (Fig.4). Especially, OVCAR-3, ES-2 and TOV112D ovarian cancer cells responded to 30μM Rupatadine with a strong antiproliferative effect ($p<0.0001$). Lower concentrations of Rupatadine (10 μM and 20 μM) diminished proliferation notably in endometrioid and clear cell ovarian cancer cells.
As a next step, we wanted to assess Rupatadines’ influence on ovarian cancer cell migration. The wound healing assay was performed showing significantly less cell migration in all cancer cell lines after 24 h compared to the control group (OVCAR-3: \( p = 0.0044 \), UWB1.289: \( p = 0.0261 \), TOV 112D: \( p < 0.0001 \) and ES-2: \( p = 0.0145 \); Fig.5).
The results of this study demonstrate the clinical importance of PAFR expression in ovarian cancer patients. Previously, it has been shown that the PAFR is overexpressed in ovarian cancer samples \(11\). To our knowledge, this is the first study evaluating the
effect of PAFR expression on long-term ovarian cancer patient's outcome. In those patients, high cytoplasmic PAFR expression was significantly associated with worse overall survival and shorter recurrence-free survival time. Raised cytoplasmic PAFR was proved to be an independent prognostic factor by a multivariate cox-analysis.

Other studies have alluded to the differences of PAFR expression in distinct ovarian cancer subtypes. In line with other studies, our data confirmed that the non-mucinous ovarian cancer patients displayed a significantly elevated PAFR staining intensity compared to the mucinous subtype. Patients with positive cytoplasmic PAFR expression demonstrated a significant correlation with histology, but more interestingly with high grading in serous cancer cells. These results were in accord with our in vitro results. When comparing the various ovarian cancer cells, the serous and the BRCA1 mutated ovarian cancer cells significantly overexpressed PAFR.

The breast cancer 1/2 (BRCA1/2) genes are important players in the pathogenesis of ovarian cancer. They have several essential caretaker roles, such as transcriptional regulation and tumor suppressors in normal epithelial cells. In 15-20% of ovarian cancer patients, a mutation in the tumor suppressor genes breast cancer 1/2 (BRCA1/2) can be found, leading to a familial accumulation of ovarian and breast cancer. BRCA1-mutation will increase the risk of developing ovarian cancer to about 40-60%, compared to the cumulative risk of about 1.3% in the general population.

In our study, we could not only show that the BRCA1 mutant cells displayed a higher PAFR expression than the rest of the epithelial OC cells but also that siRNA knockdown of PAFR in BRCA1 mutant cells significantly decreased cancer cell proliferation. The role of PAF and its receptor in BRCA1 mutated ovarian cancer cells has also been examined in other studies. Zhang et al. have focused on the molecular
pathways of PAF/PAFR, and their association with early malignant transformation in BRCA1 mutated ovarian cells\textsuperscript{24}. By the protein-protein interaction between PAFR and FAK and FAK and the Signal Transducers and Activators of Transcription (STAT1), the receptor promoted tumor progression and also contributed to early malignant transformation of BRCA1-mutant ovarian epithelial cells.

In general, the pro-inflammatory PAF/PAFR axis has been shown to play a crucial role in anti-apoptosis, tumor growth, and metastasis\textsuperscript{29-33}. The receptor induces downstream signaling via EGFR/Src/FAK/Paxillin\textsuperscript{11}. Known PAFR antagonists are amongst others: WEB-2086, Ginkgolide B, and Rupatadine\textsuperscript{16,34-36}. Rupatadine is a PAFR antagonist that is clinically used as an antihistaminic drug\textsuperscript{17,37}. Studies on Rupatadine have primarily focused on its anti-inflammatory effects, such as the inhibition of mast cell degranulation and eosinophil chemotaxis\textsuperscript{37}. This is the first study investigating Rupatadine's effect on cancer cells. We could demonstrate that Rupatadine can effectively inhibit in vitro cell proliferation and migration of clear cell, serous, BRCA1 mutant and endometrioid ovarian cancer cells. Hence, Rupatadine seems to have a significant antiproliferative effect on ovarian cancer cells.

The effect of other PAFR antagonists, such as WEB-2086 and Ginkgolide B, on ovarian cancer cells has already been studied\textsuperscript{14,24}. Compared to the two substances, Rupatadine's anti-PAF activity appears to be lower\textsuperscript{16}. However, in contrast to WEB-2086, Rupatadine is a drug that has already been tested in a multicenter phase-IV study and is clinically approved\textsuperscript{16}. Several studies have confirmed Rupatadine's long-term safety profile\textsuperscript{38}. Thus, using Rupatadine as a PAFR-antagonist and thereby reducing tumor growth in ovarian cancer is a treatment option worth considering. The following studies will need to confirm Rupatadine's antiproliferative effect on ovarian cancer in-vivo.
It is noteworthy that in melanoma and ovarian cancer cells, PAFR antagonists demonstrated to have a potentiating effect of chemotherapeutic drugs\textsuperscript{36}. Yu et al. provided evidence that PAFR antagonists sensitized ovarian cancer cells to cisplatin, and the combined treatment reduced tumor growth\textsuperscript{15}. Similarly, the combined PAFR and EGFR inhibition synergistically diminished ovarian cancer progression\textsuperscript{14}. In further studies, the combined effect of Rupatadine with other chemotherapeutic drugs should be studied, evaluating whether a combination can improve tumor therapy. Using retrospective data, it would be interesting to determine whether ovarian cancer patients who received Rupatadine as an antihistaminic drug had a better outcome. To complement our data, additional analysis should focus on the prognostic relevance of PAFR expression in BRCA1 mutant ovarian cancer specimens on long term survival.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BRCA</td>
<td>breast cancer gene</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>EOC</td>
<td>epithelial ovarian cancer</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<td>FIGO</td>
<td>International Federation of Gynecology and Obstetrics</td>
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<tr>
<td>GAPDH</td>
<td>glycerinaldehyde-3-phosphat-dehydrogenase</td>
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<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
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<td>IRScore</td>
<td>immunoreactive score</td>
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<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
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<tr>
<td>OS</td>
<td>overall survival</td>
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<tr>
<td>PAF</td>
<td>platelet activating factor</td>
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<td>PTAFR</td>
<td>platelet activating factor receptor</td>
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<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
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<tr>
<td>RFS</td>
<td>Recurrence free survival</td>
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<tr>
<td>ROC</td>
<td>receiver operating characteristic</td>
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<tr>
<td>siRNA</td>
<td>small interfering ribonucleic acid</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>Src/FAK</td>
<td>steroid receptor coactivator/focal adhesion kinase</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
</tbody>
</table>
References


Yu, Y. et al. Transactivation of epidermal growth factor receptor through platelet-activating factor/receptor in ovarian cancer cells. *Journal of*


Authors' contributions
B.C. and U.J. conceived and designed the experiments; E.D. I.H. and M.K. performed the experiments; E.D. and U.J. analyzed the data; DM and ES supervised immunohistochemistry as gynecologic pathologists and participated in immunohistochemistry analysis as well as in the design and coordination of the study. E.D., B.C. and F.T. wrote the paper. I.H., S.B., T.K., A.H., F.K., A.C., A.B., S.M. and U.J. critically reviewed the paper. All authors read and approved the final manuscript.

Additional information

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Conflict of interest
Thomas Kolben holds stock of Roche AG and his relative is employed at Roche AG. Anna Hester has received a research grant from the “Walter Schulz” foundation and advisory board, speech honoraria, and travel expenses from Roche and Pfizer. Research support, advisory board, honoraria, and travel expenses from Astra-Zeneca, Clovis, Medac, MSD, Novartis, PharmaMar, Roche, Sensor Kinesis, Tesaro, and Teva have been received by Sven Mahner and others from AstraZeneca, Medac, PharmaMar, Roche, Tesaro by Fabian Trillsch. All other authors declare no conflict of interest. All the above-mentioned companies did not influence the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Availability of data and materials
The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.
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Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Ludwig-Maximilians-University, Munich, Germany (approval numbers 227–09 and 18–392). The ovarian cancer specimens were obtained in clinically indicated surgeries and initially used for histopathological diagnostics. When the current study was performed, all diagnostic procedures were completed, and the patients’ data were anonymized. The ethical principles adopted in the Declaration of Helsinki 1975 have been respected.

Consent for publication

All authors have read the manuscript and agree to its publication.
**Figure legends**

**Fig. 1** PAFR expression varies in different ovarian cancer subtypes. (a) Raised cytoplasmic PAFR staining in serous, (b) clear cell, and (c) endometrioid cancer cells compared to the (d) mucinous subtype. (e) PAFR positive and (f) PAFR negative control in the kidney. (g) The mucinous subtype demonstrates a significantly lower cytoplasmic PAFR staining expression compared to the other histological subtypes (p=0.000) as analyzed by one-way ANOVA test.

**Fig. 2** PAFR expression is a prognostic factor in ovarian cancer patients. (a) Overall and (b) recurrence-free survival of patients whose tumors expressed cytoplasmic PAFR at high levels was compared to those with low PAFR expression by the log rank test. Kaplan-Meier survival plots were drawn. Ovarian cancer patients with elevated PAFR expression in the cytoplasm survived significantly shorter (a) and demonstrated a shorter recurrence free survival time (b) compared to the group of patients with low PAFR expression.

**Fig. 3** PAFR siRNA knock down reduces proliferation of UWB1.289 ovarian cancer cells. (a) The upregulation of PAFR gene expression was significantly decreased by the three different siRNA sequences used. (b) Compared to the UWB1.289 cells with increased PAFR expression, the PAFR knockdown cancer cells proved to proliferate significantly less. Results are represented as mean (SD) of 3 independent experiments. One-way ANOVA was employed to test for differences between groups and indicated as **p=<0.01, ***p=<0.001 and ****p=<0.0001 compared with controls.

**Fig. 4** Rupatadine decreases proliferation of ovarian cancer cells. (a) OVCAR-3, (b) UWB1.289, (c) ES-2, and (d) TOV 112D were treated with increasing concentrations
of Rupatadine. Cell viability was tested with the MTT assay. All cell lines showed a concentration dependent decrease in cell proliferation, the greatest effect obtained with 30μM of Rupatadine ((a), (c), and (d) p= <0.0001, (b) p=0.0021). 10 μM and 20 μM of Rupatadine had a significant antiproliferative effect in (c) ES-2 cells (control vs 10 μM of Rupatadine p= 0.0048 and 20μM of Rupatadine p=<0.0001) and (d) TOV 112D cells (control vs 10μM of Rupatadine p= 0.0016 and 20μM of Rupatadine p=<0.0001). Results are represented as mean (SD) of 3 independent experiments. One-way ANOVA was employed to test for differences between groups and indicated as **p= <0.01, ***p= <0.001 and ****p=<0.0001 compared with controls.

**Fig. 5** Treatment with Rupatadine reduces cell migration of ovarian cancer cells. Wound area was measured at 0 h and 24 h in the control and 30 μM Rupatadine group. Four different ovarian cancer cell lines showed significantly reduced migration after treatment compared to the control group. Columns represent the measured difference of the area covered by cells between 0 h and 24 h. Results are represented as mean (SD) of 3 independent experiments. Unpaired t-test was employed to test for differences between groups and indicated as *p= <0.05, **p= <0.01, and ****p=<0.0001 compared with controls.
Figures

Figure 1

Please see the Manuscript PDF file for the complete figure caption
Figure 2

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Figure 3
Figure 4

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Figure 5

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Supplementary Files

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