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External validation suggests Integrin beta 3 as prognostic biomarker in serous ovarian adenocarcinomas

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Abstract

Background: The majority of women with ovarian cancer are diagnosed in late stages, and the mortality rate is high. The use of biomarkers as prognostic factors may improve the treatment and clinical outcome of these patients. We performed an external validation of the potential biomarkers CLU, ITGB3, CAPG, and PRAME to determine if the expression levels are relevant to use as prognostic factors.

Methods: We analysed the gene expression of CLU, ITGB3, CAPG, and PRAME in 30 advanced staged serous adenocarcinomas with quantitative real-time polymerase chain reaction (QPCR) and the protein levels were analysed in 98 serous adenocarcinomas with western blot for semiquantitative analysis. Statistical differences in mRNA and protein expressions between tumours from survivors and tumours from deceased patients were evaluated using the Mann-Whitney U test.

Results: The gene and protein ITGB3 (Integrin beta 3) were significantly more expressed in tumours from survivors compared to tumours from deceased patients, which is in concordance with our previous results. However, no significant differences were detected for the other three genes or proteins CLU, CAPG, and PRAME.

Conclusion: The loss of ITGB3 expression in tumours from deceased patients and high expression in tumours from survivors could be used as a biomarker for patients with advanced serous tumours.

Background

Ovarian carcinoma is the fifth most common cause of cancer death among women in Western Europe and the United states [1,2]. The most common type is epithelial ovarian cancer, which has several histopathological sub-

types including serous, mucinous, endometrioid, and clear cell carcinomas. Among these, serous papillary adenocarcinoma is the most common form accounting for approximately 50% of cases [3].

The majority of women with ovarian cancer are diagnosed in late stages, and these patients are treated similarly with tumour reductive surgery followed by chemotherapy. However, even though patients with stage III-IV tumours undergo extensive therapy, the mortality rate is high, and only 30% of patients survive more than five years after diagnosis [4]. The prognostic factors used today, such as surgical stage, volume of residual tumour after primary surgery, and histologic grade, are insufficient to optimise and individualise the treatment. There is an urgent need to better classify these tumours to improve the treatment and clinical outcome of patients.

The use of novel biomarkers as prognostic factors may facilitate identification of patients who are likely to relapse and die of the disease. Expression profiles have been used to identify genes involved in ovarian tumour initiation and progression. We have previously used expression arrays to detect potential biomarkers with prognostic relevance [5]. However, there is a limited overlap of data among expression analyses of ovarian carcinomas owing to several factors [6]. First, the number of tumours studied is low and consists of different stages and mixtures of histopathological subtypes. Second, different microarray technology platforms and statistics are used, and the definition of different endpoints varies in the studies. The extensive knowledge about differences in microarray results suggests that external validations are required to verify if the data are relevant to use. Our previous analysis demonstrated differences in expression between tumours from survivors and tumours from deceased patients, including four cancer-related genes, *ITGB3*, *CLU*, *CAPG*, and *PRAME* [5]. We further established the significant differences in expression levels for the corresponding proteins [7]. Further validation of the gene and protein expressions in an external validation set is necessary, to clarify if the expression levels are usable as biomarkers. The protein expressions of these potential biomarkers may also be related to the tumours biological properties.

Integrin beta 3 (*ITGB3*) was more expressed in tumours from survivors in our previous studies. Integrins are known to participate in cell adhesion, and work as receptors in cell-surface mediated signalling, through binding with different ligands in focal adhesions [8-10]. Clusterin (*CLU*), also more expressed in tumours from survivors in our previous studies, seems to have divergent functions in cells. This is probably due to different isoforms, one secreted/cytoplasmic form (s*CLU*/c*CLU*) with chaperone activity involved in tumour progression, and one nuclear form (n*CLU*) with proapoptotic function [11-13]. Capping protein (actin filament) gelsolin-like (*CAPG*), which was more expressed in tumours from deceased patients in our studies, belongs to the gelsolin protein superfamily.

This group of proteins control actin organisation, and *CAPG* contributes to the control of actin-based motility in cells by capping the barbed ends of actin filaments [14,15]. Preferentially expressed antigen in melanoma (*PRAME*) is a repressor of retinoic acid receptor signalling [16]. In our previous studies, *PRAME* was detected as more expressed in tumours from deceased patients compared to tumours from survivors.

In this study, we performed an external validation of the four genes and proteins in a new set of advanced ovarian serous adenocarcinomas to determine if differences in expression levels are relevant to use as prognostic markers.

Methods

Patient and tumour material

In this study, 98 advanced stage (III or IV) serous papillary adenocarcinomas of the ovary were analyzed (Table 1). The tumours were randomly selected and adjusted to similar size of tumours from survivors and tumours from deceased patients. Patients who survived five years or more after the initial diagnosis were considered as survivors and all deceased patients in the study succumbed to cancer. Surgical staging of the tumours was performed according to International Federation of Gynaecology and Obstetrics (FIGO) standards, and patients with no macroscopic residual tumour were classified as radically operated. The tumours were removed at primary surgery and secured for pathology examination, RNA, and protein extraction. After surgery, patients were treated with platinum based chemotherapy, either with a combination of farmorubicine, carboplatin, and cyclophosphamide, or with a combination of paclitaxel and carboplatin. The tumours were collected from patients diagnosed between 1993 and 2003 at Sahlgrenska University Hospital, Gothenburg, Sweden, and the study was approved by the local ethics committee. A pathologist reviewed all tumours according to the treatment protocol for gynaecological malignancies in western Sweden. Specimen imprints for cytologic evaluation were performed to verify the presence of tumour cell content, and only tumours containing at least 50% tumour cells were included.

Quantitative real-time polymerase chain reaction (QPCR)

Thirty tumours from patients treated with paclitaxel and carboplatin were analysed with QPCR, performed as described by Partheen et al. [7]. Briefly, total RNA was isolated from frozen tumours by homogenisation with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and then extracted with RNeasy mini kit (Qiagen, Valencia, CA, USA). High-quality RNA was obtainable from 30 samples and used in the analysis. From each tumour sample 0.5 µg of total RNA was reverse transcribed in duplicate. Each cDNA sample was analysed once by real-time PCR, giving two data points for each tumour sample. The cDNA were

Table 1: Summary of clinicopathologic characteristics of the patients

	Tumours (from survivors/deceased patients)
Total Tumours	98 (48/50)
Tumours used in QPCR analysis	30 (15/15)
Mean age (years)	60 (57/62) (range 28-81)
FIGO Staging	
IIIa	10 (7/3)
IIIb	14 (7/7)
IIIc	73 (33/40)
IV	1 (1/0)
Surgery	
Radically operated	20 (13/7)
Residual tumour	75 (34/41)
Not available	3 (1/2)
Differentiation	
Well	15 (10/5)
Moderate	22 (10/12)
Poor	59 (26/33)
Not available	2 (2/0)
Treatment	
farmorubicine, carboplatin and cyclophosphamide	31 (16/15)
paclitaxel and carboplatin	67 (32/35)

detected with SYBR Green I. Two stable reference genes were used for normalisation, GAPDH and β -actin from the Human Endogenous Control Gene Panel (TATAA Bio-center, <http://www.tataa.com>). The efficiency of each QPCR assay was estimated from the slope of a standard curve generated from the serial dilution of purified PCR products. The assays for CLU and ITGB3 showed PCR efficiencies close to 80%, and for CAPG and PRAME 90%. These values were used for subsequent calculations. For each assay the average Ct value for each tumour sample was converted to relative copy numbers. The data were then normalised by the geometric average of the two reference genes [17].

Western blot

Western blot was performed on 98 tumour samples, each in duplicate as described by Partheen et al. [7]. Briefly, the frozen samples were homogenised with RIPA followed by centrifugation. The samples were diluted in SDS sample buffer with and without 10% 2-mercaptoethanol and heated at 97°C for 5 minutes. The unreduced samples (without 2-mercaptoethanol) were used to detect ITGB3. Total protein was loaded into each lane on a 10% Criterion™ precast gel (BIO-RAD laboratories, Hercules, CA, USA). The proteins were transferred to nitrocellulose

membranes (BIO-RAD) and blocked overnight at 4°C in 5% non-fat milk in 10 mM Tris buffered saline (TBS).

The membranes were incubated in TBS containing 0.05% Tween 20 with the following primary antibodies: chicken polyclonal to CAPG (1:3000, ab14235, Abcam, Cambridge, UK), rabbit polyclonal to PRAME (1:1000, ab32185, Abcam), mouse monoclonal to CLU (1:15 000, clone 41D, Upstate Biotechnology, Lake Placid, NY, USA), ITGB3 (1:1000, MAB1974, Chemicon, Temecula, CA, USA) and GAPDH as a relative loading control (1:15 000, ab8245, Abcam). Proteins were visualised by chemiluminescence, using horseradish peroxidase-linked (HRP) secondary antibodies; goat anti-rabbit (1:5000, ab6721, Abcam), anti-chicken (1:3000, GAYFC-HRP, Genway Biotech, San Diego, CA, USA) and anti-mouse (1:5000, sc-2005, Santa Cruz Biotechnology Inc). The membranes were exposed to Amersham Hyperfilm™ ECL (Amersham, Buckinghamshire, UK). The optical density from each band was measured using the software package Quantity One (BIO-RAD) and used for semiquantitative analysis of the proteins. The CLU antibody used detects all isoforms of the protein, and the mature form of CLU at approximately 40 kDa was analysed in this study [18]. An internal reference sample containing pooled protein from ovarian

tumours, same on each blot, was used as a standard for quantification and was given the value 1. The mean value of the duplicates was calculated and used in the analysis.

Statistical analysis

Statistical analysis of the data was performed using SPSS for Windows (version 12.0.1). The tumours used in the protein analysis were from patients treated with slightly different carboplatin-based chemotherapies. To check if the two treatment groups could be combined in the statistical analysis, treatment was tested as a 2-level factor, as well as an interaction with protein expression in logistic regression models with survival as response. Due to the skewed expression distributions, the logarithms of protein expressions were used as explanatory variables in the model, and nonparametric tests were used in the analysis of statistical differences.

Statistical differences in mRNA and protein expressions between tumours from survivors and tumours from deceased patients were evaluated using the Mann-Whitney U test. Logistic regression was performed on logarithmic values of protein expressions and the prognostic factors age, stage and surgery outcome to define if these factors contributes significantly to the prediction of survival. The relation between expressions measured with QPCR and western blot was evaluated with bivariate correlation using Spearman correlation coefficient. We used Kaplan-Meier survival curves to show differences in clinical outcome between patients with tumours that

expressed high alternatively low levels of ITGB3 mRNA or protein, using cut-off values at 0.29 and 0.43 respectively, based on the detected relative expression levels. A log-rank test was used to compare the curves. A value of $p < 0.05$ was considered to be significant.

Results

The gene ITGB3 was detected as differently expressed

The expression of *CLU*, *ITGB3*, *CAPG*, and *PRAME* was investigated to study differences in expression levels between tumours from survivors and tumours from deceased patients. The four genes were analysed with QPCR in 30 of the 98 serous adenocarcinomas from patients treated with paclitaxel and carboplatin. The number of tumours analysed was reduced, because the RNA quality did not reach the desirable level in 68 samples. The expression in tumours from survivors and in tumours from deceased patients were compared, and *ITGB3* was significantly more expressed in tumours from survivors ($p = 0.004$) (Figure 1). The best separation between the two groups was obtained with a cut-off value of the relative expression at 0.29 (Figure 1A). Only three tumours from survivors expressed *ITGB3* less than 0.29 and three tumours from deceased patients more than 0.29. No statistical differences were detected for the other three genes analysed: *CAPG* ($p = 0.24$), *CLU* ($p = 0.76$), and *PRAME* ($p = 0.52$).

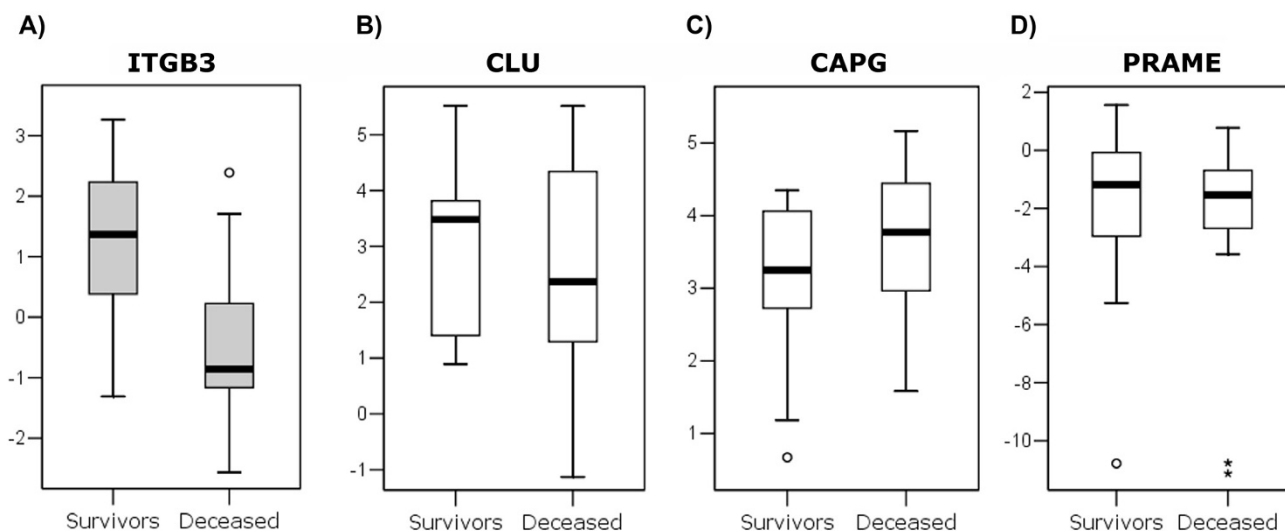


Figure 1
Relative mRNA copy numbers for the genes. Relative mRNA copy numbers as log2 values for the genes A) *ITGB3* (significantly differently expressed, $p = 0.004$) B) *CLU* C) *CAPG* and D) *PRAME*. Each plot shows the medians (centre lines), interquartile ranges (boxes), largest and smallest values (whiskers) that are not outliers (circles), or extreme values (stars) within a category.

The protein ITGB3 was detected as differently expressed

The proteins CLU, ITGB3, CAPG, and PRAME were examined with western blot for semiquantitative measurements of each protein in 98 tumours (Figure 2). Type of patient treatment could be ignored in the analysis, since no significant effects on protein expressions were detected. A significant difference in expression between tumours from survivors and tumours from deceased patients was detected for ITGB3 ($p = 0.005$), but not for the other proteins, CLU ($p = 0.15$), PRAME ($p = 0.62$), and CAPG ($p = 0.24$). The most optimal cut-off value for ITGB3 that separated survivors and deceased patients turned out to be 0.43 (logarithmic value -0.87). The logistic regression analysis using age, stage, surgery outcome and protein expressions showed that ITGB3 ($p = 0.002$) and survival ($p = 0.013$) was independent factors to predict survival. In addition, significant differences of ITGB3 expression were also detected when tumours were divided according to treatment, with $p = 0.05$ for paclitaxel and carboplatin treated patients and $p = 0.04$ for farmorubicine-, carboplatin-, and cyclophosphamide-treated patients.

The relative expression of ITGB3 mRNA and protein correlated well ($p = 0.027$). Both mRNA and protein expressions at low levels are dominated by tumours from deceased patients, as illustrated in Figure 3A. Kaplan-Meier survival curves for high versus low expression of ITGB3 mRNA and protein are shown in Figure 3B and 3C, respectively. There was a significant difference in survival for tumours with high versus low expression of the mRNA and protein.

Discussion

In this study, we performed an external validation of four potential prognostic biomarkers, ITGB3, CLU, CAPG, and PRAME, in advanced ovarian serous adenocarcinomas. We compared the gene and protein expression levels between tumours from survivors and tumours from deceased patients, and ITGB3 was the one found as differently expressed. The clinical outcome of patients with advanced ovarian adenocarcinoma is difficult to predict at an individual level, and ITGB3 could be a complementary potential biomarker for patients with serous tumours. This is of importance because an improvement of the prognostic factors may make it possible to optimise patients' treatment. The differences were detected for both

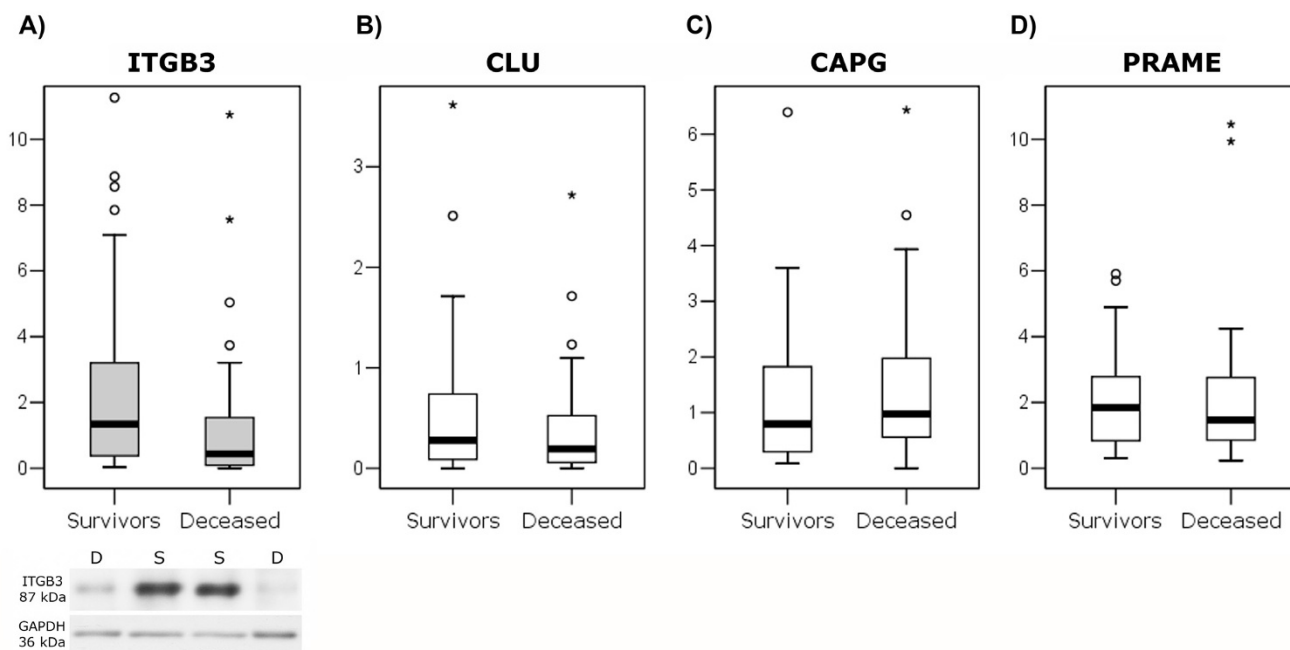


Figure 2

Relative protein expression for the proteins. Relative protein expression of A) ITGB3^a (significantly differently expressed, $p = 0.005$) B) CLU C) CAPG and D) PRAME. Each plot shows the medians (centre lines), interquartile ranges (boxes), largest and smallest values (whiskers) that are not outliers (circles), or extreme values (stars) within a category. A representative immunoblot is shown for ITGB3, where GAPDH was used as loading control and tumours from survivors (S) and deceased (D) patients are indicated. ^aIn the figure, ITGB3 values for two survivors and one deceased patients were excluded due to extremely high values.

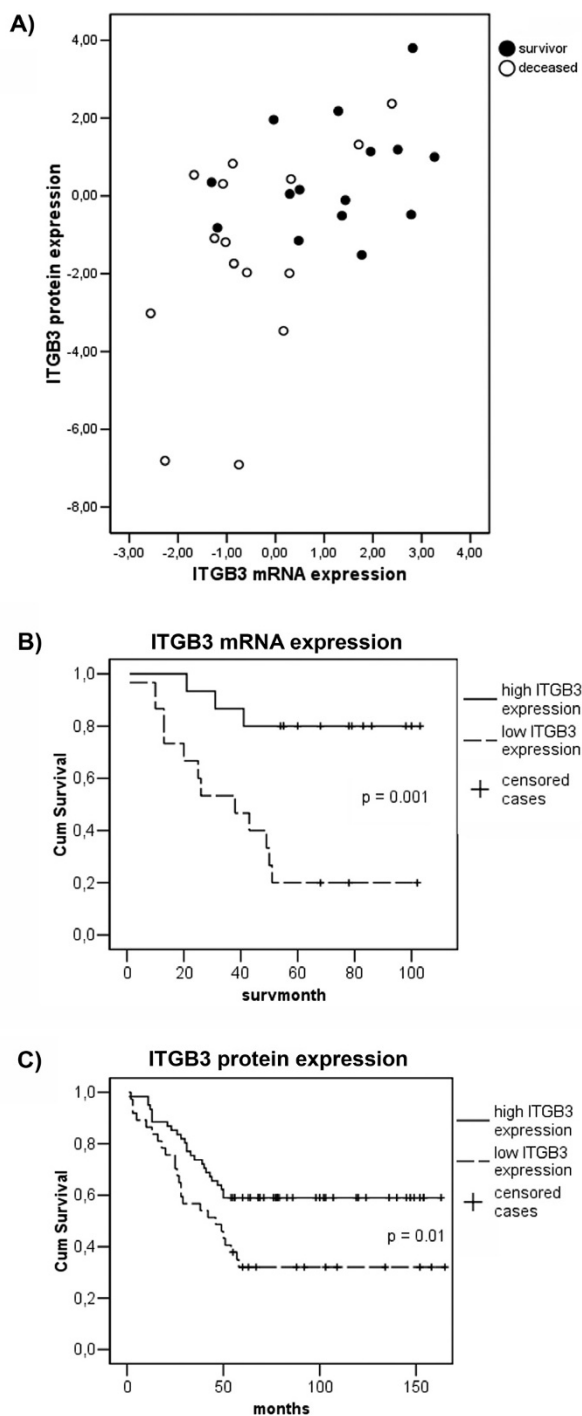


Figure 3
Visualization of differences in ITGB3 expression levels between survivors and deceased patients. A) The logarithmic relative values of protein and mRNA expressions from the same tumour plotted against each other. B) Kaplan-Meier survival curve for ITGB3 mRNA and C) the protein ITGB3.

the gene and the protein expressions, which strengthens the credibility of our results.

A limitation in many studies of biomarkers, with microarray and other methods, is the lack of follow-up with external validation of significant findings. Consequently, the insignificant results for CLU, CAPG, and PRAME in the present study highlight the importance of verifying data in an external validation set of tumours, although, the number of tumours analysed with QPCR in this study was low, and small groups may have had an impact on the results. However, earlier studies have reported CLU as down-regulated and CAPG and PRAME as up-regulated in advanced carcinomas compared to early tumours or normal tissue [19-25]. Even though we did not detect any differences in relation to survival in our analysis, the expression of CLU, CAPG, and PRAME might still be associated with the development and progression of serous ovarian adenocarcinomas. Concerning ITGB3, an evaluation in the reverse order, where the patients are classified depending on the tumours' ITGB3 expression, could determine if it is possible to distinguish patients who will respond to standard treatment from those who will not.

The gene *ITGB3* encodes the beta chain of two integral cell-surface glycoproteins, $\alpha\beta3$ and GPIIb/IIIa, of which $\alpha\beta3$ is expressed on endothelial and tumour cells [26]. As molecules involved in cell adhesion and cell signalling, integrins have an important role in various carcinomas. The expression of $\alpha\beta3$ integrins has been linked to bad prognosis for breast cancer and melanoma patients, but the relation to ovarian tumours needs further clarification [27,28]. The expression of $\alpha\beta3$ integrins has been detected as less frequent in ovarian epithelial tumours of low malignant potential (LMP) in contrast to ovarian carcinomas [29]. Controversially, Maubant et al. [30] found a significantly less frequent expression of the $\beta3$ subunit in grade 3 ovarian tumours compared to grade 1 and 2 tumours, and less expression in peritoneal metastasis compared to primary tumours. Nevertheless, they did not find any correlation to survival, as we did in our study. The use of tumours with different histological subtypes and stages in their analysis may explain the inconsistent findings compared with our results, since we used only advanced serous adenocarcinomas. Further, ITGB3 protein expression have been detected in normal ovarian epithelium and highly differentiated ovarian carcinomas, but are lacking in most of the less-differentiated tumours [31]. Regeneration of normal ovarian epithelial cells is a continuous process due to wound healing after ovulation. This process involves adhesion, spreading, and proliferation of cells, all features that could involve integrins. The expression of ITGB3 in the normal ovarian epithelium and its expression in well-differentiated ovarian carcinomas may reflect the preservation of normal cell properties.

The expression of ITGB3 in tumours from survivors in our study may indicate that these tumour cells still retain normal cell properties, and are therefore less aggressive. Moreover, $\alpha\beta3$ may have different function in various tissues, which may partly explain the divergent results of $\alpha\beta3$ expression linked to bad prognosis in other cancers, such as breast cancer and melanoma [27,28]. Therefore, the use of ITGB3 as a biomarker may only have clinical relevance in ovarian adenocarcinomas.

Normal adherent cells must be anchored to the extracellular matrix (ECM) to survive and proliferate. Ligated $\alpha\beta3$ activates cell survival pathways and suppresses proapoptotic signalling, while inactive unligated $\alpha\beta3$ promotes apoptosis [32,33]. Maubant et al. [34] used a sensitive ovarian cancer cell line and its cisplatin-resistant counterpart to study the expression of $\alpha\beta3$ and $\alpha\beta5$. They showed that three days after plating, the α subunit associated with the $\beta3$ subunit on the surface of sensitive cells, but it associated with both $\beta3$ and $\beta5$ on the surface of resistant cells. Consequently, ITGB3 could have a dual role in cancer. At its primary site, the protein favours a good prognosis, but as soon as the tumour cell has lost its attachment, the cell will be able to metastasise and progress at a distant site. This dual role of the protein may have an impact on the use of ITGB3 as a biomarker.

Finally, the function of ITGB3 as a biomarker in ovarian adenocarcinoma may be influenced by other factors in the tumours. One such factor is the up-regulation of $\alpha\beta3$ integrin in low HRG/NRG1-expressing breast cancer cell lines [35]. These cell lines are highly sensitive to treatment with paclitaxel. The up-regulation of $\alpha\beta3$ integrin and low expression of HRG/NRG1 in cancer cells could be a novel molecular marker of chemosensitivity. The correlation of HRG/NRG1 and ITGB3 expression might be relevant to study in ovarian tumours as well, or there might be other unknown factors that regulate the impact of ITGB3 as a biomarker.

Conclusion

In conclusion, ITGB3 was significantly differently expressed between tumours from survivors and tumours from deceased patients. The differences were detected for both gene and protein levels in our external validation set of advanced serous ovarian adenocarcinomas. The loss of ITGB3 expression in tumours from deceased patients and high expression in tumours from survivors could be used as a biomarker for patients with advanced serous tumours. Prospective evaluation of ITGB3 expression levels could determine if it is possible to distinguish patients who will respond to standard treatment from those who will not. It would also be worth considering a study of ITGB3 expression in stage I and stage II serous ovarian tumours, and in other histopathological subtypes. In

future, it may be that patients who respond to standard therapy could be treated with a more moderate combination of anti-cancer agents, and higher-risk patients might be offered additional chemotherapy and more frequent follow up at an initial state.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KP participated in the conception and design of the study, carried out the western blot, statistical analysis, interpretation of the data and drafted the manuscript. KL and LÖ contributed to the analysis and helped to draft the manuscript. IC carried out the western blot and contributed to the analysis of the data. KS participated in the design, and assisted in the writing of the manuscript. GH contributed to the conception and the design of the study, assisted in the writing of the manuscript, and funded the study. All authors read and approved the final manuscript.

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