VLA2 integrin expression in breast carcinomas evaluated by automated and quantitative immunohistochemistry

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Summary VLA2 is thought to be involved in the metastatic process in malignant tumours, in particular in carcinomatous cell adhesion to vessel basement membrane. VLA2 expression was immunohistochemically investigated in 204 breast carcinomas. Frozen tissue sections were probed with monoclonal anti-VLA2 using automated (Ventana ES 320 System) and quantitative (SAMBA 2005 image processor) immunoperoxidase. A positive anti-VLA2 immunoreaction was observed in 48 tumours (23.5%), within epithelial carcinomatous cells. The VLA2-positive surface in tumours varied from 3% to 20% (mean 8.75, S.D. 7.17) and was correlated with histopathological indicators and tumour expression of various antigens detected using the same method as that for VLA2. The results show that VLA2 immunorepression was independent of the tumour size, grade, type and aneuploidy, and of the nodal status. VLA2 significantly correlated with ELAM, VCAM, VLA3 and P-glycoprotein (P-gp) (P < 0.01) and inversely correlated with cathepsin D (P < 0.001), but was independent of Ki67/MIB1, p53, bcl-2, c-erbB-2, E cadherin, CD44v, CD31, oestrogen and progesterone receptors' (ER, PR) antigenic sites and pS2. The exact role, if any, of VLA2 in tumour cell dissemination remains to be elucidated and the clinical relevance of VLA2 immunodetection in breast carcinomas requires further investigation of the correlation between in antibody expression and patients' outcome and response to chemotherapy.

Keywords: VLA2 integrin; immunocytochemistry; breast carcinoma

The integrins are heterodimers consisting of non-covalently associated α and β subunits that mediate cell adhesion. As originally described (Hynes, 1987), integrins are divided into three subfamilies, each with a common β subunit capable of associating with a specific group of α subunits. In fact, there are several different β subunits, and α subunits can combine with more than one subunit (see review in Pignatelli, 1994).

Because of the complexity of the integrins family, it proves useful to group integrins according to their cell binding activity. Integrins can be segregated into three groups: those that function as cell–cell adhesion molecules, those that bind primarily to the major constituents of the basement membrane (i.e. collagen and laminin) and those that bind primarily to the extracellular matrix proteins found during early development, inflammation and wound healing (i.e. fibronectin, fibrinogen vitronectin and thrombospondin) (see review in Albelda, 1993).

The VLA2 integrin (or VLA2, very late antigen) is a receptor (145-kDa molecule) for collagen and laminin that is expressed in many tumour cell lines (see review in Albelda, 1993). In addition, VLA2 is widely expressed in normal tissue and its polarized distribution in various epithelial cells suggests that they may significantly act upon the control of cellular growth and differentiation (De Strooper et al, 1989; Zutter and Santoro, 1990; Ruoslaha, 1991). Also VLA2 is probably implicated in the complex multistep process of tumour invasion and metastasis in vitro and in vivo (Zetter, 1993). The rhabdomyosarcoma cell line, a poorly metastatic line that does not express VLA2, increases metastatic potential after either tail vein or subcutaneous inoculation when transfected with αv β3 cDNA (Chan et al, 1991; Zetter, 1993), suggesting that increased adhesion of tumour cells to subendothelial basement membrane components results in increased metastatic potential. In contrast, the down-regulation of VLA2 is observed in poorly differentiated or larger tumours of the colon (Pignatelli et al, 1991a), liver (Patriarca et al, 1993), skin (Stamp et al, 1991) and breast (Zutter et al, 1990; Pignatelli et al, 1991b; Arihiro et al, 1993), and in prostate carcinomas (Bonkhoff et al, 1993) and melanomas (Schatendorf et al, 1993), suggesting that reduced VLA2 expression may be an indicator of predisposition to the development of metastases.

However, adhesion of tumour cells to basement membrane components is not the only process thought to be involved in tumour growth and metastasis (Zutter and Santoro, 1990; Albelda, 1993; Pignatelli et al, 1994). Tumour metastasis requires: firstly, the release of the cells from the primary tumour with decreased cell–cell adhesion and reduced cell adhesion to epithelial basement membrane; secondly, the migration of tumour cells and their adhesion to extracellular matrix; and, finally, their penetration into the vessels’ walls and their arrest in the microcirculation of the distant organs, and adhesion to endothelial cells and vessel basement membrane before extravasation (Zutter and Santoro, 1990; Albelda, 1993; Pignatelli et al, 1994).

In this study, our goal was to investigate the relationship between the tumour cells’ capacity to bind to basement membrane molecules, reflected by VLA2 expression, and other steps of the metastatic process involving several other adhesion molecules or proteases, including E cadherin (cell–cell adhesion), cathepsin D
(facilitating the migration in extracellular matrix), VLA, and CD44 (adhesion to extracellular components), CD31 (angiogenesis), VCAM and ELAM (endothelial cell activation). We investigated a series of 204 breast carcinomas using automated immunohistochemistry (Ventana ES 320 device) and quantification of the immunoprecipitates by processing digitized microscopic images (SAMBA 2005 System). In addition, our objective was to investigate the prognostic significance of VLA, immunohistochemical expression in tumour cells by correlating the results with current histopathological indicators (tumour size, grade, type and axillary node status) and with immunohistochemical indicators of high degree of cell proliferation (MIB1/Ki67, p53, bcl-2, c-erbB-2).

**MATERIALS AND METHODS**

**Source of tissue samples**

The specimens were surgically obtained from 204 patients with breast carcinomas, from January 1993 to May 1994. Mean age was 56.4 years (range 32–83 years, median 56 years). For all patients, surgical resection was the primary treatment, and none received irradiation or chemotherapy preoperatively. Surgical specimens were fixed in Bouin fixative, paraffin embedded and haematoxylin–eosin- and saffronin-stained for routine microscopic diagnosis. Samples for immunodetection were taken from the representative cancerous lesions by pathologists, in the same way as for the sample used for the intraoperative microscopic diagnosis on frozen sections. Tissue samples for immunodetections were promptly dipped in liquid nitrogen and stored frozen at −80°C in the tumour library of the laboratory.

**Histopathological features**

Tumour sizes ranged from 4 to 80 mm (mean 17.9; s.d. 12.24). In 196 of 204 cases, axillary lymph node resection was performed and 62% of patients (n = 121) were node positive and 38% (n = 75) were node negative.

All carcinomas were invasive. Invasive ductal carcinomas accounted for 74% (145 of 204), lobular carcinomas for 17% (38 of 204) and invasive carcinomas of other types for 9% (20 of 204).

Tumours were graded according to Elston’s grading system (Elston et al., 1991). Grade I tumours accounted for 22% (45 of 204), grade II for 54% (109 of 204) and grade III for 24% (49 of 204). Carcinomas were also graded according to Le Doussal’s modified Bloom’s grading system (Le Doussal et al., 1989) in five grades: 8% of grade I, 30% of grade II, 31% of grade III, 27% of grade IV and 4% of grade V. Tumours were also ranked according to the Nottingham prognostic index (Galea et al., 1992), which ranged from 2.1 to 8 (mean 4.1; s.d. 2.2). Tumours were aneuploid in 58% of the cases (97/168), with a variable degree of hyperploidy (mean 5.4%, range 0–60%) (cell imprints, Feulgen staining, image analysis) (Charpin et al., 1990, 1992).

**Immunohistological staining procedures**

**Antibody sources**

Anti-human VLA, (150-kDa integrin α2 chain, anti-CD29b) mouse monoclonal (immunoglobulin G1) antibody (clone G9) was purchased from Immunotech (Marseille, France) and used diluted at 1:500 (Arihiro et al., 1993; Bonkhoff et al., 1993). The other monoclonal antibodies used were all commercially available and were used as described previously (Charpin et al., 1988a, 1994, 1995b, 1997a–d): MIB1, anti-CD31, anti-VCAM (clone 1G1), ELAM (clone 1.2 B6), VLA, (clone G9) (Immunotech), anti-p53 (Oncogene Science, Paris, France), anti-c-erbB-2 (Biogenex Menarini, Chevilly Larue, France), anti-cathepsin D (CisBio International, Gif sur Yvette, France), anti-P-glycoprotein (P-gp) (clone JSBI-N, Tebu, Le Perray en Yvelines, France), anti-ER and -PR (Abbott kits, Rungis, France), anti-pS2 (Cisbio, France), anti-CD44 V6 (clone 2F10, RD System Europe, Abingdon, Oxon, UK).

![Figure 1](image1.png) **Figure 1** VLA, expression in frozen section (anti-VLA, Immunotech) obtained using automated (Ventana) and quantitative (SAMBA) immunoperoxidase. Positive VLA, immunostaining is observed in epithelial cells of grade 2 breast ductal carcinomas

![Figure 2](image2.png) **Figure 2** VLA, expression in frozen section (anti-VLA, Immunotech) obtained using automated (Ventana) and quantitative (SAMBA) immunoperoxidase. Positive VLA, immunostaining is observed in epithelial cells of grade 3 breast ductal carcinomas

![Figure 3](image3.png) **Figure 3** VLA, expression in frozen section (anti-VLA, Immunotech) obtained using automated (Ventana) and quantitative (SAMBA) immunoperoxidase. Positive VLA, immunostaining is observed in tumour cells of lobular breast carcinomas
UK), anti-E cadherin (clone HECD1, RD System), anti-bcl-2 (clone 124) (Dako, 78196 Trappes, France).

**Automated immunohistochemistry**

Automated immunohistochemistry was used for all the monoclonal antibodies, except for anti-E cadherin (LSAB kits, Dako) (Charpin et al., 1997c) and anti-ER and anti-PR (Abbott Kits) (Charpin et al., 1988b) and was performed on consecutive sections (4 μm thick) with avidin–biotin–peroxidase complex on Ventana 320 device (Grogan et al., 1993, 1995) (Ventana) Ventana kits (Ventana Systems, Strasbourg, France) including aminothylcarbazol reagent. Sections were counterstained with haematoxylin, dehydrated and mounted in glycergel.

**Image processing and statistical analysis**

Images of immunoprecipitates were obtained using an Axioshot microscope (Zeiss, Le Peq 78230, France) and a 3CCD camera (Sony, Paris, France) and were then processed by an image analysis system (SAMBA 2005, Alcatal-TITTN, Grenoble, France) (Charpin et al., 1997a–d). The two parameters of densitometric analysis, percentage of immunostained surface (compared with counterstained surface) and mean optical density (MOD), which reflects the staining intensity (on SAMBA arbitrary units scale of 0–255) were obtained as previously reported (Charpin et al., 1988a and b, 1994, 1995a and b, 1996a and b, 1997a–d). Statistical analysis was carried out using NCSS 6.01 statistical software (Kaysville, Utah, and Deltasoft, Meylan 38200, France). Various statistical tests were used depending on the type (nominal or ordinal) and distribution (normal or not) of the variables. Consequently, parametric or non-parametric tests were applied, including the chi-square test, Student’s t-test, Kruskal–Wallis test, Mann–Whitney U-test and the calculation of correlation coefficients (Spearman’s, Kendall’s and Pearson’s tests).

**RESULTS**

**Patterns of VLA₂ distribution in cells and tissues**

Patterns of immunoreaction were heterogeneous, as shown in Figures 1–3. Anti-VLA₂ reacted with epithelial cells but not in tumour stroma. The positive immunoreactions with anti-VLA₂ in ductal carcinomas were not significantly different from lobular carcinomas or from carcinomas of other types. In invasive ductal carcinomas (grade I–III), staining was variable, but variations in positive immunoreactions observed in individual tumour were independent of tumour dedifferentiation.

**VLA₂ quantitative immunodetection**

Only 23.5% (48 of 204) of the tumours were VLA₂ positive. The distribution of anti-VLA₂-positive staining as evaluated by densitometry on tissue sections is shown in Figure 4. Positive tumour surfaces stained by anti-VLA₂, ranged from 3% to 40% (mean 8.75%; s.d. 7.2).

**VLA₂ expression and clinicopathological data**

VLA₂-immunostained surface evaluated by image analysis was independent of the patients’ age, tumour size, histological type,

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**Table 1** VLA₂ expression and clinicopathological data

|   | VLA₂ = 0 | VLA₂ > 0 |
|---|---------|---------|
| Size (mm) | | |
| ≤ 10 | 46 | 18 | χ² = 2.72 |
| 11–20 | 68 | 17 | P = 0.44 (NS) |
| 21–30 | 2213 | 9 | |
| > 30 | 2 | | |
| < 15 | 85 | 29 | χ² = 0.52 |
| ≥15 | 64 | 17 | P = 0.47 |
| Grade* | | |
| SBR (Elston’s) | | |
| 1 | 26 | 16 | χ² = 4.78 |
| 2 | 79 | 21 | P = 0.10 (NS) |
| 3 | 34 | 10 | |
| SBRM (Le Doussal’s) | | |
| 1 | 11 | 3 | |
| 2 | 41 | 16 | |
| 3 | 43 | 12 | χ² = 2.43 |
| 4 | 38 | 7 | P = 0.66 (NS) |
| 5 | 5 | 2 | |
| Group I | 95 | 31 | χ² = 1.13 |
| Group II | 43 | 9 | P = 0.29 (NS) |
| NPI (Galea) | | |
| 1 (< 3.4) | 59 | 21 | |
| 2 (3.4–5.4) | 15 | 15 | P = 0.74 (NS) |
| 3 (> 5.4) | 18 | 4 | |
| Histological types | | |
| Ductal | 110 | 38 | χ² = 0.71 |
| Lobular | 37 | 9 | P = 0.40 (NS) |
| Node status | | |
| Node positive | 93 | 28 | χ² = 0.45 |
| Node negative | 55 | 20 | P = 0.50 (NS) |
| Feulgen staining | | |
| Diploid | 49 | 16 | χ² = 0.02 |
| Aneuploid | 69 | 23 | P = 0.89 |
| No hyperploidy | 21 | 6 | χ² = 0.12 |
| Hyperploidy | 17 | 33 | P = 0.73 (NS) |

*Statistics calculated using the Mann–Whitney test. SBR, Scarff–Bloom–Richardson score; SBRM, SBR modified.

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**Figure 4** Distribution of positive VLA₂ surfaces evaluated (%) on tissue sections by computer-assisted (SAMBA) analysis of digitized microscopic coloured images.
The method of immunodetection (Table 1). Quantitative immunocytochemistry (positive surface) for VLA2, VLA2 = 0 and VLA2 > 0 (all tumours, negative tumours, and positive tumours) are shown in Table 2. The statistical tests for VLA2 immunodetection were performed using Student’s t-test. Numbers in parentheses are standard deviation values.

Correlation of VLA2 expression with quantitative immunodetection

**Distribution**
The distribution of the antigens, investigated using the same method as that for VLA2 on serial sections, was first studied by calculation of the mean positive (immunostained) surface (%) in VLA2-positive (48 of 204) and -negative (156 of 204) subsets. The results of quantitative evaluation of growth fraction (MIB1) and immunoreaction with anti-p53, anti-bcl-2, anti-c-erbB-2 protein, anti-cathepsin D, anti-CD31, anti-CD44, anti-cadherin, anti-VCAM, anti-ELAM, anti-VLA3, anti-P-gp, anti-ER and -PR antigens, and anti-p52 in VLA2-positive and -negative tumours are shown in Table 2.

**Correlation (Spearman’s and Kendall’s tests)**
VLA2 immunostaining in breast carcinomas was independent of most of the other antigens investigated (Table 3). However, a significant correlation was observed between VLA2 immunohistochemical expression and that of P-gp, VCAM, ELAM and VLA2, (Tables 3 and 4) and was inversely correlated with cathepsin D.
Table 3  Correlation of quantitative immunocytochemical assays of VLA2, to that of other antigens investigated on consecutive sections of frozen blocks, automated (Ventana) procedure and image analysis (SAMBA)

| Antigen       | VLA2 immunocytochemical expression | r | P         |
|---------------|-----------------------------------|---|-----------|
| Cathepsin D   | r = -0.296                        |   | P < 0.001 (Spearman) |
| ELAM          | r = 0.345                         |   | P < 0.01 (Spearman)  |
| VCAM          | r = 0.499                         |   | P < 0.001 (Spearman)  |
| VLA2          | r = 0.193                         |   | P < 0.01 (Spearman)  |
| P-gp          | r = 0.425                         |   | P = 0.036 (Spearman)  |
| Ki67/MIB1     | NS                                |   | NS         |
| p53           | NS                                |   | NS         |
| bcl-2         | NS                                |   | NS         |
| c-erbB-2      | NS                                |   | NS         |
| E-cadherin    | NS                                |   | NS         |
| CD44v         | NS                                |   | NS         |
| CD31          | NS                                |   | NS         |
| ER            | NS                                |   | NS         |
| PR            | NS                                |   | NS         |
| pS2           | NS                                |   | NS         |

DISCUSSION

Mortality in breast cancer is related to the development of bloodborne metastases. Tumour metastases require the release of cells from the primary site, migration through the extracellular matrix into the microvasculature and finally arrest in distant organ (Zutter and Santoro, 1990). Various molecules, especially adhesion molecules, are involved at the different steps of the metastatic process and are expressed on tumour cells or released in the extracellular compartment (Zutter and Santoro, 1990; Albelda, 1993; Pignatelli and Vessey, 1994). Thus their immunodetection in tissue sections may be a relevant tool useful in clinical practice to identify patients with significant risk of developing metastatic disease, promoted by the variations in the expression of the adhesive molecules. However, the practical relevance of immunohistochemistry can be determined provided that the technical procedures are as far as possible standardized and provided that methodological bias are optimally reduced.

Methodological bias may result from several factors. Firstly, tissue fixation and paraffin embedding may be the source of antigen damage. Although recent advances in immunohistochemistry have shown the suitability of archival paraffin tissue blocks for immunodetection after antigen retrieval (microwave), the freezing of tissue blocks, when possible, basically does not modify the structure of antigens in tissues. Secondly, inconsistencies may result from variations in the immunohistochemical methods used. In this regard, an automated device rather than in-house manual procedures provides a better guarantee of quality control, particularly with regard to reproducibility. Finally, analytical bias may result from the method used for the evaluation of the results. Semiquantitative analysis of the immunoprecipitates in tissue sections is convenient, rapid and cost effective, but its reliability hinges on observers’ experience and reproducibility. Although semiquantitative evaluation is obviously sufficient to differentiate negative vs positive reaction, it is not accurate enough to evaluate intermediate patterns of staining. In contrast, quantitative analysis by computerized processing of digitized microscopic images provides more objective data, and variations in staining are more accurately evaluated. In addition, the numeric values of parameters are more suitable for statistical analysis. In the present study, we evaluated VLA2 in optimal technical conditions using frozen tissue sections, automated immunohistochemistry and computerized analysis of digitized microscopic images, as previously used for other molecules (Charpin et al. 1988a and b, 1994, 1995a and b, 1996b, 1997a–d).

Adhesion molecules are involved in the cascade of events of the metastatic disease. Some are involved in cell–cell adhesion or in cell adhesion to epithelial basement membrane. Some are involved in adhesion to extracellular matrix and others in adhesion to the vessel wall either to activate endothelial cells or to increase binding to vessel basement membrane. VLA2, which is an α2 β1 integrin acting as a receptor for collagen and laminin, is involved in cell binding to epithelial basement membrane and also to the vessel basement membrane. These adhesive properties of tumour cells expressing VLA2 promote the development of metastases as shown by experimental data (Chan et al, 1991) and facilitate adhesion to the vessel wall. However, the decreased expression of VLA2 in human carcinomas (Zutter et al, 1990; Pignatelli et al, 1991a and b, Stamp and Pignatelli, 1991; Arihiro et al, 1993; Bonkhoff et al, 1993; Patriarca et al, 1993) is associated with higher metastatic rate or tumour dedifferentiation. In this respect, the VLA2 expression of tumour cells in early breast carcinomas could constitute a prognostic indicator.

Using optimal technical conditions (not comparable to previous studies for VLA2 immunodetection) in our series of breast carcinomas, we did not observe any significant relationship between VLA2 expression and current histopredictive indicators, such as tumour size, grade, type, degree of tumour aneuploidy and hyperploidy, or the axillary node status and the Nottingham prognostic index. These results suggest that in order to determine its exact prognostic relevance, the significance of VLA2 expression in

Table 4  VLA2 immunocytochemical expression and Cathepsin D in breast carcinomas

| Percentage of line | Percentage of column | VLA2 ≤ 5% | 5%< VLA2 ≤ 10% | 10%< VLA2 ≤ 20% | VLA2 > 20% | Total | Chi-square test |
|--------------------|----------------------|-----------|----------------|-----------------|------------|-------|----------------|
| Cathepsin D ≤ 15%  |                      | 83        | 6              | 6               | 0          | 95    |                |
| Percentage of line |                      | 87.3      | 6.3            | 6.3             | 0          |       |                |
| Percentage of column|                     | 51.6      | 24             | 60              | 0          |       |                |
| 15%< Cathepsin D < 20% |                | 25        | 9              | 1               | 0          | 35    | $\chi^2 = 13.43$  |
| Percentage of line |                      | 71.4      | 25.7           | 2.9             | 0          |       |                |
| Percentage of column|                     | 15.5      | 36             | 10              | 0          |       |                |
| Cathepsin D < 20%  |                      | 53        | 10             | 3               | 2          | 68    | $P = 0.037$     |
| Percentage of line |                      | 77.9      | 14.7           | 4.4             | 2.9        |       |                |
| Percentage of column|                     | 32.9      | 40             | 30              | 100        |       |                |
breast carcinomas requires further investigation, using the same method, taking into account correlation with the patients’ outcome.

The adhesive properties of tumour cells probably depend on the expression of adhesion molecules, which may vary independently or concomitantly. VLA₂ expression by tumour in breast carcinomas seems to be independent of cell–cell adhesion or adhesion to extracellular matrix, as VLA₁ immunostaining was independent of E cadherin or of CD44 v6 expression detected using the same method (Charpin et al., 1997b and c). However, VLA₂ significantly correlated with VLA₁ (P < 0.01), a receptor for fibronectin that also belongs to the integrin group and that may have similar regulation mechanisms.

We found that VLA₂ expression was inversely correlated with cathepsin D (Spyropoulos et al., 1989) expression in tumour sections, suggesting that the decreased capacity of tumour cells to bind to basement membrane is associated with an increased capacity of tumour cell migration in the extracellular matrix, facilitated by proteases that they produce. Thus, VLA₂ and D cathepsin seem to concomitantly promote tumour progression.

Angiogenesis in breast carcinomas favours tumour growth and facilitates entry into the circulation (Folkman, 1971; Liotta et al., 1974), and vessel counts and immunohistochemical labelling of vessels and endothelial cells have been shown to be endowed with some clinical relevance (Weidner, 1991; Charpin et al., 1997a). Our results show that VLA₂ immunocytochemical expression is independent of that of CD31, which reflects tumour stromal angiogenesis (Charpin et al., 1995b). However, VLA₂ was found to correlate (P < 0.01) with the expression of VCAM (IGSF) and ELAM (E-selectin) in tumours reflecting the activation of endothelial cells. This suggests that the increased expression of ELAM or VCAM molecules, which are receptors for ligands expressed on the surface of tumour cells, such as Sialyl Lewis determinants (Walz et al., 1990; Tiemeyer et al., 1991) or VLA₁ (Albelda et al., 1990; Elices et al., 1990; Gelhse et al., 1992), correlates with the capacity of tumour cells, to bind vessel basement membranes through VLA₂ expression.

Some experimental studies have shown that collagen-induced morphogenesis and expression of α2 integrin subunit is inhibited in c-erbB-2-transfected MTSV1-7 human mammary epithelial cells (D’Souza et al., 1993), suggesting that in human breast carcinomas the expression of c-erbB-2 product could correlate with integrin expression. However, in our study, VLA₂ / α2 β1 integrin did not significantly correlate with c-erbB-2, both molecules being detected according to the same immunohistochemical procedures on consecutive sections.

Recently, we also used this method to detect p53, bcl-2 and Ki67 antigens in breast carcinomas (Charpin et al., 1996a, 1997b and d). These antigens have been reported to correlate with tumour growth and tumour cell proliferative activity. Similar to C-erbB2 expression, the expression of these molecules did not correlate with VLA₂ expression, suggesting that cell proliferation and adhesive properties are regulated differently.

In conclusion, our results show that VLA₂ immunohistochemical expression in breast carcinomas is independent of most current histoprogenic indicators and of the tumour cells expression of some adhesion molecules, such as E cadherins. However, VLA₂ expression correlates with the expression in tumours of adhesion molecules of the same family, such as VLA₁ integrin or another family, such as E-selectin (ELAM) or IGSF (VCAM).

The prognostic significance of VLA₂ expression in tumours remains to be demonstrated by correlations with the patients’ outcome.

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