Combined expression of KLK4, KLK5, KLK6, and KLK7 by ovarian cancer cells leads to decreased adhesion and paclitaxel–induced chemoresistance

Daniela Loessner¹,³, Verena MC Quent²,⁴, Julia Kraemer¹,³, Eva C Weber¹,³, Dietmar W Hutmacher²,⁴, Viktor Magdolen⁵ and Judith A Clements¹,³*

¹Cancer and ²Regenerative Medicine Programs, Institute of Health and Biomedical Innovation, ³School of Biomedical Sciences, Faculty of Health and ⁴Faculty of Science and Engineering, Queensland University of Technology, 60 Musk Avenue, Kelvin Grove, Brisbane, Queensland 4059, Australia; ⁵Clinical Research Unit, Department of Obstetrics and Gynecology, Technical University of Munich, Ismaninger Str. 22, D–81675, Germany

Running title: Kallikrein proteases alter cell adhesion and chemoresistance

*Corresponding author:
Judith A Clements
Institute of Health and Biomedical Innovation
Queensland University of Technology
60 Musk Avenue, Kelvin Grove, Brisbane, Queensland 4059, Australia
Phone: +61–7–3138–6198
Fax: +61–7–3138–6039
E–mail: j.clements@qut.edu.au
Abstract

Objective: Chemoresistance is a critical feature of advanced ovarian cancer with only 30% of patients surviving longer than 5 years. We have previously shown that four kallikrein–related (KLK) peptidases, KLK4, KLK5, KLK6 and KLK7 (KLK4–7), are implicated in peritoneal invasion and tumour growth, but underlying mechanisms were not identified. We also reported that KLK7 overexpression confers chemoresistance to paclitaxel, and cell survival via integrins. In this study, we further explored the functional consequences of overexpression of all four KLKs (KLK4–7) simultaneously in the ovarian cancer cell line, OV–MZ–6, and its impact on integrin expression and signalling, cell adhesion and survival as contributors to chemoresistance and metastatic progression.

Methods: Quantitative gene and protein expression analyses, confocal microscopy, cell adhesion and chemosensitivity assays were performed.

Results: Expression of α5β1/αvβ3 integrins was downregulated upon combined stable KLK4–7 overexpression in OV–MZ–6 cells. Accordingly, the adhesion of these cells to vitronectin and fibronectin, the extracellular matrix binding proteins of α5β1/αvβ3 integrins and two predominant proteins of the peritoneal matrix, was decreased. KLK4–7–transfected cells were more resistant to paclitaxel (10–100 nmol/L: 38–54%), but not to carboplatin, which was associated with decreased apoptotic stimuli. However, the KLK4–7–induced paclitaxel resistance was not blocked by the MEK1/2 inhibitor, U0126.

Conclusions: This study demonstrates that combined KLK4–7 expression by ovarian cancer cells promotes reduced integrin expression with consequently less cell–matrix attachment, and insensitivity to paclitaxel mediated by complex integrin and MAPK independent interactions, indicative of a malignant phenotype and disease progression suggesting a role for these KLKs in this process.

Keywords: adhesion, chemoresistance, integrins, kallikreins, ovarian cancer
**Introduction**

Ovarian cancer is the most common malignancy among gynaecological cancers and is the major cause of all female cancer deaths [1, 2]. The predominant cancer type is epithelial ovarian carcinoma (EOC). More than 75% of ovarian cancer patients are diagnosed at a late–stage with evidence of metastatic spread within the peritoneal cavity and pelvic region. Cytoreductive surgery and chemotherapy is the standard treatment for advanced ovarian cancer. Although platinum–taxane combination therapies have improved, the 5 year survival rate of patients is only 30% with the overall mortality due to relapse and chemoresistance unchanged for decades. The main reasons for unsuccessful therapies are a limited understanding of the heterogeneous pathobiology of this disease, the mechanisms underlying drug resistance and a limited number of prognostic and therapeutic biomarkers [2-4].

Currently, the serum biomarker CA125 is clinically used to indicate the presence of ovarian cancer, although not all patients have elevated CA125 levels and its detection can result in false positives. Due to the low specificity and sensitivity, its use for monitoring treatment responses and predicting prognosis after treatment is limited. Therefore, novel ovarian cancer–specific serum biomarkers are needed to detect this disease and to follow up patient outcomes [5]. Cancer–related proteases, such as kallikrein–related peptidases (KLK; proteins encoded by KLK genes), are under investigation as potential diagnostic indicators for ovarian cancer and prognostic biomarkers for disease progression [6-9]. Among the fifteen human KLKs, twelve, including KLK4, KLK5, KLK6, and KLK7 (KLK4–7), are up–regulated in cancerous ovarian tissues, serum and ascites fluid from patients, and cell lines [6, 10, 11]. KLK5 serves as a biomarker for early detection of ovarian cancer and its levels in serum or ascites fluid did not correlate with the established cancer biomarker CA125, thus it provides independent information from CA125 in this disease [12]. KLK4–7 overexpression is associated with late–stage and high–grade disease, shorter disease–free and overall survival rates [12]. Elevated serum levels of KLK4–7 are associated with non–responsiveness of ovarian cancer patients to paclitaxel [9, 12-14]. Both KLK/KLK4 and KLK/KLK7 are also associated with multicellular aggregation and paclitaxel resistance in ovarian cancer in vitro [13, 14]. KLKs likely play a crucial role throughout the progression of ovarian cancer by contributing to invasion, metastasis and
modification of the tumour microenvironment via degradation and/or activation of extracellular matrix (ECM) and other proteins leading to altered cell–cell/cell–ECM interactions, ECM remodelling, cell survival and differentiation [15]. However, the precise signalling pathways involved are not yet fully elucidated.

Cell–cell/cell–ECM adhesion molecules, such as integrin cell adhesion and signalling receptors are also critical factors in tumour progression. Integrin signalling is involved in multiple functions of tumour cells, such as adhesion, invasion, proliferation, survival and apoptosis, thereby affecting tumour growth and metastasis [16]. Integrins are non–covalently associated heterodimeric cell surface receptors promoting bidirectional signal transduction via preferential binding to distinct ECM proteins, such as fibronectin, vitronectin and collagens. Although transmembrane integrins lack kinase activity, by clustering they recruit and activate kinases, such as focal adhesion kinase (FAK) and src family kinases (SFK), to form focal adhesions and crosstalk with other cell surface receptors, thereby activating the MAPK signalling cascade [17]. Of particular interest, it has been reported for various cancer types including ovarian cancer, that integrins mediate tumour–ECM interactions, like matrix adhesion and alteration of apoptotic mediators, leading to drug resistance [18]. Different integrins contribute to tumour progression and chemoresistance of ovarian cancer. For example, αvβ3, α5β1 and α2β1 integrins are upregulated in late–stage ovarian cancer [19-21].

The objective of this study was to determine the functional consequences on cell adhesion, cell survival, integrin expression and related signalling responses towards the commonly used chemo–agents paclitaxel and carboplatin upon stable KLK4–7 overexpression in the EOC cell line OV–MZ–6 [22, 23]. In this previously generated cell line, combined KLK4–7 transfection promoted increased invasion in vitro and enhanced tumour growth in an EOC mouse model compared to vector controls [22], although the underlying mechanisms involved were not studied. An advantage of this model is that simultaneous expression of KLK4, KLK5, KLK6, and KLK7 in EOC cells allows their proposed activation cascades which have been described in vitro by pro–KLK fusion protein digestions on cleavage of the pro–region [24] to proceed. For instance, KLK4 and KLK5 autoactivate; active KLK4 activates KLK5 and KLK6; active KLK5 activates KLK6 and KLK7; and KLK6 partially activates KLK5 [24]. Although one should be
circumspect in extrapolation of this data to an *in vivo* environment, especially considering many additional potential interactions of (pro)KLKs within the extracellular proteolytic web, several tissue–specific KLK cascades have been described in detail, for instance, within the central nervous system, semen or skin [24-27]. In fact, we only saw the marked tumorigenic effect when the four KLKs (KLK4–7), and not the individual KLKs or other combinations, were overexpressed in OV–MZ–6 cells [22]. Therefore, it is not unreasonable to assume that an activation cascade, dependent on combined expression of certain KLKs, akin to that described by Yoon and colleagues [24], is operational in these cells.

We demonstrate that stable overexpression of KLK4–7 by EOC cells promoted decreased cell adhesion to ECM proteins and integrin expression as well as paclitaxel–induced chemoresistance accompanied by differential expression of drug–resistance associated genes representative of a malignant phenotype and disease progression. Our results also showed that integrin and MAPK independent pathways may mediate the cellular responses of KLK4–7–expressing EOC cells that contribute to their chemoresistance.
**Materials and Methods**

**DNA constructs, generation of stable cell lines and cell culture.** Human KLK4, KLK5, KLK6, and KLK7 cDNA including pre-pro-domains were isolated from EOC tissue, stable KLK-expressing OV–MZ–6 cells generated using G418–selection (1 g/ml; Invitrogen, Mulgrave, VIC, Australia) and grown in DMEM (Invitrogen) containing 10% (v/v) FCS as described previously [22]. The human EOC cell line OVCAR–3 was purchased from American Type Culture Collection (ATCC). Cells were cultured at 37°C/5% CO\(_2\) until reaching confluency of 60–80% and harvested with EDTA (0.48 mmol/L; Invitrogen).

**Cell adhesion assays.** Attachment of cells onto the ECM proteins (10 µg/ml) fibronectin (FN) (BD Biosciences, Eight Mile Plains, QLD, Australia), vitronectin (VN) (Promega, Alexandria, NSW, Australia), collagen (Col) type I (Sigma–Aldrich, Castle Hill, NSW, Australia) and type IV (Calbiochem, South Granville, NSW, Australia) and the effect of blocking β1 (#P2D5)/αv (#AV1) integrin antibodies (Chemicon, North Ryde, NSW, Australia) or mouse IgG (10 µg/ml; Sigma–Aldrich) was measured as reported previously [13]. For each condition, three different biological assays were conducted in triplicate and normalised to BSA–coated plates and non–blocked controls.

**Cell survival post paclitaxel/carboplatin/U0126 treatment.** Chemosensitivity assays using anti–cancer agents (Sigma–Aldrich) were performed as reported previously [28]. Briefly, 1 day after seeding (3.0x10\(^4\) cells) onto 96–well plates (Perkin Elmer, Melbourne, VIC, Australia), cells were treated with paclitaxel (0, 0.01, 0.1, 1, 2.5, 5, 10, 50, 100 nmol/L), carboplatin (0, 1, 5, 10, 50, 100 µmol/L) for 3 days, and in 1 day intervals using paclitaxel (100 nmol/L), carboplatin (50 µmol/L) or in combination; control agents (DMSO (Merck, Kilsyth, VIC, Australia) or H\(_2\)O). For inhibition, 1 day before seeding, cells were serum–starved with media containing 1% (v/v) FSC cells, and then pre–treated with the MEK1/2 inhibitor (U0126; 50 µmol/L; Cell Signaling Technology, Arundel, QLD, Australia) or control agent (DMSO) for 2 h followed by paclitaxel (100 nmol/L) over 3 days. Cell survival post anti–cancer agents or inhibition was detected using CyQuant®/AlamarBlue® assays (Invitrogen) and DNA content/metabolic activity calculated as percentage of fluorescence in non–treated control samples. The percentage of OV–
Vector cells was set to 100% for each dose and the proportion of OV–KLK4–7 cell survival calculated from this reference level. For each condition, three different biological assays were conducted in triplicate.

**Confocal laser scanning microscopy (CLSM).** Cell monolayers grown on glass coverslips were processed as described earlier [28]. Primary [$\alpha_5$ (1:50), $\beta_1$ (1:200), $\alpha_5\beta_1$ (1:50; #JBS5) integrins (Chemicon); $\alpha$–tubulin (1:200; #236–10501; Invitrogen); KLK4 (1:200), KLK7 (1:20), both generated in Prof Magdolen’s laboratory (Technical University of Munich, Germany); KLK5 (1:100; (#193318; R&D Systems, Gymea, NSW, Australia); KLK6 (1:100; (#265508; R&D Systems); cytokeratin8 (1:300; #M20; #EP1628Y; Abcam, Waterloo, NSW, Australia); annexin V (1:200; Abcam)] and secondary [Alexa488/568–conjugated anti–mouse/rabbit IgG (1:1000; Invitrogen)] antibodies in 1% (w/v) BSA/PBS were incubated for 1 h each. Secondary antibody only or mouse IgG served as negative controls. F–actin filaments were stained with rhodamine415–conjugated phalloidin (0.3 U/ml; Invitrogen) and nuclei with DAPI (2.5 mg/ml; Invitrogen) in 1% (w/v) BSA/PBS. Additionally, cells were labelled with propidium iodide (1:2000; Invitrogen). Immunofluorescence was visualized and photographed using a confocal microscope (TCS SP5 II, Leica) with a 20/40x immersion oil objective applying the Leica Microsystems LAS AF software (version 1.8.2 build 1465). Cells were imaged at five different positions from three different biological assays.

**Quantitative RT–PCR (RT–qPCR).** Equal amounts (1 µg) of total RNA extracted from cells using TRIzol® were treated with DNase 1, reverse transcribed using random hexamers and cDNA synthesized with SuperScript III. RT–qPCR was performed in triplicate with SYBR Green chemistry (all PCR reagents from Invitrogen) on an ABI7300 thermal cycler (Applied Biosystems). Reaction setup and normalization applying the standard curve method ($R^2$ 0.99–0.96) were conducted as reported previously [28]. Gene specific primers are listed in Supplementary Table 1.

**Flow cytometry.** Cells (4x10$^5$) were blocked with 1% (w/v) BSA/PBS for 30 min and treated with fluorescein isothiocyanate–conjugated $\alpha_5$ (1:250; #SAM–1)/$\beta_1$ (1:500; #P4G11) integrins and phycoerythrin–conjugated $\alpha_v\beta_3$ integrin (1:500; #LM609) antibodies (Chemicon) for 1.5 h,
washed twice with PBS and resuspended in 0.4 ml PBS for analysis with CXP software (version 2.0) on a flow cytometer FC500 (Beckman Coulter). Mouse IgG (1:500) incubated for 1 h, followed by Alexa488/568–conjugated anti–mouse IgG (1:1000), incubated for 30 min, served as control. Flow cytometric analyses were performed under identical instrumental settings analysing 2x10⁴ events. Signals from subcellular debris were eliminated during data acquisition by gating. Values of mean fluorescence intensities of triplicates are depicted and normalized to gated cell numbers and vector controls.

**Western blotting.** To determine activation of signalling pathways, cells were serum–starved over 24 h and treated with paclitaxel (100 nmol/L)/carboplatin (5 µmol/L)/combination of both agents for 5 min or for inhibition studies with blocking β1 integrin antibody (10 µg/ml), Src (SU6656; 10 µmol/L; Invitrogen) or MEK1/2 inhibitors (U0126; 50 µmol/L) and control agent (DMSO) for 2 h. Cells were collected in lysis buffer [50 mmol/L HEPES (pH 7.5); 150 mmol/L NaCl; 1 mmol/L EDTA; 10% (v/v) glycerol; 1% (v/v) triton X–100; 10 mmol/L sodium pyrophosphate; 0.1% (w/v) protease inhibitor cocktail (Roche Diagnostic, Castle Hill, NSW, Australia); 10 mmol/L sodium fluoride; 2 mmol/L sodium vanadate] and protein concentrations analysed by the bicinchoninic acid assay (Invitrogen). Cell lysates (20 µg) were electrophoresed on 10% SDS–PAGE, transferred onto nitrocellulose membranes and treated with Odyssey® blocking buffer (LI–COR Biosciences, Surrey Hills, VIC, Australia). Membranes were incubated with primary [phospho–p44/42 MAPK (1:1000; #E10; Thr202/Tyr204; Cell Signaling Technology); p44/42 MAPK (1:1000; Cell Signaling Technology); phospho–Src (1:250; #9A6; Tyr416; Chemicon); Src (1:250; #GD11; Chemicon); α5 integrin (1:500); β1 integrin (1:500); GAPDH (1:10 000; Abcam)] and secondary [IRDye 680/800–conjugated rabbit/mouse IgG (1:5000; LI–COR Biosciences)] antibodies overnight at 4°C and 1 h at room temperature respectively. Images were taken using the Odyssey system (LI–COR Biosciences). Signal intensities were recorded by densitometry and averaged from at least three different Western blot analyses.

**Statistics.** Statistical analyses were carried out using an ANOVA and Student’s t–test with the software ‘R’ (version 2.14.0). Results with ‘P’ value less than 0.05 were considered to indicate statistically significant differences (* – P<0.05; ** – P<0.01; *** – P<0.001).
Results

Combined KLK4–7 expression leads to decreased cell adhesion and integrin expression

Expression of all four KLKs in the transfected OV–MZ–6 cell line (OV–KLK4–7) was confirmed by RT–qPCR (Figure 1A) and immunofluorescence (Figure 1B) at similar levels to that reported [22]. Notably, the expression levels of KLK4–7 are in comparable properties to that seen endogenously in the OVCAR–3 EOC cells (Figure 1A). OV–MZ–6 cells transfected with empty vector (‘OV–Vector’) and non–transfected parental OV–MZ–6 cells behaved identically in all assays. In our previous study, we detected increased invasion through Matrigel, but no difference in cell proliferation for OV–KLK4–7 compared to OV–Vector cells [22]. Herein, we sought to further explore the mechanisms underlying this invasive ability contributing to metastatic progression. Analysis of their adhesive capacity to ECM proteins, indicative of the EOC microenvironment, showed that attachment of OV–KLK4–7 cells to fibronectin (FN) and vitronectin (VN) was significantly decreased compared to vector controls, but unchanged on type–I (Col I) and type–IV (Col IV) collagens (Figure 2A). Using a blocking β1 integrin antibody to the FN receptor, α5β1 integrin, the adhesive behaviour of both OV–KLK4–7/OV–Vector cells to FN was reduced compared to IgG controls (Figure 2B; left panel). Upon αv integrin blocking of the VN receptor, αvβ3 integrin, attachment of both OV–KLK4–7/OV–Vector cells to VN was reduced, with OV–KLK4–7 cells being less adhesive than vector controls. Adhesion of OV–KLK4–7 cells on FN increased by αv integrin blocking compared to vector controls (Figure 2B; right panel).

Next, we wanted to examine whether the reduced adhesive ability upon combined KLK4–7 expression was linked to an altered integrin expression. Indeed, significantly decreased mRNA levels of αv/α5/β1 integrins in OV–KLK4–7 compared to OV–Vector cells were detected by RT–qPCR; β3 integrin did not change (Figure 2C). A similar finding was apparent at the protein level on performing flow cytometry. Both OV–KLK4–7/OV–Vector cells expressed high levels of α5/β1 integrins (OV–KLK4–7/OV–Vector: α5 – 2.8±0.7/3.3±0.7 units, β1 – 8.4±0.4/10.6±0.8 units) and minor amounts of αvβ3 integrin (OV–KLK4–7/OV–Vector: 0.004±0.003/0.013±0.004 units; Figure 2D; left panel). Upon normalisation to vector controls, expression of αvβ3/β1 integrins in OV–KLK4–7 cells declined significantly, with α5 integrin showing a clear trend to
lower levels (Figure 2D; right panel). Using confocal laser scanning microscopy (CLSM), less transmembrane fluorescent staining of α5/β1/α5β1 integrins was visualised in OV–KLK4–7 compared to OV–Vector cells (Figure 2E); αvβ3 integrin staining was not detectable (data not shown). These results suggest that the invasive ability of KLK4–7–expressing EOC cells is due, at least in part, to a decreased adhesive capacity to VN/FN, known components of the peritoneal ECM, and mediated by decreased α5/β1/αvβ3 integrin expression.

Paclitaxel–induced chemoresistance of OV–KLK4–7 cells

Ovarian cancer cells have an enhanced resistance to anti–cancer agents, including paclitaxel and cisplatin/carboplatin [13, 29, 30]. Previously, it was shown that KLK4 and KLK7, in particular, play a role in chemoresistance of EOC cells [13, 14]. Hence, we determined the effect of the chemo–agents, paclitaxel and carboplatin, on OV–KLK4–7 cells. Exposure to paclitaxel (0.01–100 nmol/L) over 3 days caused a strong reduction of cell survival. However, compared to vector controls, a significant increased cell survival at a dose of 10–100 nmol/L in OV–KLK4–7 cells was observed (10 nmol/L – 38%, 50 nmol/L – 49%, 100 nmol/L – 54% equivalent to the proportion of OV–Vector cell survival; Figure 3A). In contrast, the combined expression of KLK4–7 in OV–MZ–6 cells did not change the chemosensitivity of these cells to carboplatin (1–100 µmol/L) treatment at a dose of 10–100 µmol/L (Figure 3B).

Since platinum–taxane–based combination chemotherapy is more routinely given to patients, we determined the effects of this combined treatment on OV–KLK4–7 cells. Hereby, chemoresistance of OV–KLK4–7 cells to paclitaxel (100 nmol/L) was confirmed either when given on day 1 followed by 2 days with media only (P–M–M; 118% equivalent to the proportion of OV–Vector cell survival) or on day 3 after 2 days with media only (M–M–P; 24% equivalent to the proportion of OV–Vector cell survival). Paclitaxel–induced cell death was not further increased upon combined treatment with carboplatin either when cells were treated on day 1 with paclitaxel followed by media only on day 2 and carboplatin (50 µmol/L) on day 3 (P–M–C; 114% compared to vector controls) or in combination with paxlitaxel on day 3 (C–M–P; 20% equivalent to the proportion of OV–Vector cell survival; Figure 3C). Accordingly, carboplatin had no effect whether added on day 1 or day 3 alone (C–M–M/M–M–C; Figure 3C).
To determine that the induced changes to paclitaxel sensitivity were indicative of that observed in both cell types, paclitaxel–induced cellular effects were further examined. Paclitaxel enhances the assembly of stable microtubules from tubulin dimers and inhibits their depolymerisation resulting in abnormal microtubules [31], microtubule bundles and micronuclei as shown in both OV–KLK4–7/OV–Vector paclitaxel–treated cells by CLSM (Figure 4A). Consistent with other reports [32, 33], the OV–MZ–6 cells used here, whether expressing KLK4–7 or not, exhibited nuclei with variable morphologies on treatment with paclitaxel. About half of the population exhibited typical oval–shaped nuclei (indicated by yellow arrows), whereas the other half showed lobulated and multiple micronuclei (indicated by green arrows; Figure 4A).

On paclitaxel (100 nmol/L) treatment over 0–48 h (Figure 4B), caspase8 (indicative of apoptosis [34]) mRNA levels were significantly decreased in the chemoresistant OV–KLK4–7 cells and increased in the chemosensitive OV–Vector cells. The cytoskeletal marker cytokeratin8 revealed a similar trend as caspase8 showing a significant downregulation in chemoresistant OV–KLK4–7 cells and upregulation in chemosensitive OV–Vector cells after treatment. In contrast, cytokeratin18 was strongly induced in both OV–KLK4–7 (7–fold)/OV–Vector (3–fold) cells after treatment, with higher levels in OV–KLK4–7 cells (Figure 4B). Paclitaxel–induced cell death was also depicted by propidium iodide and annexin V fluorescent staining, with less prominent appearance in chemoresistant OV–KLK4–7 cells (Figure 4C). These results indicate that combined KLK4–7 expression alters paclitaxel sensitivity, as reflected in these key cellular markers, in the EOC cell line OV–MZ–6.

**Chemoresponse of OV–KLK4–7 cells is only partially mediated by β1 integrin or MAPK signalling**

Given that α5β1 and αvβ3 integrins play a role in the decreased ECM adhesion of OV–KLK4–7 cells and that integrin–mediated pathways have also been implicated in chemosensitivity [16, 18], we next assessed integrin mRNA expression levels upon paclitaxel treatment (100 nmol/L) over 0–48 h. Whereas the levels of α5/αv/β1/β3 integrin (representative of cell survival [16]) and the ECM proteins FN/VN (data not shown) did not change in both OV–KLK4–7/OV–Vector cells after treatment, the mRNA level of the β1 integrin–related factor CXCR4 was significantly decreased in chemoresistant OV–KLK4–7 cells compared to OV–Vector cells after treatment (Table 1). In contrast, in Western blot and densitometry analyses, β1 integrin protein expression
was significantly upregulated in OV–KLK4–7 cells compared to vector controls upon paclitaxel treatment (Figure 5A, B); α5 integrin expression did not change (data not shown). The altered β1 integrin level was confirmed by flow cytometry, with αvβ3 integrin being marginally increased in OV–KLK4–7 cells compared to vector controls upon paclitaxel treatment; α5 integrin levels did not change (data not shown). These results suggest that the increased chemoresistance of KLK4–7–expressing EOC cells to paclitaxel maybe only partially due to integrin–mediated cell survival.

Next, we wanted to elucidate other signalling mechanisms leading to the enhanced survival of OV–KLK4–7 cells involved in paclitaxel–induced chemoresistance. Western blot and densitometry analyses were performed to detect the activation of potentially involved signalling pathways, such as MAPK, Src, and FAK. Basal expression levels of MAPK, Src (Figure 5A) and FAK (data not shown) were comparable in both OV–KLK4–7/OV–Vector cells without treatment. Upon administration of paclitaxel, MAPK, in particular ERK1, showed significantly increased phosphorylation in OV–KLK4–7 cells compared to vector controls (Figure 5A, B). Src (Figure 5A, B) and FAK (data not shown) were not activated with these treatments.

To further support these and our earlier findings, MAPK, Src and integrin–related pathways were blocked by pre–exposure for 2 hr of cells before paclitaxel treatment to a blocking β1 integrin antibody and specific Src (SU6656) and MEK1/2 (U0126) inhibitors. While β1 integrin and Src inhibition had no effect in either cell line, with or without paclitaxel treatment, MEK1/2 inhibition blocked ERK1/2 phosphorylation of both OV–KLK4–7/OV–Vector cells independent of paclitaxel treatment (Figure 5C). As MAPK signalling was altered upon paclitaxel treatment in line with other reports [35], we further examined the effect of MEK1/2 inhibition on cell survival after paclitaxel administration. Survival of OV–KLK4–7 cells was significantly enhanced upon MEK1/2 inhibition with U0126 before (10 nmol/L – 23%, 100 nmol/L – 54% equivalent to the proportion of OV–Vector cell survival) and after (10 nmol/L – 56%, 100 nmol/L – 86% equivalent to the proportion of OV–Vector cell survival) paclitaxel treatment compared to vector controls (Figure 5D). MEK1/2 inhibition alone reduced the growth rates of both OV–KLK4–7/OV–Vector cells over a time course of 3 days, with OV–KLK4–7 cells showing a slight increased proliferation (data not shown). These results indicate that the MAPK
pathway, independent of β1 integrin and Src, is not the major player in the cellular responses of KLK4–7–expressing EOC cells that contributes to their chemoresistance.
Discussion

The focus of this study was to further dissect the crucial function of kallikreins in ovarian cancer progression and chemoresistance upon crosstalk with integrins and other cell survival factors. The data presented here further elaborates our previous studies [13, 22] showing critical effects for KLK4–7 in peritoneal dissemination and paclitaxel chemoresistance (KLK7 alone). Herewith, our observation of reduced adhesion to the EOC peritoneal matrix proteins, FN and VN, mediated by decreased levels of α5/β1/αvβ3 integrins, further supports a role for KLK4–7 in peritoneal migration and invasion. We also show that paclitaxel–induced chemoresistance was accompanied by differential expression of drug–resistance associated genes and by an activation of MAPK signalling that was enhanced in KLK4–7–expressing cells. However, additional networks initiated by cell surface receptors independent of the MAPK cascade are in play given the lack of the effect of the MEK1/2 (U0126) inhibitor.

Role of KLKs, the ECM and integrins in ovarian cancer progression

The underlying mechanisms by which the kallikrein peptidases are involved in the pathogenesis and progression of ovarian cancer are still not fully understood. Proteolytic factors are crucial during intra–peritoneal metastasis of ovarian cancer and required at multiple stages, especially for initial detachment of cancer cells from the surface of the ovary and peritoneal invasion. The involvement of KLKs in these processes to date has been most specifically related to the suggested degradation of ECM substrates based on biochemical analyses [36]. For instance, KLK4 has been shown to degrade fibrinogen [37] and FN, VN (unpublished data), KLK5 and KLK6 degrade FN, VN, Col I, Col IV, and KLK7 degrades FN [15]. Our KLK4–7 data indicate that, as with MMPs [38], a more complex regulatory event that influences integrin levels and binding capacity to specific ECM proteins (VN/FN), both key elements in cancer metastasis. KLK4–7–expression in EOC cells did not alter attachment to Col I and Col IV, but attachment decreased to FN and VN.

Integrins are strongly implicated in EOC dissemination, adhesion onto the mesothelial lining of the peritoneal cavity and subsequent invasion of the organs of the small pelvis [16, 17, 39]. The observed reduced cell adhesion of KLK4–7–transfected cells to FN and VN, which was further decreased by functional blocking αv/β1 integrin antibodies, indicates an interaction between the
KLK and integrin pathways. These data are also consistent with our previous report that KLK7–
SKOV–3 EOC cells form multicellular aggregates in 3–dimensional suspension via an α5/β1
integrin–mediated process [13]. Reduced E–cadherin levels also stimulate α5/β1 integrin
expression, cell adhesion, invasion and MAPK signalling, in particular via ERK1 in ovarian
cancer cells [40]. Reduced E–cadherin expression has also been linked to the action of other
proteases, such as MMP–9, that are enzymatic contributors to EOC disease progression [3, 39,
41]. KLK4 (unpublished data), KLK6 [42] and KLK7 [43] can cleave the ectodomain of E–
cadherin, thus suggesting a role in maintenance of this phenotype and EOC cell shedding from
the primary tumour into the peritoneal cavity [15, 40]. However, the EOC cell line used to
overexpress KLK4–7 in our study, OV–MZ–6, does not express E–cadherin [28], thus this
pathway may be constitutively active to maintain cell responses [35]. In this regard, we detected
basal phosphorylated MAPK levels in these cells. These data further demonstrate that the
KLK4–7 peptidases, both individually and as a group, have specific regulatory roles in the
complex EOC microenvironment.

**Differential expression of paclitaxel–resistance associated factors**

The OV–MZ–6 cell line was established from a patient with an advanced cystadeno ovarian
carcinoma after surgery without having received any chemotherapy [23] and was used to
overexpress KLK4–7. The observed paclitaxel–resistance, indicative of a decreased cell survival,
was detectable at a dose close to peak plasma concentration, approximately 5 nmol/L paclitaxel,
with significant differences for the control and transfected OV–MZ–6 cell line seen at 10 to 100
nmol/L paclitaxel.

We reported earlier elevated α5β1 integrin levels upon KLK7 expression in SKOV–3 EOC cells,
in EOC cells derived from ascites and in tumour tissues, as well as an KLK7 association with
poor response of patients to chemotherapy [13]. Although combined KLK4–7 overexpression in
untreated OV–MZ–6 EOC cells displayed reduced α5/β1 integrin levels compared to vector
controls, paclitaxel treatment caused an increase in β1 integrin levels in the paclitaxel–insensitive
cells. These results in a different EOC cell line expand on our previous data [13] and elaborate a
KLK4–7 functional role partially in conjunction with selected members of the integrin axis in
chemoresponsiveness of EOC cells. Although resistance to paclitaxel is not elucidated by a
single genetic event or mechanism but rather due to alignment of multiple factors mediating cell
growth and survival [44]. The crosstalk of integrins with growth factor and/or cytokine receptors
has cancer–promoting effects contributing to tumour metastasis and drug–resistance [16]. The β1
integrin–dependent pathway associated with chemokine (C–X–C–motif) receptor 4 (CXCR4)
was identified to be elevated in advanced ovarian cancer [45]. Cell survival signalling via
integrin–CXCR4 is as part of the tumour–associated microenvironment which in turn conferred
drug–resistance and tumour cell growth [46]. We detected decreased CXCR4 levels in
paclitaxel–resistant KLK4–7–expressing cells and increased levels in paclitaxel–sensitive vector
controls upon paclitaxel treatment; its ligand, stromal–derived factor–1 (SDF–1/CXCL12) did
not change (data not shown).
Adhesion to ECM components is another chemoresistance–promoting event. KLK4–7–
expressing cells capable of attachment to FN did not protect them from apoptosis induced by
paclitaxel nor did the blocking β1 integrin antibody reduce apoptosis (data not shown). ERK–
duced apoptosis can occur due to cell detachment caused by decreased integrin levels [35]. The
composition of cell–ECM interactions is far more complex, and protective tumour
microenvironmental effects from paclitaxel–induced apoptosis are dependent on specific ECM
proteins, integrin heterodimers and cell type [18, 47]. In fact, levels of integrin subunits vary
between drug–resistant subpopulations [48]. Expression of αvβ3 integrin made cells partially
resistant to paclitaxel [49]. However, αvβ3 integrin was downregulated upon KLK4–7
expression and did not alter upon paclitaxel treatment and β1 integrin increased marginally upon
treatment. As shown by others, no association between β1/β3 integrin subunits and survival rate
was found in patients treated with platinum–based chemotherapy [50]. Overall, the direct
relationship between the KLK4–7 peptidases, integrins and chemoresistance still needs to be
further elucidated.

It was shown that nanomolar concentrations of paclitaxel can affect the shape of the nucleus,
whereas micromolar doses promote stress–induced pro–apoptotic pathways, associated with
drug–resistance in ovarian cancer [32, 33]. Regulation of cytokeratins also plays a role in drug–
resistance [51]. Paclitaxel–treated KLK4–7–expressing cells showed a downregulation of
cytokeratin8 and a minor increase in vector controls, while cytokeratin18 was upregulated
independent of KLK4–7 expression. In line with other data, paclitaxel–resistant cancer cells
revealed higher cytokeratin18 expression than paclitaxel–sensitive controls [52]. The
intermediate filaments cytokeratin8/18 and vimentin are co-expressed [23], and increased vimentin levels are associated with chemoresistance [51], but the molecular mechanism between drug–resistance and intermediate filaments still requires further clarification. It was postulated that increased invasive behaviour of epithelial cancer cells expressing both cytokeratins and vimentin may have a selective survival advantage [51]. Collectively, our data reported here and earlier [13, 22], underpin this hypothesis linking enhanced invasion and chemoresistance upon KLK expression in EOC cells with the simultaneous presence of intermediate filaments and vimentin.

KLKs promote MAPK and other signalling cascades in paclitaxel–treated EOC cells

We demonstrated that paclitaxel treatment of KLK4–7–expressing cells was associated with increased phosphorylation of ERK1 which occurs via a β1 integrin independent pathway as we only detected a slight increase in β1 integrin expression. Moreover, paclitaxel treatment of paclitaxel–insensitive vector controls decreased the activation of ERK1. Activation of ERK1/2, in particular ERK1, is linked to chemoresistance [18]. Another study showed that ERK1 was also activated upon paclitaxel treatment of EOC cells, but at much higher concentrations [53]. Since, however, the β1 integrin blocking antibody had no effect on ERK1/2 signalling upon paclitaxel treatment, ERK1 activation in our study is unlikely mediated to any large extent by integrin signalling.

KLKs may be involved in activation of the ERK1/2 pathway via either direct activation of protease–activated receptors (PARs) or indirectly via epidermal growth factor receptor (EGFR) transactivation of PARs [37, 54-57]. Consequently, PAR–stimulated ERK1/2 activation influences cell proliferation and survival [54]. It was shown that EGFR expression and activity is upregulated upon integrin engagement with FAK and MAPK exhibiting a synergistic response in the crosstalk between integrins and EGFR in EOC cells [17]. The involvement of EGFR in paclitaxel–resistance in EOC cells was reported previously by others [58].

ERK activity has also been involved in apoptosis, for example due to transiently enhanced apoptotic activities induced by various anti–cancer compounds, including paclitaxel [35]. ERK1/2 inhibition of paclitaxel–treated cancer cells prevented cell death [35], an effect also observed in our study showing that the KLK4–7–induced paclitaxel resistance was not blocked by the MEK1/2 (U0126) inhibitor. This is in line with other reports showing that ERK1/2
inhibition reduced the ability of paclitaxel to mediate cell death in cancer cells, thus providing protection from paclitaxel–induced apoptosis [59]. The MAPK pathway is known for its oncogenic potential to increase cell survival by mediating anti–apoptotic stimuli, but recent data also suggest that aberrant ERK activation can induce apoptosis [35].

Recent data have demonstrated that a phase II trial using the MEK inhibitor selumentinib (AZD6244), reducing the activity of the MAPK pathway by blocking both MEK1 and MEK2 enzymes, given to patients who had failed multiple rounds of cytotoxic chemotherapy, showed 81% disease control (Cancer Discovery, 2012). Therefore, a combination therapy using MEK and kallikrein inhibitors, depending on the kallikrein expression of the tumour tissue, would be a novel approach to treat patients who become chemoresistant to the current therapy regime for advanced ovarian cancer potentially slowing down disease progression.

In conclusion, we demonstrated that KLK4, KLK5, KLK6, and KLK7, when co–expressed in an EOC cell line representative of late stage disease, functionally contribute to a ECM–integrin crosstalk and activation of MAPK signalling implicated in chemoresistance to the clinically–administered drug, paclitaxel, but not to carboplatin. The complex interplay with factors independent of integrin and MAPK cascades supports a role for these KLKs as partners in proteolytically critical cascades promoting ovarian cancer progression. Our study further underscores their potential as bioindicators for advanced disease, characterised by peritoneal dissemination and invasion, and their value as key therapeutic targets.
Acknowledgements

The authors are grateful to Dr Leonore de Boer from the Cell Imaging Facility of the Institute of Health and Biomedical Innovation (IHBI) for her microscopy assistance. This study was supported by the National Health and Medical Research Council (NHMRC) of Australia, an Early Career Research Award, IHBI, Queensland University of Technology, Australia and the German Federal Ministry of Education and Research, Leading Edge Cluster m4.
Conflict of Interest Statement

The authors declare that there are no conflicts of interest.
References


Lengyel E. Ovarian cancer development and metastasis. Am J Pathol 2010;177: 1053-64.


**Figure legends**

**Figure 1:** Levels of KLK expression quantified by RT–qPCR and CLSM. A. KLK4, KLK5, KLK6 and KLK7 mRNA expression in combined OV–KLK4–7–transfected cells was confirmed by RT–qPCR, with OVCAR–3 cells showing endogenous levels (n=3; SEM). Parental OV–MZ–6 and OV–Vector cells were negative for KLK expression (n.d., not detected). B. On immunofluorescent staining, KLK4, KLK6 and KLK7 expression was shown in OV–KLK4–7 cells with low levels of KLK5 and negative staining in vector controls. Cytokeratin8 was stained with its respective primary and Alexa568–conjugated antibody, nuclei with DAPI, and KLKs with respective primary and Alexa488–conjugated antibodies. Scale bars, 20 µm.

**Figure 2:** Altered cell adhesion and integrin expression of OV–KLK4–7 cells. A. Cell adhesion of OV–KLK4–7 cells to FN and VN was decreased compared to vector controls. No difference in the adhesive behaviour between OV–KLK4–7 and OV–Vector to Col I and Col IV was observed. B. Using a blocking β1 integrin antibody, cell attachment of both OV–KLK4–7/OV–Vector cells to FN was reduced compared to the IgG control. Additionally, a statistically significant reduction of cell adhesion for OV–Vector cells to VN was observed. Blocking of αv integrin did not inhibit cell adhesion to FN, but reduced cell adhesion of both OV–KLK4–7/OV–Vector cells to VN compared to the IgG control, with OV–KLK4–7 cells being less adhesive than vector controls. OV–KLK4–7 cells were more adhesive to FN after αv integrin inhibition (n=3; SEM; * – P<0.05; ** – P<0.01; *** – P<0.001). C. RT–qPCR showed decreased mRNA levels of αv/α5/β1 integrins in OV–KLK4–7 cells compared to vector controls; β3 integrin did not change (n=3; SEM; ** – P<0.01; *** – P<0.001). D. Flow cytometrical analyses showed that OV–KLK4–7 and OV–Vector cells expressed high α5/β1 and very low αvβ3 integrin levels. Normalisation of flow cytometry data to OV–Vector cells revealed that OV–KLK4–7 cells have significantly decreased αvβ3/β1 integrin levels (n=3; SEM; * – P<0.05; ** – P<0.01). E. Presence of α5/β1/α5β1 integrins in both OV–KLK4–7/OV–Vector cells was further confirmed by immunofluorescent staining visualised by CLSM using the respective primary and Alexa488–conjugated secondary antibodies. Cell actin filaments were stained with rhodamine415–conjugated phalloidin, nuclei with DAPI. Scale bars, 10 µm.
Figure 3: Paclitaxel–induced chemoresistance of OV–KLK4–7 cells. A. Administration of paclitaxel enhanced OV–KLK4–7 cell survival (10/50/100 nmol/L) compared to vector controls. For a dose of 10/50/100 nmol/L, the OV–Vector cell survival was 24/14/13% and for OV–KLK4–7 cells 33/21/20%; setting 24/14/13% to 100%, an OV–KLK4–7 cell survival difference of 38/49/54% compared to OV–Vector was reached (n=3; SEM; * – P<0.05; ** – P<0.01). B. Both OV–KLK4–7/OV–Vector cells responded equally to carboplatin treatment (n=3; SEM). C. Exposure of paclitaxel (100 nmol/L) on day 1 followed by media/carboplatin (50 µmol/L) on day 3 (P–M–M/P–M–C) caused less OV–KLK4–7 cell death compared to vector controls. Presence of carboplatin/media on day 1 followed by media/paclitaxel/carboplatin on day 3 (C–M–M/C–M–P/M–M–C) increased cell survival, with OV–KLK4–7 cells being less responsive to paclitaxel compared to vector controls (n=3; SEM; * – P<0.05; ** – P<0.01; *** – P<0.001).

Figure 4: Paclitaxel–induced chemoresistance of OV–KLK4–7 cells due to decreased apoptotic expression. A. Stable microtubules (indicated by white arrows) formed by tubulin dimers upon paclitaxel treatment of both OV–KLK4–7/OV–Vector cells over 48 h were immunofluorescently visualized by CLSM using anti–α–tubulin and Alexa488–conjugated secondary antibody. Both, typical oval–shaped nuclei (indicated by yellow arrows) as well as lobulated and multiple micronuclei were detected (indicated by green arrows). Cell actin filaments were stained with rhodamine415–conjugated phalloidin, nuclei with DAPI. Scale bars, 25 µm. B. Expression of caspase8 and cytokeratin8 was decreased on 48 h paclitaxel treatment in chemoresistant OV–KLK4–7 cells, while expression of cytokeratin18 was enhanced in both OV–KLK4–7/OV–Vector cells (n=3/4; SEM; * – P<0.05; ** – P<0.01). C. Stable microtubules (indicated by white arrows) formed by tubulin dimers upon paclitaxel treatment over 48 h of both OV–KLK4–7/OV–Vector cells were immunofluorescently visualized by CLSM using anti–α–tubulin and Alexa488–conjugated secondary antibody. Propidium iodide was used to stain double–stranded nucleic acids as it penetrates cell membranes of dead cells. Nuclei were counterstained with DAPI. Additionally, annexin V was used to stain apoptotic cells due to loss of cell membrane integrity with anti–annexin V and Alexa488–conjugated secondary antibody. Scale bars, 25 µm.
Figure 5: Paclitaxel–induced chemoresistance of OV–KLK4–7 cells due to phosphorylation of ERK. A. Western blot analyses revealed enhanced ERK1/2 phosphorylation and increased β1 integrin expression in OV–KLK4–7 cells due to paclitaxel treatment compared to vector controls. Src activation was minimal and did not change upon chemotreatment. B. Paclitaxel treatment caused significant activation of ERK1 and enhanced β1 integrin expression in OV–KLK4–7 cells compared to OV–Vector cells as indicated by densitometry of Western blots represented in panel A; no altered expression and phosphorylation of Src was detected after treatment (n=3; SEM; * – $P<0.05$; ** – $P<0.01$). C. Inhibition experiments using β1 integrin blocking antibody, Src (SU6656) and MEK1/2 (U0126) inhibitors pre–exposed to cells before chemotreatment showed that the MEK1/2 pathway was involved in the response of both OV–KLK4–7/OV–Vector cells to paclitaxel. D. Chemosensitivity assays demonstrated enhanced OV–KLK4–7 cell survival upon MEK1/2 inhibition and paclitaxel treatment (10/100 nmol/L) compared to vector controls (n=3; SEM; * – $P<0.05$; ** – $P<0.01$; ***/### – $P<0.001$).
Table 1. Gene expression performed by RT–qPCR upon paclitaxel treatment. Relative mRNA expression of integrins and the integrin–related factor CXCR4 (n=3; SEM; * – \( P<0.05 \)).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cell type</th>
<th>No treatment (± SEM)</th>
<th>Paxlitaxel treatment (± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha_5 ) integrin</td>
<td>OV–Vector</td>
<td>5.02( \times10^{-3} ) ± 9.62( \times10^{-4} )</td>
<td>5.16( \times10^{-3} ) ± 9.14( \times10^{-4} )</td>
</tr>
<tr>
<td></td>
<td>OV–KLK4–7</td>
<td>3.76( \times10^{-3} ) ± 4.59( \times10^{-4} )</td>
<td>3.55( \times10^{-3} ) ± 3.56( \times10^{-4} )</td>
</tr>
<tr>
<td>( \alpha_\nu ) integrin</td>
<td>OV–Vector</td>
<td>1.49( \times10^{-4} ) ± 9.98( \times10^{-5} )</td>
<td>9.80( \times10^{-5} ) ± 3.37( \times10^{-5} )</td>
</tr>
<tr>
<td></td>
<td>OV–KLK4–7</td>
<td>5.68( \times10^{-5} ) ± 2.97( \times10^{-5} )</td>
<td>4.64( \times10^{-5} ) ± 7.95( \times10^{-6} )</td>
</tr>
<tr>
<td>( \beta_1 ) integrin</td>
<td>OV–Vector</td>
<td>1.14( \times10^{-4} ) ± 8.56( \times10^{-6} )</td>
<td>1.50( \times10^{-4} ) ± 2.04( \times10^{-5} )</td>
</tr>
<tr>
<td></td>
<td>OV–KLK4–7</td>
<td>5.86( \times10^{-5} ) ± 6.79( \times10^{-6} )</td>
<td>5.01( \times10^{-5} ) ± 3.01( \times10^{-6} )</td>
</tr>
<tr>
<td>( \beta_3 ) integrin</td>
<td>OV–Vector</td>
<td>4.06( \times10^{-5} ) ± 1.81( \times10^{-5} )</td>
<td>3.66( \times10^{-5} ) ± 1.50( \times10^{-5} )</td>
</tr>
<tr>
<td></td>
<td>OV–KLK4–7</td>
<td>3.84( \times10^{-5} ) ± 1.69( \times10^{-5} )</td>
<td>2.95( \times10^{-5} ) ± 6.81( \times10^{-6} )</td>
</tr>
<tr>
<td>CXCR4</td>
<td>OV–Vector</td>
<td>1.04( \times10^{-3} ) ± 3.57( \times10^{-4} )</td>
<td>1.09( \times10^{-3} ) ± 9.75( \times10^{-5} )</td>
</tr>
<tr>
<td></td>
<td>OV–KLK4–7</td>
<td>1.21( \times10^{-3} ) ± 4.68( \times10^{-4} )</td>
<td>6.35( \times10^{-4} ) ± 1.87( \times10^{-4} )*</td>
</tr>
</tbody>
</table>