Expression of VLA-2, VLA-3, and α_v integrin receptors in uveal melanoma: association with microvascular architecture of the tumour and prognostic value

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Abstract

Background—The interaction of the integrin receptors with their ligands (collagen, laminin, fibronectin, and others) has a crucial role during the reorganisation of the extracellular matrix and the metastatic process. The presence of particular vascular patterns in uveal melanoma is associated with the development of metastases. There is some evidence that interactions between the tumour cells and the extracellular matrix are responsible for the shape of these patterns.

Methods—The expression of VLA-2, VLA-3, and α_v integrin receptors was examined by immunohistochemistry on paraffin embedded tumour specimens from 92 uveal melanomas (iris melanomas excluded). Possible correlations between these results and the tumour vascular patterns, the histological features of the tumours as well as the clinical outcome of the patients, were investigated.

Results—The expression of VLA-2 in tumours was associated with the presence of vascular networks ($p = 0.05$). Tumours with less than 25% VLA-3 positive cells infiltrated the sclera more frequently than those with more than 25% VLA-3 cell positivity ($p = 0.05$). Tumours expressing less than 50% α_v positive cells were associated with the mixed or epithelioid cell type ($p = 0.05$) and, with less statistical precision, with the presence of extraocular growth ($p = 0.07$). The univariate logistic regression analysis showed that the risk of developing metastases within the first 5 years after diagnosis did not depend on the expression of the integrin receptors investigated.

Conclusion—The potential biological importance of the associations between integrin expression and the histopathological features of the tumours found in the present study remains to be elucidated in future experiments. The immunohistochemical detection of VLA-2, VLA-3, and α_v integrins had no prognostic value in our preliminary report.

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Uveal melanomas are the most common primary intraocular malignancies in adults. Despite the successful treatment of uveal melanomas by

various techniques—enucleation, radiotherapy, and local resection—the overall mortality after 5 and 10 years is approximately 30% and 50%, respectively.^{1–3} The survival is strictly related to the metastatic spread of the tumour that occurs haematogenously and preferentially to the liver. Numerous variables have been disclosed to predict a high risk of developing metastases in patients with uveal melanoma. These include the tumour size, histological cell type, monosomy 3, nucleolar size, extrascleral tumour extension, ciliary body involvement, age at diagnosis, lymphocytic infiltration, and others.^{1–4–9} In particular, the presence of microvascular networks composed of either so called “back to back loops”, which encircle microdomains of the tumour, or parallel vessels with cross linking, are associated with death from metastatic melanoma.¹⁰ The formation of these vascular patterns may result from reciprocal interactions between the tumour cell and the extracellular matrix.¹⁰

Integrins form a family of membrane spanning cell surface proteins that promote cell-cell and cell-matrix adhesion. The integrins are composed of two different subunits, the α and β chains. Fourteen different α and eight β chains have been detected to date. The α and β chains in various combinations form a multitude of receptors: some of them have affinity to components of the extracellular matrix—collagen, laminin, fibronectin, and vitronectin—and others to cell bound ligands on leucocytes and endothelial cells.^{11–12} The integrin superfamily of VLA (very late activation antigens) receptors comprises heterodimeric proteins containing the $\beta 1$ chain and the $\alpha 1$ –7 chain. The VLA integrin receptors have been postulated to have a substantial role in the metastatic cascade with an altered pattern of VLA phosphorylation status and/or VLA surface expression in invasive and metastatic tumour cells.^{13–14}

The expression of VLA-2,¹⁵ VLA-6,¹⁶ and $\alpha_v\beta_3$ ¹⁷ integrins was associated with tumour progression in cutaneous melanomas. A correlation between integrin expression and tumour invasiveness or cell type was not found in two previous histopathological studies of 32¹⁸ and 12¹⁹ uveal melanomas, respectively. MacNeil and co-workers investigated the role of tamoxifen in inhibiting the attachment of uveal melanoma cells to components of the extracellular matrix in vitro.²⁰

In the present study, we evaluated the microvascular architecture of uveal melanomas

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and the expression of the VLA-2, VLA-3, and α_v (CD-51) integrin receptors. Possible associations between these factors were statistically analysed. In addition, we investigated the impact of these variables on the clinical outcome of patients in a case-control study model and compared them with known histological prognostic markers.

Materials and methods

The case-control study was performed on 37 patients (mean age at diagnosis 62 years) who developed metastases within the first 5 years after diagnosis of uveal melanoma. Fifty five patients (mean age at diagnosis 58 years) who survived the first 5 years after diagnosis without metastases served as a control group. Cases of iris melanomas were excluded from the study. All patients underwent enucleation as single therapy. The enucleated eyes were fixed in 4% buffered formalin and embedded in paraffin. Both the study group and control group consisted of consecutive patients in alphabetical order with known outcome status and available tissue blocks obtained from the medical archives of the department of ophthalmology (1972–97) of the University of Essen. Seven patients were excluded because they died of another cause. Time between diagnosis and enucleation never exceeded 10 days; thus, evaluation of follow up from the time of first diagnosis and time of surgical intervention were considered comparable. Follow up data were selected by contacting the general practitioner or the local ophthalmologist.

By conventional histology, each tumour was classified according to cell type (spindle cell versus mixed and epithelioid cell type). Its location and degree of extension were noted. For the evaluation of the vascular architecture, conventional PAS (periodic acid Schiff) staining without haematoxylin counterstaining was performed and vascular patterns were classified according to the guidelines of Folberg *et al.*²¹ The histological variables were determined by two examiners independently (HS and GA), who were masked to the patient outcome.

Routine histology of the 92 uveal melanomas enrolled in the present study showed a dimension greater than 10 mm in 74 tumours (80.4%). This selection bias occurred since smaller melanomas were treated by conservative eye salvaging therapy. Thirty three of the tumours (35.9%) demonstrated ciliary body involvement. Extraocular extension of the tumour was present in seven (7.6%) cases, scleral infiltration in 28 (30.4%) tumours. The uveal melanomas consisted of 47 (51.1%) spindle B and 45 (48.9%) mixed and epithelioid tumours.

Immunohistochemical staining was performed with anti- α -2 integrin (clone P1E6, Chemicon Int, USA), anti- α -3 integrin (clone P1B5, Chemicon Int, USA) and anti- α -V integrin (clone VNR147, Chemicon Int, USA) monoclonal antibodies on paraffin embedded tissues. Further antibodies for integrins suitable for paraffin sections were not available at the time of this study. Briefly, sections were cut

at 5–6 μ m from each block and were mounted on aminopropyltriethoxysilane coated slides, dried overnight at 37°C, deparaffinised, and rehydrated. Thermal induced antigen retrieval was performed by microwave irradiation (2 $\times$ 5 minutes; 750 W; in target retrieval solution, Dako, Hamburg, Germany). The slides were blocked for 30 minutes with rabbit serum (Dako). After incubating with the primary mouse anti-human monoclonal antibodies overnight, the slides were washed and incubated with a secondary rabbit anti-mouse antibody (Dako) for 30 minutes, and subsequently with the StrepABComplex/AP (Dako) for 20 minutes. The reaction was made visible by new fuchsin (Sigma, Deisenhofen, Germany). The red staining allowed easy detection of immunoreactivity in pigmented tumours. Counterstaining was achieved with Gill's haematoxylin (Sigma), and the sections were mounted with Aquatex (Sigma).

Sections from skin (VLA-2) and tonsils (VLA-3 and α_v) served as positive controls. Negative controls were performed by incubating the slides with PBS instead of the primary antibody. The optimal dilutions for the reagents were assessed in a series of stainings performed before this study: anti-VLA-2 1:50, anti-VLA-3 1:150, and anti- α_v 1:50.

The evaluation of the immunohistochemical staining was also performed with a light microscope (Zeiss, magnification $\times$ 125–400) by two independent investigators (HS and GA). All of the tumour section was evaluated and the positivity was assessed using a semiquantitative scale: 0 = no positive cells in the total, 1+ = 1–25% positive cells, 2+ = 26–50% positive cells, 3+ = 51–75% positive cells, and 4+ = 76–100% positive cells.

Odds ratio estimates (OR), 95% confidence intervals (95% CI), and p values for the expression of integrins and histological characteristics were calculated according to standard methods. Adjusted ORs were estimated using multiple logistic regression models, controlling for known risk factors.²² The Pearson's test was used to assess the association between the expression of integrins and histopathological characteristics.

Results

HISTOPATHOLOGY AND IMMUNOHISTOCHEMISTRY
The evaluation of the vascular patterns was unsuccessful in three tumours as a result of heavy pigmentation. In the remaining 89 tumours, the distribution of the prognostically significant patterns²⁰ was as followed: 25 tumours (28.1%) showed vascular networks; seven tumours (7.9%) had vascular loops without networks; eight tumours (9.0%) demonstrated parallel vessels with cross linking without any of the previous characteristics.

The results of the immunohistochemical staining are summarised in Table 1. Agreement was achieved in more than 90% of the cases; interobserver error was estimated to be 7%. Owing to technical problems (fixation and heavy pigmentation), the staining for VLA-2 was unsuccessful in two cases; similarly, VLA-3 staining was unsuccessful in seven tumours

Table 1 Immunohistochemical detection of integrins in uveal melanomas

	0	1–25%*	26–50%	51–75%	76–100%
VLA-2 (n=90)	34 (37.8%)	34 (37.8%)	11 (12.2%)	7 (7.8%)	4 (4.4%)
VLA-3 (n=85)	—	17 (20%)	26 (30.6%)	23 (27.1%)	19 (22.3%)
α_v (n=89)	11 (12.4%)	23 (25.8%)	24 (27%)	14 (15.7%)	17 (19.1%)

*Percentage of the tumour cells stained positive.

and α_v staining in three tumours. Consequently, these patients were excluded from the subsequent statistical evaluations.

ASSOCIATIONS BETWEEN INTEGRINS, VASCULAR PATTERNS, AND HISTOLOGICAL FEATURES

The univariate analysis of the results demonstrated a correlation between tumour vascular patterns and ciliary body involvement as well as vascular patterns and histological cell type (Table 2). No associations were found between the vascular patterns and the remaining clinicopathological features—that is, patient age or sex, greatest tumour diameter, extraocular growth, and scleral infiltration.

The investigation of the associations between the immunohistochemical markers and the histological features of the tumours demonstrated a positive association between VLA-2 expression and tumour vascular patterns (Table 2). Twenty out of 25 tumours with vascular networks were found in VLA-2 positive tumours. In contrast, only five of the 31 VLA-2 negative tumours demonstrated vascular networks ($p = 0.05$).

An association was also found between VLA-3 expression and the scleral infiltration demonstrated by the uveal melanomas. Tumours with less than 25% of VLA-3 positive cells infiltrated the sclera more frequently than tumours with more than 25% of VLA-3 positive cells ($p = 0.05$).

Table 2 Correlations between vascular patterns, histological features, and VLA-2

	Vascular networks (n = 25)		Loops and networks (n = 32)	
Histological cell type				
Spindle (n = 46)	8	p=0.02	12	p=0.04
Mixed and epithelioid (n = 43)	17		20	
Ciliary body involvement				
Yes (n = 31)	18	p=0.0001	20	p=0.0004
No (n = 58)	7		12	
VLA-2 expression				
Negative (n = 31)	5	p=0.05	8	p=0.1
Positive (n = 56)	20		24	

Table 3 Univariate analysis estimating the risk for developing metastases

Variable	Odds ratio	95% Confidence interval	p Value
Extraocular growth	10.4	1.2–90.1	0.03
Scleral infiltration	5.4	2.1–14.1	0.0006
Vascular patterns:			
Vascular networks	3.1	1.2–8	0.02
Loops and networks*	5.3	2.1–13.7	0.0005
Loops, networks, and cross linking †	5.2	2.1–13.2	0.0004
Ciliary body involvement	3.8	1.5–9.3	0.003
Histological cell type (mixed and epithelioid)	2.5	1–5.8	0.04
Greatest tumour diameter (continuous)	1.1	1–1.2	0.01
Sex (male)	1.3	0.6–3.1	0.5
Age (>60 years)	1.9	0.8–4.4	0.1
VLA-2 (positive)	0.5	0.2–1.3	0.2
VLA-3 (<25% positive cells)	0.7	0.2–1.9	0.5
α_v (<50% positive cells)	1.2	0.5–2.9	0.7

*Tumours with vascular networks (n=25) and tumours with vascular loops without networks (n=7). †As above (*) plus tumours with parallel vessels with cross linking (n=8).

The expression of α_v correlated with the histological cell type of the tumours. Mixed or epithelioid cell type uveal melanomas tended to demonstrate less than 50% positivity for α_v ($p = 0.05$). In addition, all tumours with extraocular growth expressed less than 50% of α_v positive cells. Owing to the small number of the cases, this observation did not obtain statistical significance ($p = 0.07$).

PREDICTIVE VALUE OF THE INVESTIGATED VARIABLES FOR THE DEVELOPMENT OF METASTASES

The univariate analysis demonstrated that the risk of developing metastases depended on the cell type (mixed and epithelioid), the presence of extraocular growth, vascular patterns, scleral infiltration, ciliary body involvement, and greatest tumour diameter. The remaining factors—that is, patient sex and age, VLA-2, VLA-3, and α_v expression, did not demonstrate any predictive value (Table 3). Given the sample size the multivariate analyses was limited for a maximal three variables and, thus, did not present helpful results (data not shown).

Discussion

In the current study, we found an association between the expression of VLA-2 and the presence of vascular networks in uveal melanomas. These networks were found more often in VLA-2 positive tumours. In a previous in vitro study, cultured uveal melanoma cells (spindle B and epithelioid) infiltrated type I collagen gels, whereas the spindle A cells remained on the gel surface.²³ Interestingly, only those cells which infiltrated the gels expressed the type VI collagen.²³ The authors concluded that the ability of invasive melanoma cells to produce an element of the extracellular matrix (that is, collagen VI) associated with tissue remodelling may help to explain the association between vascular networks and the development of metastases.²³ Our observation that VLA-2, which is a receptor for collagen type I–VI, is more strongly expressed in uveal melanomas with vascular networks than in those without networks may support this assumption. The production and deposition of ECM elements alone is not sufficient to remodel the environment around the tumour cells; the interaction between these elements and their receptors is also a prerequisite for this process.²⁴

The loss of VLA-3 and α_v expression in uveal melanoma was associated in the present study with the infiltration of the sclera and the extraocular growth of the tumour, respectively. In addition, the loss of α_v expression was associated with an unfavourable cell type (mixed and epithelioid). In the only previous immunohistochemical study on integrins in uveal melanomas, ten Berge *et al*¹⁸ found a wide

expression of VLA-3 (more than 75% of the tumour cells) in 27 out of 32 (84%) primary tumours but there was no correlation with cell type or invasion of the sclera. They also found that only eight out of 32 (25%) primary tumours demonstrated less than 50% α_v positivity and that there was no association between α_v expression and cell type or scleral invasion.¹⁸ These results are in contrast with our findings listed in Table 1. These differences, however, may be explained by the different material (fresh frozen sections *v* paraffin embedded material), the different staining procedures (peroxidase based ABC system *v* alkaline phosphatase based ABC system), and the different monoclonal antibodies (for α_v : NKI-M7 *v* VNR 147) used. Further, the differences in the distribution of these integrin receptors within the tumour specimens in these two studies may also explain the contrasting findings with respect to integrin expression and cell type or scleral invasion.

The presence of vascular networks and loops in uveal melanomas was associated in the current investigation with the tumour location (involvement of the ciliary body) and the unfavourable cell type (mixed and epithelioid). The same observation was made in two previous studies^{25, 26} which suggested that the aggressive behaviour of ciliary body melanomas is related to the tendency for vascular networks to develop in this location.²⁶

Although VLA-2, VLA-3, and α_v expression in uveal melanoma correlated with vascular networks, scleral infiltration, and cell type, respectively, none of the integrin receptors showed a predictive impact for the development of metastases in the logistic regression analyses. The relative small number of tumours in our study, however, did not allow multiple regression models with several factors. In addition, the 5 year metastasis free survival sectioning level used in the present study might be too short for the evaluation of prognostic factors since a considerable proportion of patients develop metastases even after 5 years. Therefore, our statements should be considered as preliminary. The potential biological implications of the integrin receptors in uveal melanoma could not be determined by the present immunohistochemical study. For this information more sophisticated experimental approaches are needed.

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