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Loss of VLA-2 Collagen Receptor in Breast Carcinoma, Facilitating Invasion and Metastasis

Koji Arihiro,[1] Kouki Inai,[1] Kanji Kurihara,[1] Shinpei Takeda,[1] Mayumi Kaneko,[1] Katsumasa Kuroi[2] and Tetsuya Toge[2]

[1]*Second Department of Pathology, Hiroshima University School of Medicine and* [2]*Department of Surgery, Research Institute for Nuclear Medicine and Biology, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734*

The integrin VLA-2 as a collagen receptor and VLA-5 as a fibronectin receptor were detected immunohistochemically in normal, benign tumor and carcinoma tissues of the breast. Both proteins were also detected by Western-blot analysis in some carcinoma cases. Epithelial and myoepithelial cells of both normal breast and benign tumor were in all cases immunoreactive for VLA-2 in the plasma membrane. Carcinoma cells in the invasive component were not immunoreactive for VLA-2 in 31 (46%) of 67 cases. Carcinoma cells in the intraductal components were negative for VLA-2 in only 4 (11%) of 36 cases, while 20 cases (56%) showed weak expression and 12 cases (33%) showed strong expression. Metastatic carcinoma cells in the lymph nodes of 6 cases showed no immunoreactivity except in one case, whereas, again with the exception of one case, the carcinoma cells in the primary tumors did show VLA-2 expression. With regard to VLA-5, there was no difference in its expression in the invasive components and the intraductal components. These findings suggest that the loss of VLA-2 plays a role in the invasion and metastasis of breast carcinoma.

Key words: Breast carcinoma — Invasion — Collagen receptor — VLA-2 — VLA-5

In recent years many studies have been made concerning the mechanism of the invasion of carcinoma cells *in vivo* and *in vitro*. Although these studies have indicated that adhesion molecules such as the cadherin family,[1] immunoglobulin superfamily, integrin family, selectin family and so on,[2] metalloproteinases such as type IV collagenase, some cathepsins and so on[3,4] and motility factors including various cytokines[5] possibly play a role in invasion, the true mechanism has not yet been ascertained.

Of the above molecules, integrins are known to be located in the plasma membrane of various cells and to play a role in the interaction between cells and the extracellular matrices. There have been some reports suggesting that these integrins may contribute to the differentiation of carcinoma cells or to the grade of differentiation of carcinomas in the breast,[6–9] colon[10,11] and skin.[12] We have shown that the loss of VLA-6 plays a role in the invasion of breast carcinoma, and that VLA-6 laminin receptor and laminin may contribute to tubular differentiation in breast carcinoma cells.

In the present study, the expressions of VLA-2 and VLA-5 were immunohistochemically examined in normal, benign breast lesions and carcinomas, and particular cases were also examined by Western-blot analysis. It is known that VLA-2 with a molecular weight of 145 kilodaltons (kD) can adhere to both collagen[13–15] and laminin,[16] and that VLA-5 with a molecular weight of 125 kD is one of the fibronectin receptors.[17,18] Both epithelial and myoepithelial cells showed immunoreactivity to an anti-VLA-2 antibody (12F1), but other tissues, including the basal cells of the epidermis, ciliated columnar and alveolar epithelial cells of the lung, alveolar and ductal epithelial cells of the salivary gland, columnar epithelial cells of the intestines, tubular epithelial cells of the kidney, transitional epithelial cells of urinary bladder, Schwann cells, astrocytes, endothelial cells and fibroblasts of human tissue, were positive.[19,20] On the other hand, positive immunoreactivity for VLA-5 has been noted in the thymocytes,[21] human placenta,[22] human fibroblasts, fibrosarcoma cells,[23] osteosarcoma cells[24,25] and other culture cells[26] but immunoreactivity in the mammary epithelial cells seems to be very weak.[6,8]

To evaluate the roles of VLA-2 collagen receptor and VLA-5 fibronectin receptor in invasive growth, we were particularly interested in the immunohistochemical difference of their localization in the invasive component and in the intraductal component of breast carcinoma, and we tried to detect both VLA-2 and VLA-5 by Western-blot analysis in some cases.

MATERIALS AND METHODS

Tissues Breast carcinoma tissues surgically removed from 77 female patients were obtained, as well as 25 breast tissues with benign lesion, including fibroadenoma (13 cases), intraductal papilloma (1 case), phyllodes tumor (1 case), and fibrocystic disease (7 cases). Normal breast tissues from three females were also evaluated as controls. The tissues were freshly frozen in OCT com-

pound (Miles Laboratories, Elkhart, IN) at −20°C. Serial frozen sections, of 5 μm thickness, were subsequently placed on neoprene-coated glass slides and stored at −80°C until use.

Immunohistochemistry The following antibodies were used for immunohistochemical staining: mouse IgG monoclonal antibody for α2 subunit of CDw49b VLA-2 (Immunotech S. A., Marseilles, France), rabbit polyclonal antibody for α5 subunit of CDw49e VLA-5 (Chemicon, Temecula, USA) and mouse IgG monoclonal antibody for type IV collagen (DAKO, Glostrup, Denmark).

Prior to immunohistochemical staining, the sections were fixed in acetone at −20°C for 5 min. The avidin-biotinylated-peroxidase complex (ABC) method was employed,[27] using a Histofine SAB-PO kit (Nichirei, Tokyo). In order to inhibit endogenous peroxidase activity, the sections were incubated at room temperature for 5 min in 0.01 mol/liter phosphate-buffered saline (PBS) containing 0.1% hydrogen peroxide. The incubation with antibodies was performed at 37°C for six hours within the incubator (Ikemoto Co., Tokyo), and the specimens were rinsed for 10 min with PBS. The sections were counterstained with 2% methyl green.

Western-blot analysis Western blotting was carried out as described previously.[28,29] Protein samples (50 μg) were subjected to 7.5% polyacrylamide gel electrophoresis followed by electrotransfer onto nitrocellulose filters. The antibodies used were the same as those used for immunohistochemistry. For detection of the immunocomplex, the ECL Western blotting detection system (Amersham, Aylesbury, UK) was used.

RESULTS

Normal, benign tumor tissues and fibrocystic disease (Table I) In the normal breast tissue, the lateral and basal plasma membranes of the epithelial and myoepithelial cells in the ducts and acini were positive for immunoreactivity for VLA-2. The myoepithelial cells were more strongly positive than the ductal and acinar epithelial cells.

The lateral and basal plasma membranes of the epithelial and myoepithelial cells in the benign tumor tissue (Fig. 1) were strongly positive for immunoreactivity for

Fig. 1. Myoepithelial cells of fibroadenoma of breast showed strong immunoreactivity for VLA-2. The intensity in the tubular epithelial cells was weaker than in the myoepithelial cells (immunohistochemical stain; original magnification ×100).

Table I. Expressions of VLA-2 and VLA-5 in Normal Breast and Benign Lesions

Lesions	Total no. of lesions	No. of lesions with VLA-2 (%)	No. of lesions with VLA-5 (%)
Normal breast tissue	3	3 (100)	3 (100)
Fibroadenoma	13	13 (100)	9 (69)
Intraductal papilloma	1	1 (100)	0 (0)
Phyllodes tumor	1	1 (100)	1 (100)
Fibrocystic disease	7	7 (100)	3 (43)
Epitheliosis	4	4 (100)	3 (100)
Blunt duct adenosis	5	5 (100)	0 (0)
Microcyst	1	1 (100)	0 (0)
Apocrine metaplasia	3	3 (100)	3 (100)

VLA-2. In the case of fibrocystic disease, every single epithelial layer in the blunt duct adenosis, microcyst, apocrine metaplasia and hyperplastic epithelial layer in the epitheliosis showed weak immunoreactivity for VLA-2. In these lesions the myoepithelial cells were more strongly positive than the epithelial cells.

With regard to VLA-5, the plasma membranes of the epithelial and of the myoepithelial cells in the ducts and acini of the normal breast tissue were weakly positive for immunoreactivity for VLA-5. In other lesions, a weak positive immunoreactivity for VLA-5 was noted in the plasma membrane of the epithelial layer and myoepithelial cells with various frequencies.

Table II. Comparison of Expression of VLA-2 between Intraductal and Invasive Components of Breast Carcinoma

	Total no. of lesions	No. of lesions with VLA-2 (%)		
		(−)	(+)	(++)
Intraductal component	36	4 (11)	20 (56)	12 (33)
Invasive component	67	31 (46)	30 (45)	6 (9)

$P<0.01$.

Breast cancer tissues Immunohistochemical results were evaluated as follows: when no immunoreactivity in any epithelial cell was noted, the outcome was regarded as negative (−), and when an intermediate intensity of staining between negativity and strong positivity was noted, it was regarded as weakly positive (+), while when a strong intensity of staining similar to that in the myoepithelial cells of normal breast tissue was noted, it was regarded as strongly positive (++).

(1) Correlation between VLA-2 and invasion of carcinoma (Table II): Each of the cases examined in frozen section contained an invasive component and/or an intraductal component of carcinoma and, therefore, the

Fig. 2. Intraductal carcinoma cells, particularly basal membrane of the cells, of breast carcinoma showed strong immunoreactivity for VLA-2 (immunohistochemical stain; original magnification ×100).

Fig. 3. The trabeculae of carcinoma cells in the invasive component of breast carcinoma showed weak immunoreactivity for VLA-2 (immunohistochemical stain; original magnification ×100).

lesions were broadly divided into 36 intraductal components and 67 invasive components of carcinoma, independent of the histological type of carcinoma. In a comparison between these two types of component, 32 of 36 intraductal components (89%) were positive for VLA-2, with 12 intraductal components showing strong positivity (Fig. 2), while 31 of 67 invasive components (46%) were negative for VLA-2 (Fig. 3), and only 6 lesions showed strong positivity. A statistically significant difference (chi square: $P<0.01$) was found between the loss of VLA-2 and the invasion of carcinomas.

In a total of 27 cases, both intraductal and invasive components were noted together in one frozen section. Table III summarizes the correlation of the expression of VLA-2 between the intraductal and invasive component in these cases. The intensity of immunoreactivity for VLA-2 in the intraductal components was the same as or stronger than that in the invasive components. No case showing a stronger immunoreactivity for VLA-2 in the intraductal than in the invasive components was noted.

Table III. Comparison of Expression of VLA-2 between Intraductal and Invasive Components in One Section of 27 Cases

VLA-2 in the intraductal component	Total no. of cases	No. of cases with VLA-2 in the invasive component (%)		
		(−)	(+)	(++)
(−)	4	4 (15)	0 (0)	0 (0)
(+)	13	9 (33)	4 (15)	0 (0)
(++)	10	1 (4)	6 (22)	3 (11)

(2) Correlation between VLA-2 and histological types, differentiation of carcinoma, lymph node metastasis and expression of type IV collagen (Table IV): The histological types of carcinoma were evaluated in representative specimens of the tumor, fixed with formalin and stained with hematoxylin and eosin. Fifty-five of 77 examined cases of breast carcinoma (71%) showed positive immunoreactivity for VLA-2. There was no correlation between the expression of VLA-2 in the carcinoma cells and the carcinoma's histological type.

The grade of differentiation in the invasive component of 67 carcinomas was evaluated according to the criterion of Bloom and Richardson,[30] and they were divided into three categories: low, moderate and high. The expression of VLA-2 in the carcinoma cells was not correlated to the grade of differentiation.

Fifty-six cases with an invasive component could be evaluated with regard to a correlation between the expression of VLA-2 and lymph node status. The cases associated with lymph node metastasis seemed to show a trend toward a more frequent expression of VLA-2 in the invasive component of the carcinoma than the cases not associated with lymph node metastasis. However, no statistically significant correlation between them was noted.

The correlation between expression of VLA-2 in the invasive component and of type IV collagen around the carcinoma cell nests could be examined in 63 cases. Immunoreactivity for type IV collagen around the carci-

Table IV. Correlation between Expression of VLA-2 in Carcinoma Cells and Histological Type of Carcinoma, Differentiation of Carcinomas, Lymph Node Metastasis, Expression of Type IV Collagen around Carcinoma Cell Nests

	No. of examined cases	No. of cases with VLA-2 (%)	
		(−)	(+)
Histological type			
Intraductal carcinoma	5	1 (20)	4 (80)
Invasive ductal carcinoma	64	18 (28)	46 (72)
Mucinous carcinoma	2	1 (50)	1 (50)
Medullary carcinoma	1	0 (0)	1 (100)
Invasive lobular carcinoma	5	2 (40)	3 (60)
Grade of differentiation			
Low	51	24 (47)	27 (53)
Moderate	10	4 (40)	6 (60)
High	6	3 (50)	3 (50)
Lymph node metastasis			
(−)	33	17 (52)	16 (48)
(+)	23	8 (35)	15 (65)
Type IV collagen			
(−)	16	8 (50)	8 (50)
(+)	47	21 (45)	26 (55)

Fig. 4. Strong but fragmented expression of type IV collagen was noted along carcinoma cell nests and small rounded expression of it in basement membrane of capillary (small arrow) in the invasive component (immunohistochemical stain; original magnification ×100).

noma cell nests was noted in 47 (75%) of 63 invasive components (Fig. 4). No statistically significant difference between VLA-2 and type IV collagen expression was noted.

(3) Comparison of VLA-2 expression in primary carcinoma and lymph node metastasis (Table V): The expression of VLA-2 in both the primary carcinoma and its lymph node metastasis could be evaluated in 6 cases. The histological type in all 6 cases was invasive ductal carcinoma but the frozen section of one case (No. 88) did not include an invasive component because its histological type was invasive ductal carcinoma with a predominant intraductal component (WHO classification). No positive immunoreactivity for VLA-2 was noted in any of the cases except one in which a weak expression was seen in the cytoplasm of the carcinoma cells of the lymph node. The expression of VLA-2 in the primary carcinoma was

Table V. Comparison of VLA-2 Expression in 6 Primary Carcinomas and Their Accompanying Lymph Node Metastases

Case no.	Age	Histological type	VLA-2		
			IN	ST	LN
33	50	IDC	N	+	−
37	69	IDC	N	−	−
78	78	IDC	N	+	−
53	69	IDC	N	++	C+/−
74	41	IDC	N	+	−
88	45	IDC	+	N	−

Abbreviations: IDC, invasive ductal carcinoma; IN, intraductal component; ST, stromal invasive component; LN, lymph node; N, the component was not included; C, cytoplasm.

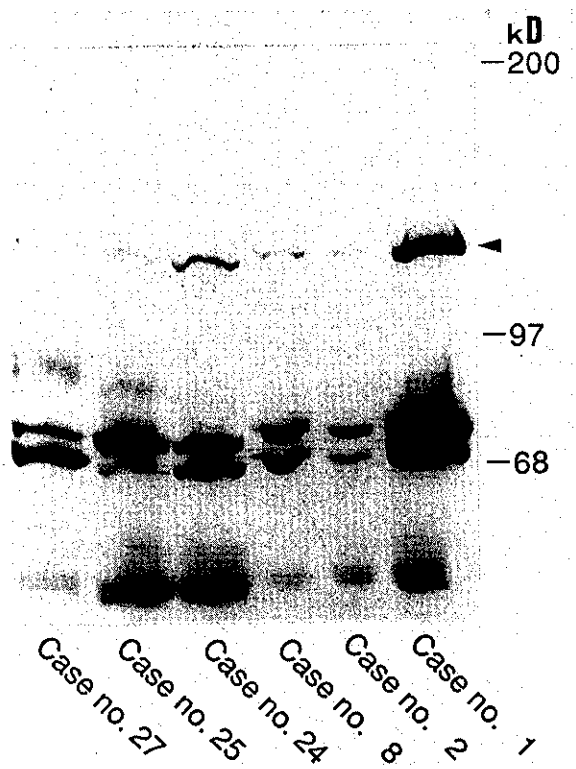

Fig. 5. Expression of VLA-2 in human breast carcinoma tissues by Western blotting. Case no. 1 showed a dense and wide band. Case no. 24 showed a weak and thin band. Cases no. 2, 8 and 25 each showed a fine and fragmented band. Case no. 27 showed no band.

stronger than that in the lymph node with metastasis except in one case, in which VLA-2 expression was noted in neither the intraductal nor invasive components.

(4) Correlation between VLA-5 and invasion of carcinoma, histological type, differentiation of carcinoma,

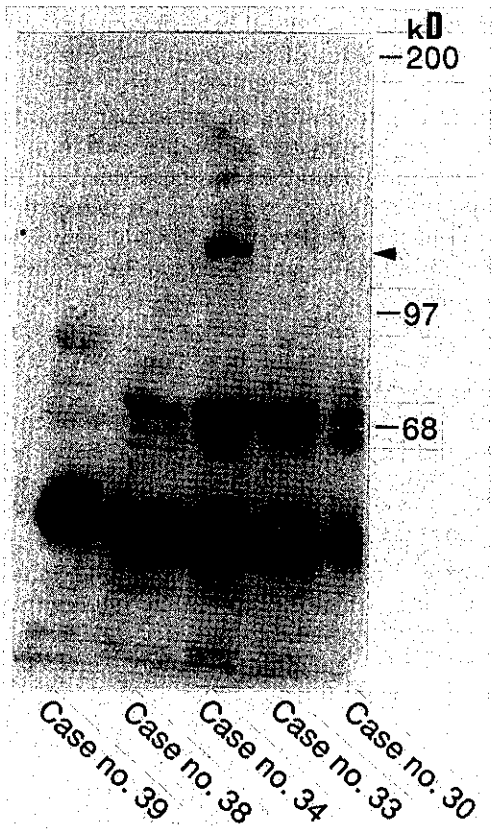

Fig. 6. Expression of VLA-5 in human breast carcinoma tissues by Western blotting. Case no. 34 showed a dense but thin band. Other cases showed no band.

lymph node metastasis: There was weak positive immunoreactivity for VLA-5 in the plasma membrane of carcinoma cells in both the intraductal and invasive components. No correlation among VLA-5 expression, invasion of carcinoma, histological type, differentiation of carcinoma and lymph node metastasis was noted.

(5) Confirmation of expression of both VLA-2 and VLA-5 by Western-blot analysis: Twenty-eight cases of frozen carcinoma tissue could be evaluated by Western-blot analysis. Slight to dense bands consistent with VLA-2 were noted in 25 (89%) of 28 cases (Fig. 5), and slight to intermediate bands consistent with VLA-5 were observed in 13 (48%) of 27 cases (Fig. 6).

DISCUSSION

The observation in the present study that the myoepithelial cells in normal ducts and acini showed a strong expression of VLA-2 collagen receptor may indicate an anchoring of these cells by the collagen receptor to type IV collagen which is one of the components of the basement membrane.[31,32] In benign tumors and fibrocystic disease, the myoepithelial cells retained VLA-2 collagen receptor, which presumably provided structural stability. On the contrary, loss of VLA-2 collagen receptor in some of the carcinoma cells in the invasive component may imply decreased structural stability and increased invasive potency. We have confirmed that VLA-6 laminin receptor showed a similar pattern of expression to that of VLA-2, so the expression of both VLA-2 and VLA-6 may contribute cooperatively to structural stability.

Various changes of expression of VLA-2 have been observed in breast carcinoma as well as colon carcinoma and skin carcinoma.[6–12] For example, a decrease in expression not only of VLA-2 but also of VLA-3, VLA-5 and vitronectin receptor were noted in poorly differentiated adenocarcinoma of the breast,[6,7] and the expression of VLA-2 in the cytoplasm of carcinoma cells was correlated to the loss of tubular structures, which meant a lower grade of differentiation of carcinomas, in comparison with its expression in the membrane of carcinoma cells.[9] These differences from the results of the present report may be due to different methods of evaluation, namely, in previous reports VLA-2 expression was evaluated without consideration of its location (in the invasive component or the intraductal component), but in the present study its expression in the invasive component was compared with clinicopathological factors. On the other hand, some reports[8,9] have indicated that VLA-2 expression in the primary carcinoma does not show any correlation with either the histological grade or lymph node metastasis. These results seem to be in accord with that of the present report, namely, the expression of VLA-2 was not correlated to the histological type, the grade of differentiation in the invasive components or lymph node metastasis.

With regard to colon carcinoma, it has been pointed out that low expression of VLA-2 as well as VLA-3 in carcinoma cells is correlated to poor differentiation,[10] lymph node metastasis and Duke's grade.[11] The difference may be due to organ specificity, but the exact reason is not known and further investigation is necessary.

As stated in the previous section, the carcinoma cells in the lymph nodes of metastatic foci showed no expression of VLA-2 except in one case (in this case the VLA-2 expressed in the cytoplasm of carcinoma cells may partly or completely lack binding activity to collagen). Loss of VLA-2 in the metastatic carcinoma cells involving the lymph nodes may be a result of metastatic growth at other sites. But it is speculated that the carcinoma cells with no VLA-2 expression have a higher metastatic potency and that the loss of it may play a direct and/or indirect role in metastasis to the lymph node.

In a previous report[8] it was pointed out that some carcinoma cells expressed VLA-6 laminin receptor in the invasive component of breast carcinoma, and that the expression of VLA-6 laminin receptor was concordant with the production of laminin in breast carcinoma cells, while, in the present study, no correlation was found between the expression of VLA-2 collagen receptor of carcinoma cells in the invasive component and that of type IV collagen around carcinoma cell nests. These results suggest that the expression of both VLA-2 on carcinoma cells in the invasive components and of type IV collagen around carcinoma cell nests may be independently regulated and that the interaction between VLA-2 collagen receptor and type IV collagen may be different in function from that between VLA-6 laminin receptor and laminin.

With regard to VLA-5 fibronectin receptor, only a small amount of VLA-5 was seen in carcinoma cells *in vivo*. Previous reports noted a weak expression of VLA-5 in breast carcinoma cells,[6,8] and this decreased expression was correlated to poor differentiation of carcinoma.[6] Furthermore, it is indicated that transformed cultured cells show both a reduction of VLA-5 synthesis and an altered localization of VLA-5.[23,33] With regard to its ligand, previous reports noted a marked expression of fibronectin in the stroma of the invasive component of breast carcinomas[34,35] and we have confirmed this (data not shown). These results indicate that VLA-5 expressed in the carcinoma cells of the breast may have no significant effect on the biological behavior of the breast carcinoma, though a lot of fibronectin is expressed in the stroma.

Furthermore, to confirm the molecular weights of the epitopes recognized by the antibodies used in the present study, Western-blot analysis was performed. In the present study, the molecular weight of the detected band of VLA-2 seemed to be less than 145 kD, which was the expected molecular weight. So, it can be speculated that an incomplete VLA-2 molecule was synthesized or that denaturing of the molecule took place during the procedure. On the other hand, a band consistent with VLA-5 could also be detected.

On the basis of the results in the present study, VLA-2 collagen receptor might be correlated to breast carcinoma invasion out of the ducts, and the decreased expression of VLA-2 as well as VLA-6 might be one of the changes of the cellular membrane involved in the process of invasion. However, there are so many adhesion molecules, which have multiple binding capacities to the extracellular matrices or other cells, expressed on carcinoma cells that these adhesion molecules may cooperate in their functions. So further investigation, not only of the relation between the various cell adhesion molecules and their ligands, but also of the change of cytoskeleton[36-40] and the induction of metalloproteinases,[41] which seem to be regulated by the binding of the receptors to their ligands, should be undertaken.

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