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Expression of the VLA β1 Integrin Family in Bladder Cancer

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Integrins are a family of transmembrane heterodimers, many of which function as receptors for extracellular matrix molecules and play a role in adherence to and motility on matrix components. Because of these functions, integrins are suspected of participating in metastatic processes. We investigated the expression of β1 integrins in human bladder cancer cell lines and tissues. Expression of β1 integrins on cultured bladder cancer cell lines was evaluated by flow cytometry, of 8 cell lines tested, α1 was found in 4, α2 and α3 in all 8, α4 in 1, and α5 in 3. These results were in sharp contrast to the expression detected by immunostaining tissues containing normal urothelium and low stage (noninvasive) and high stage (invasive) bladder cancers. All normal urothelial tissues tested expressed α2 and α3 and none expressed α1, α4, or α5. Similarly, a majority (77%) of low stage (noninvasive) bladder cancers stained positively for α3, whereas only 6 of 13 expressed α2 and none expressed α1, α4, or α5. Among invasive bladder cancers, α1 was detected in 7%, α2 in 24%, α3 in 68%, α5 in 10%, and α4 was not found in any samples. These results indicate that integrin expression in cultured human bladder cancer cell lines does not represent expression observed in tissue samples and may reflect adaption to or selection during tissue culture conditions. A progressive loss of α2 expression is seen from normal urothelial cells through invasive bladder cancers. This loss may contribute to an invasive phenotype by a loss of the cell-cell adherence function mediated by the α2β1 and α3β1 integrins. (Am J Pathol 1994, 144:1016–1022)

Bladder cancer arises from the urothelial cells lining the urinary tract. Bladder cancer occurs essentially as two forms: a low stage, noninvasive type that is most common and prone to recurrence and a high stage invasive type that is frequently associated with metastasis.[1] The study of bladder cancers therefore provides the opportunity to evaluate the biological differences between the two functionally different cancers arising from the same precursor cells.

Integrins are a family of transmembrane heterodimers, many of which function as receptors for extracellular matrix molecules.[2,3] The VLA, or β1 integrin family, includes heterodimers composed of the β1 subunit associated with the α1, α2, α3, α4, α5, αv, α6, and recently described α7 and α8 subunits, although the α6 and αv subunits can also be found in association with other β subunits.[3] The β1 integrin family is found on a wide variety of tissues and includes the classical receptors for the extracellular matrix components laminin, collagen IV, and fibronectin and function in adherence to and motility on these matrix components. Because of these functions, integrins are suspected of participating in metastatic processes.[2,3]

We have previously shown in normal and cancerous bladder tissue that the α6 subunit of the integrin family is expressed exclusively in association with the β4 subunit and that the α6β4 integrin is overexpressed in the majority of the bladder cancer tissue specimens tested, showing the strongest expression in invasive bladder cancers.[4] Because of these findings of altered expression of one type of integrin in bladder cancers, we studied the expression of members of the β1 family on bladder cancer cell lines and normal urothelium and cancerous tissue sections and here report our observations.

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Materials and Methods

Tissue Culture

The cell lines used were RT4, J82, 253J, and T24, frequently studied human bladder cancer cell lines,[5] and UM-UC-2, UM-UC-3, UM-UC-9, and UM-UC-13, which are cell lines established in this laboratory.[4,6–8] All cells were maintained in minimal essential medium supplemented with 2% fetal calf serum and 8% newborn calf serum.

Antibodies

All antibodies used were mouse monoclonal antibodies. TS1/7, anti-α1, and anti-β1 were obtained from T Cell Diagnostics, Cambridge, MA; anti-α2 and anti-α4 were obtained from AMAC, Westbrook, ME; J143, anti-α3 was obtained from Signet, Dedham, MA; and anti-α5 and anti-αv were obtained from Telios, San Diego, CA. Included as a positive control in the analysis was BQ16, anti-α6, prepared in our laboratory,[4] and MOPC-21, a mouse IgG1 myeloma protein obtained from Cappel Organon Teknika, Durham, NC, was used as a negative control.

Flow Cytometry

Flow cytometry was performed as previously described.[4] Briefly, the cultured cells were trypsinized, washed, and incubated with appropriately diluted primary antibodies for 1 hour on ice. Primary antibodies included MOPC-21 as a negative control and BQ16, anti-α6, as a positive control.[4] Then the cells were washed twice with phosphate-buffered saline containing 5% fetal calf serum (FBS/PBS). The cells were resuspended in secondary antibody. Because all the primary antibodies were mouse monoclonal antibodies, fluorescein isothiocyanate-conjugated goat anti-mouse IgG, A, and M (Cappel Organon Teknika) was used. The cells were incubated with secondary antibody for 1 hour on ice in the dark, washed twice with FBS/PBS, and evaluated by flow cytometry (Coulter Epics C, Coulter Diagnostics, Hialeah, FL).

Tissue Staining

Tissue staining by immunoperoxidase and immunofluorescence techniques was performed as previously described.[4] Briefly, frozen tissue sections were fixed for 5 minutes in ice-cold acetone, allowed to air dry, rehydrated in PBS, and blocked for 30 minutes in blocking serum (10% horse serum in PBS). The sections were blotted and incubated for 1 hour in appropriately diluted primary antibody. Immunoperoxidase staining was performed by the avidin-biotin-peroxidase technique using a commercially available kit (Vectastain, Vector Laboratories, Inc., Burlingame, CA). The sections were washed in PBS and incubated for 30 minutes with secondary biotinylated horse anti-mouse antibody. The sections were washed in PBS then endogenous peroxidase was inactivated by incubation for 30 minutes in 0.3% peroxide in methanol. After washing the sections in PBS, the sections were incubated for 1 hour with avidin-biotin-peroxidase complex. The sections were washed in PBS and the peroxidase reaction was developed with diaminobenzidine containing 0.03% peroxide for 5 minutes. The sections were then rinsed in water and counterstained with hematoxylin and microscopically evaluated. For immunofluorescence staining, after fixation, rehydration, blocking, and incubation with primary antibody, the sections were washed in PBS and incubated for 1 hour in the dark with fluorescein isothiocyanate goat anti-mouse IgG, A, and M (Cappel Organon Teknika). After washing in PBS, the sections were mounted in glycerine and evaluated by fluorescence microscopy.

Results

Preliminary studies were performed with antibodies to the α1, α2, α3, α4, α5, and αv subunits. No staining with antibody to the αv subunit was detected on either human bladder cancer cell lines (8 tested) or tissue sections (11 specimens tested) in the preliminary study, therefore αv was excluded from further evaluation. Frequently, the staining for the α6 subunit has been included in other studies as indicating the expression of VLA-6 or α6β1, whereas we have clearly shown that α6 staining in normal urothelium and bladder cancer tissues is completely associated with β4 expression.[4] Furthermore, studies with cultured cells indicate that α6 preferentially associates with β4 when the β4 chain is present.[9,10] Therefore, we did not include studies with α6 in this report. Antibodies to the α7 and α8 subunits were not available for evaluation.

Integrin expression on cultured human bladder cancer cell lines was examined by flow cytometry, with the results shown in Figure 1. Of the eight cell lines examined, α1 was detected in 4, α2 and α3 in all 8, α5 in 7, and α4 in only 1.

The pattern of expression of the α subunits of the β1 integrin family on human bladder cancer cell lines is in contrast to the expression detected on tissues, as summarized in Table 1. In tissues, normal urothelial cells did not express α1, α4, or α5, whereas showing

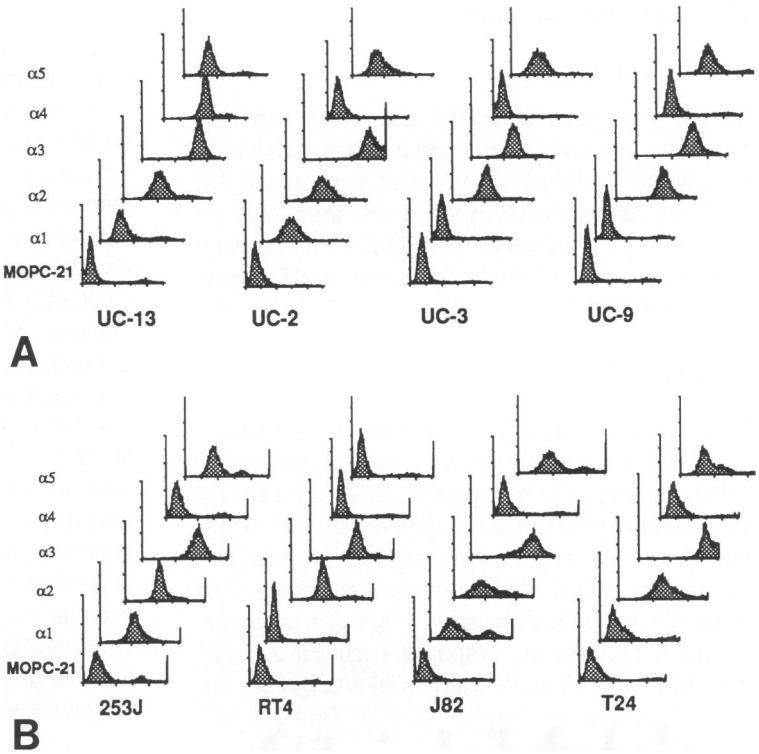

Figure 1. *Flow cytometry of cultured human bladder cancer cell lines. The x-axis is the channel number (log relative fluorescence intensity); on the right (y-axis) is cell number. Thus, the greater number of positive cells results in a shift of the cell peak to the right. Antibodies to the different α-subunits are indicated on the left; MOPC-21 is a negative control. The cell line tested is indicated at the bottom of the column.*

Table 1. *Integrin Expression in Normal and Neoplastic Bladder Tissues*

	Tissue		
Integrin	Normal*	Low Stage (Ta/T1)	High Stage (T2-T4)
α1	0/12 (0)	0/6 (0)	2/28 (7)
α2	13/13 (100)	6/13 (46)	8/34 (24)
α3	12/12 (100)	10/13 (77)	19/28 (68)
α4	0/12 (0)	0/11 (0)	0/25 (0)
α5	0/12 (0)	0/12 (0)	3/31 (10)

* First figure is the number positive/number tested; figure in parentheses is the percentage positive.

strong staining for α2 and α3. Representative staining of ureter specimens is shown in Figure 2. Similar staining was observed in normal bladder specimens. The α1 reactivity seen in tissues was limited to the muscle fibers below the lamina propria. Expression of α2 and α3 appeared to be limited to the more basal layers of cells, with the most superficial layers being less positive. Furthermore, staining in normal urothelial cells with α2 and α3 is strong at the cell borders. Low stage (Ta/T1) bladder cancer tissue specimens showed expression of α3 (77%) and α2 (46%), whereas none expressed α1, α4, or α5. Most invasive (T2/T4) bladder cancer tissue specimens stained strongly for α3 (19 of 28), whereas fewer showed expression of α2 (3 of 21), α1 (2 of 28), or α5 (3/31) and none expressed α4. Representative staining is shown in Figure 3. The staining for α2 and α3, when present in tumor tissues, appeared to be diffuse throughout the tumor.

Discussion

In this paper, we report the expression of the subunits of the β1 integrin family in human bladder cancer cell lines and on normal and neoplastic bladder tissues. Of special note is the lack of a relationship between the integrin expression on bladder cancer cell lines compared with the bladder cancer tissue specimens. All of the bladder cancer cell lines, with the exception of RT4, originated from invasive bladder cancers.[4–8] However, all of the bladder cancer cell lines show strong expression of α2, whereas bladder cancer specimens show progressive loss of α2 expression from normal bladder specimens, which are all α2 positive, through invasive bladder cancers in which only 24% are positive. Similarly, 7 of 8 of the human bladder cancer cell lines expressed α5 and 4 of 8 expressed α1, whereas none of the normal urothelial specimens expressed either subunit and only 10 and 7% of invasive bladder cancer tissue specimens expressed these integrin subunits, respectively. The overexpression of integrins in the cultured cell lines may reflect selection of subpopulations or adaptation to culture conditions, especially to culture as adher-

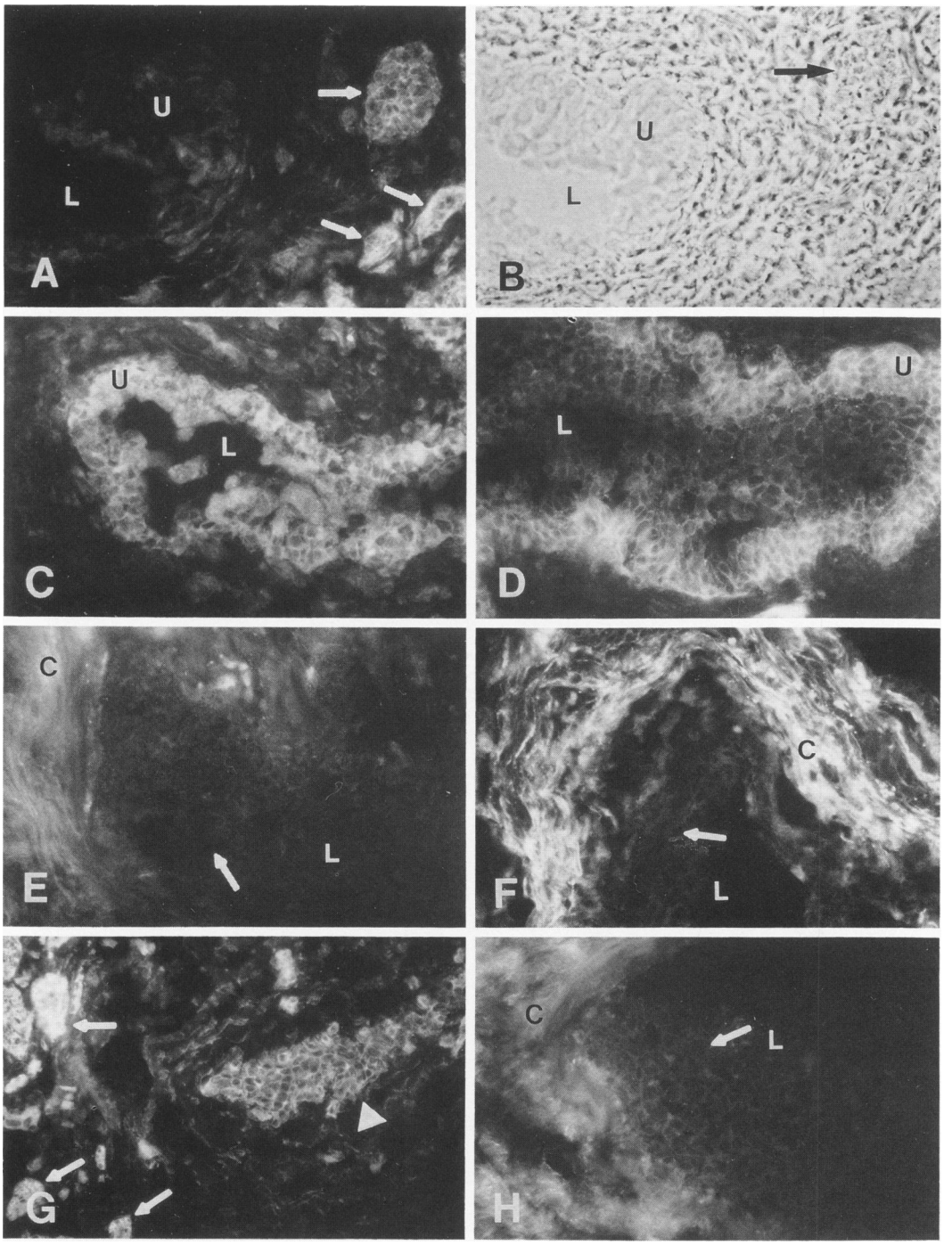

Figure 2. A *and* B: *Fluorescence staining of normal ureter with anti-α1 shown as fluorescence* (A) *and as phase contrast* (B) *for comparison* (×200). *Only the muscle fibers show staining, as indicated by the* white arrows *in* A *and the* black arrow in B. *L indicates the lumen and U marks the urothelial cells that are not stained with anti-α1.* C: *Fluorescence staining of normal ureter for α2* (×160). *L indicates the lumen and U marks the urothelial cells that are strongly stained.* D: *Fluorescence staining of normal ureter for α3* (×160). *L indicates the lumen and U marks the urothelial cells that are strongly stained.* E: *Fluorescence staining for α4* (×200). *L indicates the lumen and C marks the positively stained connective tissue. The* white arrow *indicates the location of the urothelial cells that are not stained.* F: *Fluorescence staining for α5* (×200) *showing only staining of the connective tissue in the lamina propria. L indicates the lumen and C marks the positively stained connective tissue. The* white arrow *indicates the location of the urothelial cells that are not stained.* G: *Fluorescence staining for β1* (×160) *showing staining of urothelial cells* (*marked with the large* arrowhead) *and the submucosal muscle fibers* (*marked with the* arrows). H: *Fluorescence staining with MOPC-21, a negative control* (×200). *The lumen is labeled with L and the connective tissue in the lamina propria showing some background staining is marked with C. The* arrow *indicates the location of the urothelial cells that are not stained.*

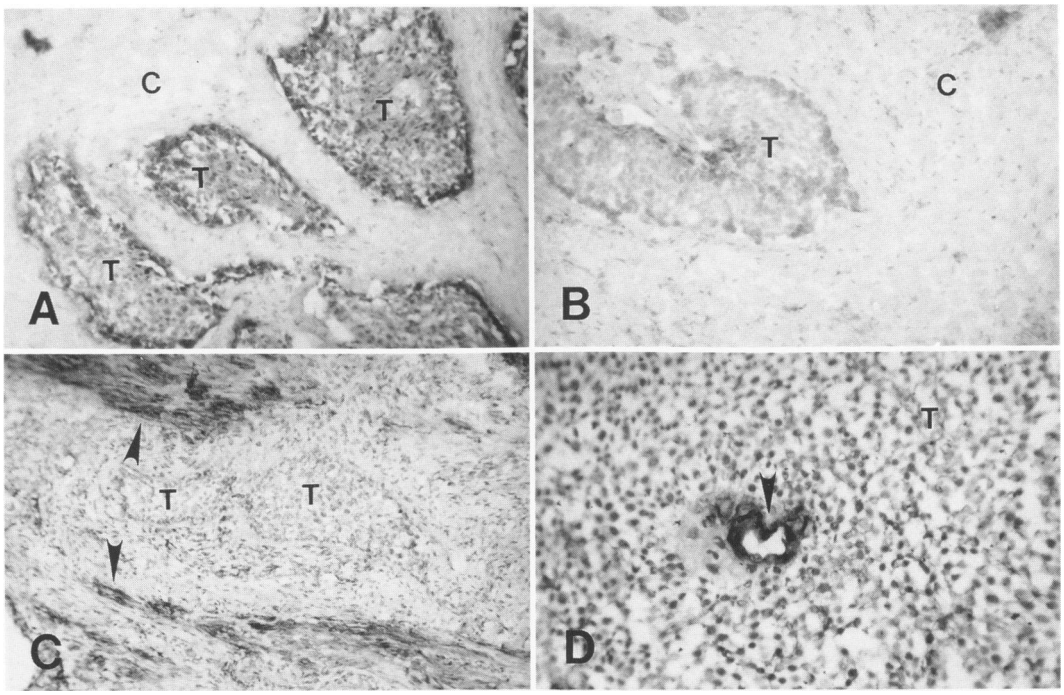

Figure 3. **A** *and* **B**: *Invasive bladder tumor specimen stained with anti-α3* (**A**) *or with negative control MOPC-21* (**B**: *both ×80*). *The tumor islands are marked with T and the surrounding connective tissue is marked with a C.* **C**: *Invasive bladder tumor specimen stained with anti-α5 showing only stain of connective tissue* (*marked with the* **arrowheads**) *and not of the tumor* (*labeled with T; ×64*). **D**: *Staining of low stage* (*noninvasive*) *bladder cancer specimen with anti-α1 showing only staining of the blood vessel* (*marked with an* **arrowhead**) *and not of the surrounding cancer cells* (*marked with T; ×160*). *Similar staining of blood vessels but not of cancer cells was seen with anti-α4.*

ent cells, as previously reported for the expression of $\alpha3\beta1$.[11] Equally clear from these data is the fact that the integrin expression on these cell lines does not reflect or represent the integrin expression in either normal or cancerous tissues. These results call into question numerous studies on integrin expression that have been performed solely on cultured cell lines. For example, studies of Bacillus Calmette-Guerin, a highly effective treatment for superficial bladder cancer, have been performed using the bladder cancer cell line T24. Internalization of the bacteria was shown to be due to the $\alpha5\beta1$ integrin.[12] As we have shown, neither normal urothelial cells nor low stage bladder cancer in tissues express the $\alpha5\beta1$ integrin, therefore it is unlikely $\alpha5\beta1$ functions in this manner in patients with low stage bladder cancer treated with Bacillus Calmette-Guerin, unless the inflammation associated with the introduction of live bacteria into the bladder causes the induction of $\alpha5$ expression. Other integrins (such as the αv family) with fibronectin-binding activity may be involved as well.

The other striking finding in this report is the progressive loss of $\alpha2\beta1$ expression ranging from expression on all normal samples to 46% of low stage cancers and only 24% of high stage bladder cancers. The underlying genetic alteration resulting in loss of $\alpha2$ expression in bladder cancer requires further investigation. Several recent reports have indicated that $\alpha2\beta1$ and $\alpha3\beta1$ bind to each other, functioning as cell-cell adhesion molecules in normal epithelial tissues.[13–15] The localization of $\alpha2$ and $\alpha3$ staining at the cell borders in normal urothelial tissues suggest that these integrins also function as cell-cell adhesion molecules in this tissue. In this respect, $\alpha3\beta1$ and $\alpha2\beta1$ may function similar to and together with molecules like E-cadherin in maintaining normal tissue architecture and contributing to cell-cell communication.[2] Loss of cell-cell adhesion via E-cadherin is known to be associated with increased invasion in bladder cancer.[16] The loss of $\alpha2$ expression may reduce cell-cell adherence and promote invasive potential. Furthermore, the progressive loss of $\alpha2\beta1$ expression with continuing high levels of $\alpha3\beta1$ expression may free the $\alpha3\beta1$ integrin from the cell contact function and allow participation of this integrin in migration and invasion. In support of this concept is the observation that the introduction of $\alpha2\beta1$ expression in a cell line that lacked both $\alpha2$ and $\alpha3$ expression resulted in greater metastatic potential in a mouse model when compared with the parental cell line.[17]

In a large survey of tumors, Miettinen et al[18] reported a difference between mesenchymal and epi-

thelial tumors. The mesenchymal tumors and normal tissues showed a stronger expression of α1 and α5, whereas both normal and cancerous epithelial tissues showed stronger expression of α2 and α3. However, many reports have indicated alterations in integrin expression in cancers compared with the normal tissue of origin. Two studies of breast cancer have observed a loss of α2 expression compared with normal tissue,[19,20] one study reported no change of α2 and α3 expression,[21] and a fourth study showed altered patterns of α2, α3, α5, and α6 (probably α6β1 because these authors demonstrated that β4 was not present) in infiltrating ductal carcinomas, mucinous cancers, and lobular cancers of the breast compared with the normal distribution.[22] A loss of α2 expression in ductal carcinomas and lobular carcinomas of the breast was reported in the latter study.[22]

Loss of α2 reactivity in advanced colon tumors has also been previously described in two studies,[23,24] one of these studies also found that α3 staining was lost in these tumors.[24] Neuroblastomas exhibit decreased α2 and α3 expression in the less differentiated forms.[25] Finally, loss of α2 and α3 subunits was reported in squamous cell carcinomas and was especially striking in tumors that were poorly differentiated.[26]

In contrast, other reports indicate increased expression of β1 integrins in some cancers. Increased staining for α2 and α6 was found in prostate cancer in one study,[27] increased expression of α3 was seen in pancreatic carcinomas,[28] increased levels of α2 and α3 were observed in basal cell carcinomas,[29] and increased expression of α6β1 was detected in renal cell carcinomas.[30] These reports support the concept that different tumor types show different patterns of integrin expression.[18] Several problems prevent the direct comparison of these studies: different antibodies and staining techniques were used, and, in general, reactivity with integrin antibodies is not preserved in formalin-fixed, paraffin-embedded materials, contributing to the difficulty in reproducing the studies. Nevertheless, these studies continue to suggest that alterations in integrin expression may play a biological role in tumors. Further studies with larger sample numbers and careful attention to pathological detail should clarify this role.

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