Ulex europeus Agglutinin-I Binding as a Potential Prognostic Marker in Ovarian Cancer

KATHARINA BLONSKI[1], KARIN MILDE-LANGOSCH[2], ANA-MARIA BAMBERGER[2], TINA OSTERHOLZ[3], CHRISTIAN UTLER[3], JÜRGEN BERGER[4], THOMAS LÖNING[2] and UDO SCHUMACHER[1]

[1]Department of Anatomy II: Experimental Morphology, [2]Institute of Gynaecopathology, [3]Department of Gynaecology, and [4]Institute of Mathematics and Data Processing in Medicine, University Medical Center Hamburg-Eppendorf, 20246 Hamburg, Germany

Abstract. *Background: Ovarian cancer represents the malignant tumour of the female genital tract with the worst prognosis, mainly caused by early intraperitoneal spread. Cell-to-cell and cell-to-matrix interactions play a functionally important role in this spread and are both mediated by the cell membrane. Changes in the glycosylation of the cell membrane, as detected by lectin histochemistry, are sometimes associated with a poor prognosis. Patients and Methods: The expression of lectin binding of 164 ovarian cancer patients was analysed and the staining results were correlated with the clinical data of the patients. Results: The univariate and multivariate statistical analysis revealed an independent prognostic significance for Ulex europeus agglutinin-I (UEA-I) binding. Conclusion: These findings indicate that UEA-I binding can serve as a prognostic factor in ovarian cancer.*

Although it represents just 5% of malignancies in women, ovarian cancer causes more deaths than any other malignancy of the female genital reproduction tract (1). Late detection and early intraperitoneal spread contribute primarily to the high death toll of this cancer.

The metastatic process is composed of a sequence of single steps, so far inadequately understood, starting with the loosening of individual tumour cells or small clusters from the primary tumour. Once they are enabled to migrate and do so, they interact with the surrounding extracellular matrix and the neighbouring cells. If they have migrated into the peritoneal cavity, they have to survive as single cells or small lumps of cells to adhere to the mesothelial cells that form the peritoneal lining. After they have accomplished adherence, they proliferate locally and form a secondary tumour mass. Cell-to-cell and cell-to-matrix interactions of these cells, mediated by the cell membrane, are of particular importance in this process. On the outside of the cell membrane, carbohydrate chains are covalently linked to both lipids and proteins, collectively called glycoconjugates. As metastasis represents a breakdown of the normal intercellular communication, many studies have analysed the carbohydrate residues of malignant cells in order to detect abnormalities in the sugar residues indicative of metastasis. Lectin histochemistry has been employed to analyse these carbohydrate residues.

Lectins are carbohydrate-binding proteins of non-immune origin that agglutinate cells and/or precipitate polysaccharides or glycoconjugates (2). The present study focuses on the lectin *Ulex europeus* agglutinin-I (UEA-I). The source of this lectin is in its latin name *Ulex europaeus* with the common name gorse or furze. The inhibitory carbohydrate is α-L-fucose (fuc).

UEA-I has already been used to study glycoconjugate changes in malignant tumours. In breast cancer research tumours with a distinctive binding for UEA-I were associated with a shorter patient survival (3, 4) and in endometrial carcinoma the high affinity of tumour cells for UEA-I was associated with a poor outcome (5). Furthermore, a comparative study of non-malignant human prostate and prostatic carcinoma showed an increased expression of fucose residues in prostatic carcinomas (6). In squamous cell carcinomas of the uterine cervix diffuse UEA-I binding correlated with poor survival of the patients (7).

Binding for UEA-I has not been evaluated for prognostic significance in ovarian cancer tissue yet. Therefore this study investigated UEA-I binding in epithelial ovarian cancer tissue and then correlated the staining results with the patients' prognosis.

Correspondence to: Prof. Dr. med. Udo Schumacher, FRCPath, CBiol, FIBiol, DSc, Zentrum für Experimentelle Medizin, Institut für Anatomie II: Experimentelle Morphologie, Universitätsklinikum Hamburg-Eppendorf, Martinistr. 52, 20246 Hamburg, Germany. Tel: +49 40 42803 3586, Fax: +49 40 42803 5427, e-mail: uschumac@uke.uni-hamburg.de

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Patients and Methods

One hundred and sixty-four cases of ovarian cancer were investigated for UEA-I binding. The formalin-fixed and paraffin wax-embedded tissue blocks were retrieved from the files of the University Hospital Hamburg-Eppendorf (UKE), Institute of Gynaecopathology. The blocks originated from patients treated in the period from 1985 to 2002 at the Clinics of Gynaecology, UKE. For comparative reasons, six non-malignant ovarian tissue blocks of four patients were investigated (in two cases both ovaries were investigated).

Patients. The tumour tissue of 164 patients with ovarian cancer was investigated. The patients' ages ranged from 24- to 83-years-old (mean age was 57.50 years). All patient characteristics are shown in Table I. All tumours were classified as epithelial tumours and were further subdivided according to the current WHO Classification (8).

Lectin histochemistry. Sections from formalin-fixed and wax-embedded tissue blocks were used. Lectin histochemistry was performed using biotin-conjugated lectin. From the paraffin wax-embedded tissue blocks 5-μm sections were cut and applied to Adhesion Micro Slides (HistoBond®; Medite GmbH, Burgdorf, Germany). Slides were then deparaffinized in xylene and rehydrated in a series of graded ethanols to distilled water. Trizma Base (Sigma, Steinheim, Germany), sodium chloride (J.T. Baker, Deventer, Netherlands) and hydrochloric acid (Merck, Darmstadt, Germany) in distilled water were used to make up Tris-buffered saline (TBS, pH 7.6). The slides were incubated in 0.1% trypsin (Biochrom KG, Berlin, Germany) dissolved in TBS (pH 7.6) with calcium chloride (1 mM) and magnesium chloride added (1 mM) (Merck) (lectin buffer, pH 7.6) and were incubated at 37˚C for 15 min. To stop the trypsin digestion, the slides were washed in running tap water for 10 min. Slides were then washed three times in lectin buffer for 5 min. Incubation with biotin-conjugated lectin (10 mg/ml; Sigma, Darmstadt, Germany) in lectin buffer followed in a humid chamber for 1 h at room temperature. In between each step of the procedure the slides were washed in TBS. Alkaline phosphatase-labelled streptavidin was used for visualisation of the biotin-conjugated lectin binding sites. The slides were incubated with Vectastain® ABC KIT solution (Vectastain®, Vector, ABC KIT, Burlingame, CA, USA) in a humid chamber for 30 min. Naphthol-AS-biphosphate (Sigma, Darmstadt, Germany) together with hexatozized New fuchsin was then used as a substrate and the slides were incubated in the dark for 20 min. To stop the reaction, the slides were washed in running tap water and were then transferred to distilled water. Slides were counterstained with Mayer's hemalum solution (Merck) and covered using a resinous permanent mounting medium (Clariøn, Biømeda, Foster City, CA, USA).

In each staining procedure lectin incubation was omitted for one slide which was thus used as negative control. Sugar specificity for UEA-I was tested with fuc at 100 mM (Sigma, Steinheim, Germany) before incubating the slides with the lectin. As a general stain, a section of each paraffin wax block was also stained with hematoxylin and eosin.

Analysis of the staining pattern. The staining of the tumour cells was assessed using a semi-quantitative scale. A negative symbol (–) was assigned if fewer than 5% of the tumour cells were stained. A plus (+) was assigned if 5 to 50% of the tumour cells were stained, a double plus (++) if more than 50% of the tumour cells were stained. Two observers analysed the slides independently. Cases in which opinions differed were discussed in a meeting and a consensus was achieved. The slides were examined under a Zeiss Axioplan photomicroscope (Carl Zeiss, Jena GmbH, Germany) and photographed with the Axiocam MRc5 (Zeiss, Munich, Germany).

Table I. *Patient characteristics.*

Stage	Frequency in % (number)
Stage I	11 (18)
Stage II	7.9 (13)
Stage III	50.6 (83)
Stage IV	30.5 (50)
Grade	**Frequency in % (number)**
G1	12.2 (20)
G2	29.9 (49)
G3	57.9 (95)
Tumor entity	**Frequency in % (number)**
Serous	77.4 (127)
Mucinous	9.8 (16)
Endometrioid	6.1 (10)
Undifferentiated	6.1 (10)
Clear cell	0.6 (1)

Statistical methods. The clinical course of all patients was followed up. The overall survival time was defined as the interval from the date of diagnosis to the date of death or to the last date of information for living patients. Relapse-free time was defined as the interval from the date of diagnosis to the date of the first progression of the disease. The overall survival time and relapse-free time was recorded and described together with the data of the clinical stage, tumour grade, histological tumour entity and patient's age. The staining results for UEA-I were added. A Chi-square test was used to analyse for possible associations between the lectin and the clinical and histological data. All variables were analysed using the Cox regression model to evaluate the prognostic significance of the factors. Cox-regression analysis was performed using SPSS for Windows version 9 (SPSS Inc, Chicago, Illinois, USA). A univariate analysis for each factor separately was first performed. A p-value of less than 0.05 was defined as statistically significant. These statistically significant variables were further included in a multivariate analysis using forward variables selection (likelihood ratio). In addition, survival curves according to the Kaplan-Meier method (9) were carried out for statistically significant variables.

Results

Statistical analysis of patient characteristics. The clinical course of all 164 patients was followed up for a maximum time of 152 months (mean 26.79 months; standard

Table II. *Analysis for overall survival (total number of cases 164).*

Factor	*P*-value		
	Univariate	Multivariate	Exp (β) (95% CI)
UEA-I	0.018	0.032	–
UEA-I + (*vs.* neg.)	0.006	0.012	2.1 (1.1-3.8)
UEA-I ++ (*vs.* neg.)	ns	ns	–
Stage I+II	<0.0005	<0.0005	–
Stage III (*vs.* I+II)	ns	0.001	4.5 (1.7-11.5)
Stage IV (*vs.* I+II)	<0.0005	<0.0005	7.3 (2.8-18.6)
Age	0.013	0.042	1.6 (1.0-2.6)
Grade	ns	–	–
Histology	ns	–	–

Exp (β): Hazard ratio; CI: confidence interval; ns: non-significant.

Table III. *Analysis for relapse-free time (total number of cases 164).*

Factor	*P*-value		
	Univariate	Multivariate	Exp (β) (95% CI)
UEA-I	ns	ns	–
UEA-I + (*vs.* neg.)	0.033	ns	–
UEA-I ++ (*vs.* neg.)	ns	ns	–
Stage I+II	<0.0005	<0.0005	–
Stage III (*vs.* I+II)	ns	<0.0005	6.2 (2.5-15.7)
Stage IV (*vs.* I+II)	<0.0005	<0.0005	9.6 (3.7-24.6)
Age	0.015	0.029	1.7 (1.0-2.6)
Grade	ns	–	–
Histology	ns	–	–

Exp (β): Hazard ratio; CI: confidence interval; ns: non-significant.

deviation 24 months). Eighty-two patients (50%) died during follow-up time; 71 patients (43.3%) did not relapse during follow-up time while the majority, namely 93 patients (56.7%), did.

In univariate analysis, stage ($p<0.0005$) and age ($p=0.013$) were significantly associated with overall survival (Table II) and relapse-free time (for stage: $p<0.0005$; for age: $p=0.015$) (Table III). Neither the grade nor the histological tumour type had any significant influence on overall survival or relapse-free time.

In multivariate analysis, the stepwise forward variables selection (Analysis of Likelihood) was used and revealed stage and age as independent statistically significant if correlated to patients' overall survival (stage, $p<0.0005$; age, $p=0.042$) (Table II) and relapse-free time (stage, $p<0.0005$; age, $p=0.029$) (Table III).

Binding characteristics and statistical analysis of lectin binding. The tumours of 43 patients (26.2%) were assigned as UEA-I^- (Figure 1), 72 tumours (43.9%) were assigned as UEA-I^+ and 49 tumours (29.9%) were assigned as UEA-I^{++} (Figure 2).

For non-malignant ovarian tissue, UEA-I histochemistry binding sites were demonstrated in a different intensity patterns in the blood vessels, erythrocytes (five cases), the mucus of the cysts and in the fallopian tube. For all control slides without lectin incubation no staining was observed. The sugar specificity controls always resulted in a complete inhibition of the staining.

Univariate analysis revealed that UEA-I had significant influence on patients' overall survival ($p=0.018$) (Table II). Analysis of UEA-I binding assigned with one plus (+) *versus* no binding (–) was significantly associated with overall survival ($p=0.006$), as well as relapse-free survival ($p=0.033$) (Table III). Multivariate Cox regression analysis using the stepwise forward variables selection (analysis of likelihood) demonstrated statistically significant results for UEA-I binding ($p=0.032$).

In addition, survival curves according to the Kaplan-Meier method (9) show that patients with tumours with 5 to 50% positively labelled tumour cells with UEA-I (n=72) had a significantly poorer survival than patients whose tumours were UEA-I negative (n=43) (Figure 3).

The mean survival time for the group of UEA-I-negative tumours (n=43) was 74 months (median standard error (SE) 13, 95% CI: 49-98 months); for the group of patients with 5 to 50% of the UEA-I-labelled tumour cells the mean survival time was 42 months (SE 7, 95% CI: 28-56 months) and for the group of patients with more than 50% of the UEA-I-labelled tumour cells mean survival time was 41 months (SE 4, 95% CI: 33-50 months).

Discussion

The present study was undertaken to investigate the lectin binding sites of UEA-I in ovarian cancer and to relate the expression to overall survival and relapse-free time.

This study revealed that fucose (fuc) residues, specific for UEA-I are overexpressed in ovarian tumours that were associated with a shorter overall and relapse-free survival. UEA-I^+ tumours were of higher malignancy than UEA-I^- tumours hence we conclude that these carbohydrate residues were associated with a high

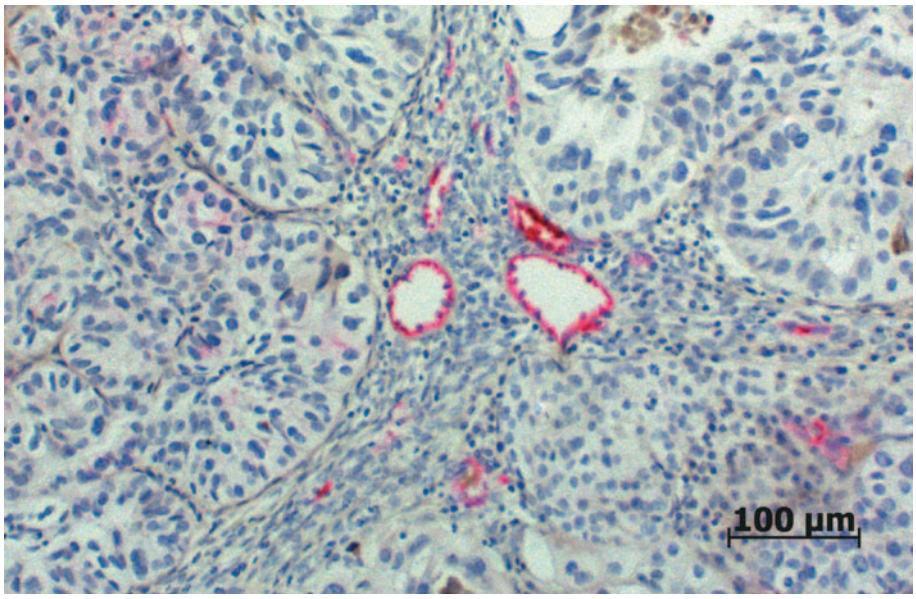

Figure 1. *Ovarian cancer cells without UEA-I-binding sites. UEA-I-positive blood vessels are located between the UEA-I-negative ovarian cancer cells. The tumour was derived from a 63-year-old cancer patient, who was still alive after nine months of follow-up time. The staging of this serous type tumour was G3 and stage II.*

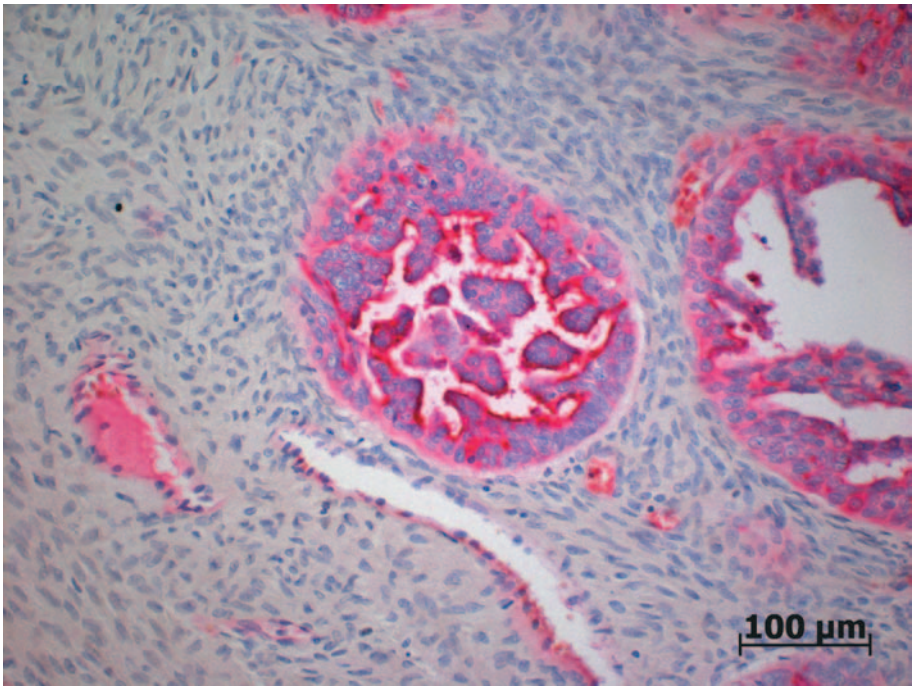

Figure 2. *UEA-I positive ovarian cancer cells. This serous type tumour was derived from a 62-year-old cancer patient who died one month after diagnosis. The classification of the tumour was G2 and stage III.*

malignant potential in the ovarian cancer cells. However, UEA-I^{++} tumours were not associated with overall survival or relapse-free time. It is hence possible that the terminal fuc residues detected by UEA-I are present in different core glycans. However, a definitive explanation cannot yet be given.

The factors of stage and age were also of prognostic significance: stage had the highest prognostic impact for

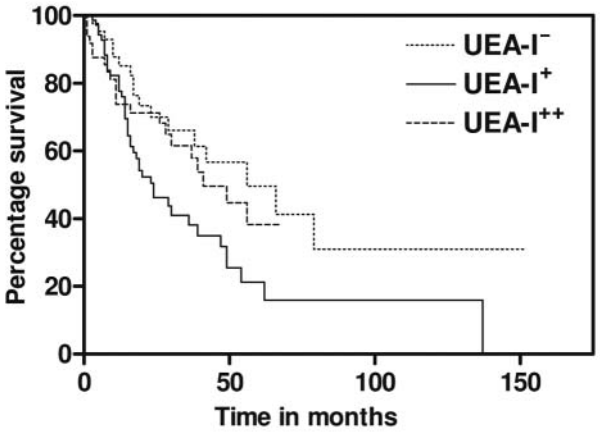

Figure 3. *Kaplan-Meier plot of overall survival for UEA-I binding.*

overall survival and relapse-free time (Tables II and III) so that this factor is deservedly the most commonly used factor in clinical everyday use.

Other studies also reported an association between UEA-I binding and poor prognosis. In studies of breast cancer (3, 4), endometrial carcinoma (5), squamous cell carcinoma of the uterine cervix (7) and prostatic carcinoma (6), an increased expression of UEA-I-binding sites was associated with a higher malignant potential of the cancer cells. However, this does not apply to all types of cancers. In oral squamous cell carcinoma, it was shown that tumours with UEA-I-binding sites showed a better prognosis and lower metastasis rate than tumours without UEA-I binding sites (10). By comparing the design of our study with the above-mentioned studies, all the other studies used different scoring systems for the analysis of the staining results. Most studies differentiated only between negative and positive tumour cell staining. But in our case, a more precise three-scale system was used. Therefore a direct comparison of the results is unfavorable.

Nevertheless, the association of fuc residues of the UEA-I^+ tumours and their high malignancy is evidence of a functional role in metastases for these carbohydrate chains, according to findings in many other studies. Stubbs *et al.* showed that core fucosylation had a dramatic effect on the conformation of *N*-linked oligosaccharides, so that α-1,6-fucosylation of *N*-glycans altered the functions of different glycoproteins (such as growth factor receptors, adhesion molecules and extracellular matrices) (11). The α-1,6-fucose is transferred to the innermost GlcNac residue of complex *N*-glycans by the enzyme α-1,6-fucosyltransferase (α-1,6FucT). A comparison of serous adenocarcinoma of the ovaries with non-malignant ovarian tissue in a study by Takahashi *et al.* in 2000 (12), the serous adenocarcinoma showed a higher α-1,6-fucosyltransferase (α1,6FucT) activity. Using lectin blot analysis with *Lens culinaris* agglutinin (LCA) the serous adenocarcinoma tissues also contained more α-1,6-fucose residues than normal ovaries and further immunohistochemical studies of serous adenocarcinoma tissues suggest that the expression of 1,6FucT is increased in tumour cells (12). These results are evidence for a change of conformation of *N*-glycans by α-1,6-fucose residues in ovarian cancer cells. This alteration in cell surface oligosaccharides can then contribute to the malignant potential of the cells.

Another study investigated a rat mammary adenocarcinoma cell line on the basis of nuclear magnetic resonance (NMR) spectroscopy. The approach was to treat the rat mammary adenocarcinoma cell line with fucosidase first before the cells were subcutaneously injected in rats. The study revealed that the injected fucosidase-treated cells metastasised in only 20% of cases, whereas the untreated cells metastasised in 80%. Apparently, the removal of the surface fuc by enzyme treatment decreased the metastatic characteristics of the cells (13). Fuc residues also play a major role in cell adhesion, in particular in selectin-mediated adhesion. In a study by Xia *et al.* (14), the importance of fuc for this function was demonstrated. Human umbilical cord blood cells with a defect in binding to P-selectin were unable to roll on endothelial selectins in bone marrow vessels of nonobese diabetic/severe combined immune deficiency (NOD/SCID) mice. When treated with GDP-fucose and a 1-3-fucosyltransferase IV these cells improved binding to P- and E-selectin, hence improved cell rolling under flow. The P- and E-selectin-mediated cell rolling is a common feature in leucocyte recruitment during inflammation and possibly metastasis. In selectin-mediated cell rolling the selectin binds to sialylated, fucosylated or sulfated glycans (15). As fuc seems to be mainly involved in this mechanism, it is possible that fuc-positive ovarian cancer cells may more easily attach to the peritoneum lining the peritoneal cavity. The above-mentioned phenomenon would support the spread of the ovarian cancer cells in the peritoneal cavity, which is a particular feature in ovarian cancer, which could contribute to a higher malignant potential of these cells.

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