Expression of the Adherens Junction Protein Vinculin in Human Basal and Squamous Cell Tumors: Relationship to Invasiveness and Metastatic Potential

BEATRIZ LIFSCHITZ-MERCER, MD, BERNARD CZERNOBILSKY, MD, EDITH FELDBERG, MD, AND BENJAMIN GEIGER, PHD

The acquisition of an invasive or metastatic phenotype in malignant neoplasms is often correlated with reduced cellular adhesiveness. We investigated the expression of the adhesion-associated cytoplasmic protein, vinculin, in normal and neoplastic human squamous epithelia, as well as in metastases of squamous cell carcinomas, and correlated the results with invasiveness and metastatic potential. Tissue samples from various tumors were examined, including basal cell carcinomas (BCC), keratoacanthomas, and squamous cell carcinomas (SCC). In addition, lymph node metastases from nine of the SCC were tested in this study. Our results indicate that most BCC, keratoacanthomas, and in situ SCC display strong positive staining for vinculin. The level of immunolabeling for vinculin and its pattern of distribution in the low malignant, nonmetastasizing lesions was similar to those observed in normal squamous epithelia. In contrast, in SCC, which are invasive and possess metastatic potential, as well as in their metastases, vinculin labeling was negative or poor, irrespective of their degree of differentiation. In conclusion, poor vinculin labeling in tumors of squamous epithelial origin examined here appears to be related to the metastatic potential of the tumor. Vinculin immunostaining of primary tumors originating in stratified squamous epithelia may thus be of value in helping to determine the metastatic potential of these neoplasms. HUM PATHOL 28:1230–1236. Copyright © 1997 by W.B. Saunders Company

Key words: **Vinculin, cell adhesion, squamous, basal cell carcinoma.**

Abbreviations: **BCC, basal cell carcinoma; SCC, squamous cell carcinoma.**

The deterioration of cell-cell and cell-matrix adhesions, and the loss of contact responsiveness, are among the most common manifestations of malignant transformation.[1-7] These changes are often associated with the loss of tissue integrity, the acquisition of anaplastic morphology, and the invasion into blood vessels and neighboring tissues. In addition, transformed cells often fail to respond to adhesion-mediated signals affecting growth and differentiation. Most normal cells are known to be "anchorage dependent," namely, to require direct interaction with the extracellular matrix to proliferate or even to survive. Many malignant cells were shown to be anchorage independent and grow in suspension or in semi-solid medium.[8] They also fail to undergo growth arrest when reaching high density, as do normal cells, a phenomenon known as "density-dependent growth arrest" or "contact inhibition."

The recent progress in the characterization of the molecular mechanisms underlying cell adhesion has shed much light on possible molecular targets, the modulation of which might be involved in the loss of contact responsiveness and the acquisition of a "transformed phenotype." Several possible mechanisms may be responsible for the apparent downregulation of cell adhesion. One of them is a putative overphosphorylation of junctional molecules on tyrosyl residues, which apparently leads to the disruption of junctional structures. It has been shown, for example, that overexpression of $pp60^{v\text{-}src}$ in Rous Sarcoma virus–infected cells leads to phosphorylation of focal contacts, followed by their deterioration[9,10] and that cell-cell junctions and cell-matrix focal contacts are the primary targets for vanadate- or growth factor–stimulated tyrosine phosphorylation in epithelial and mesenchymal cells, respectively.[11]

An alternative mechanism that might be related to the reduced adhesiveness of cancer cells is the altered expression of different adhesion-associated proteins, including vinculin, α-actinin, cadherins, and catenins.[1,6,12] Notably, malignant transformation and, in particular, the acquisition of an invasive or metastatic phenotype were commonly associated with reduced cellular adhesiveness caused by low expression of these proteins.[4-7,13] It was proposed that these changes may be responsible for the higher tendency of the malignant cells to detach from the primary tumors, invade neighboring tissues, penetrate into blood vessel and lymphatics, and extravasate in target organs to form metastases.[4-7,14] Changes in the adhesive properties could also contribute to the reduced contact responsiveness characteristic of cancer cells.[15]

In the current study, we investigated the expression of the adherens junction protein, vinculin, in certain human malignant tumors. We show that normal squamous epithelia, as well as a variety of neoplasms arising in stratified squamous epithelium and lacking metastasizing potential, display high levels of vinculin labeling. Conversely, squamous tumors with metastatic potential exhibited considerably lower vinculin levels. Moreover, lymph node metastases derived from these tumors were typically devoid of vinculin labeling. On the basis of these results, we propose that the reduction in vinculin expression in human squamous carcinomas may lead

From the Department of Pathology, Sourasky Medical Center, Tel Aviv, Israel; the Department of Pathology, Kaplan Hospital, Rehovot, Israel; and the Department of Molecular Cell Biology, The Weizmann Institute of Science, Rehovot, Israel. Accepted for publication February 13, 1997.

Supported by a grant from the Minerva Foundation, Munich.

Address correspondence and reprint requests to Benjamin Geiger, PhD, Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot 76100, Israel.

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to the acquisition of a highly invasive and metastatic phenotype, highlighting the role of vinculin in maintaining normal cell adhesions and suggesting that its downregulation may play a central role in the acquisition of a "malignant phenotype."

MATERIALS AND METHODS

Tissues

Tissue samples used in this study were obtained from routine operative procedures, fixed in 4% buffered formaldehyde, embedded in paraffin, cut at 4 μm thickness, and stained with hematoxylin and eosin. Unstained slides were used for immunoperoxidase.

The tumors studied included 15 basal cell carcinomas (BCC), three of which were of the morphea type, one of the adenoid type, and one of the keratotic type, five keratoacanthomas, six in situ squamous cell carcinomas (SCC; Bowen's disease), and 15 invasive SCC of the skin as well as five cases of invasive SCC from the larynx, eight from the nasopharynx, and two from the upper esophagus. Five of the invasive SCC of the skin were well differentiated, six moderately, and the remaining four were poorly differentiated tumors. Three of the tumors from the upper respiratory and digestive tracts were well differentiated, seven moderately, and five were poorly differentiated. In addition, nine regional lymph nodes with metastases from laryngeal, nasopharyngeal, and esophageal SCC were examined. In two of them the primary tumors in the larynx and esophagus were among those studied here, and in the remaining seven cases, only the lymph node metastases were available.

Normal tissues studied here as controls included the normal skin surrounding the various skin tumors as well as normal mucosa at a distance from the laryngeal, nasopharyngeal, and esophageal carcinomas.

The staining intensity for vinculin was graded by at least three independent observers as undetectable (UD), weak (+), or strong (++). The strong staining of blood vessels, present in all of the tissues, served as an internal positive control. In addition, we distinguished between uniform labeling, namely, presence of labeling in almost all of the cells and focal labeling, present only in isolated groups of cells throughout the lesion. The subcellular distribution of the labeling was either diffuse throughout the cytoplasm or associated with the plasma membrane along cell-cell junctions.

Immunoperoxidase Labeling

Tissue sections were labeled for vinculin by the biotin-streptavidin method using monoclonal anti-vinculin antibodies (hVin Sigma Immunochemicals, St. Louis, MO) and the Zymed Streptavidin peroxidase kit.[16] Stained specimens were examined microscopically and the labeling intensity evaluated by at least three independent observers.

RESULTS

Immunoperoxidase Labeling for Vinculin of Normal Human Tissues

Immunoperoxidase labeling of a large variety of normal human tissues for vinculin showed intense labeling of essentially all squamous epithelia, smooth muscle, hair follicle epithelia, and myoepithelial cells. Labeling of simple epithelia, however, was weak and often undetectable. The labeling in stratified epithelia was particularly prominent in the suprabasal cells of the epidermis (Fig 1A, B), as well as in various nonkeratinizing squamous epithelia, such as in the esophagus. The staining was largely cytoplasmic and was often enriched at cell borders and intercellular bridges (Fig 1C, D). Prominent labeling was also noted in vascular smooth muscle (Fig 1A, B). In fact, the staining of blood vessels served as a most useful internal control for vinculin labeling. Strong vinculin labeling was also noted in myoepithelial cells associated with sweat glands (Fig 1E).

Immunoperoxidase Labeling for Vinculin of Human Tumors

In the BCC samples, positive staining for vinculin was observed. In 12 of 15 tumors tested, the intensity of labeling was strong, and in three cases, moderate (Fig 2A, Table 1). Of the intensely labeled samples, eight, including those classified as morphea-type BCC (Fig 2B), displayed uniform staining, whereas in four cases the staining was nonuniform, with focal intense labeling. Often, intensely labeled mesenchymal cells were detected in the stroma around the tumor (Fig 2A).

Keratoacanthomas displayed an intense and largely uniform labeling in all five cases studied (Fig 2C, D, and Table 1). Again, positively labeled stromal cells were commonly detected around the tumors.

SCC samples, however, showed highly variable levels and patterns of vinculin labeling. In five of six cases of in situ SCC (Bowen's disease) (Fig 3A), intensive labeling was observed. However, only two cases showed uniform labeling, whereas in three cases, nonuniform labeling was observed. The other case showed weak and nonuniform labeling. Furthermore, invasive SCC of the skin showed significantly lower levels of vinculin labeling (Fig 3B, C, D). Of 15 cases studied, five were essentially negative, and six additional cases showed weak uniform labeling. Of the other four cases that displayed intense labeling, three were highly nonuniform, with negative regions alternating with positively labeled patches, often displaying strongly stained horn pearls (Fig 3C).

Another group of invasive SCC, derived from larynx, nasopharynx, and the upper esophagus, showed similar labeling patterns. Of the 15 cases examined, nine were essentially negative, three showed weak uniform labeling, and the rest were highly nonuniform (Table 2, Fig 3E). Most of the lymph node metastases from SCC were apparently vinculin negative (six of nine cases) (Fig 3F), two additional cases displayed weak uniform labeling, and one case was heterogenous with negative areas and some strongly labeled foci. There was no correlation between vinculin staining and degree of histological differentiation in any of the tumors.

DISCUSSION

In the current study, we have examined the expression of vinculin in different carcinomas of stratified epithelial origin. The results presented here indi-

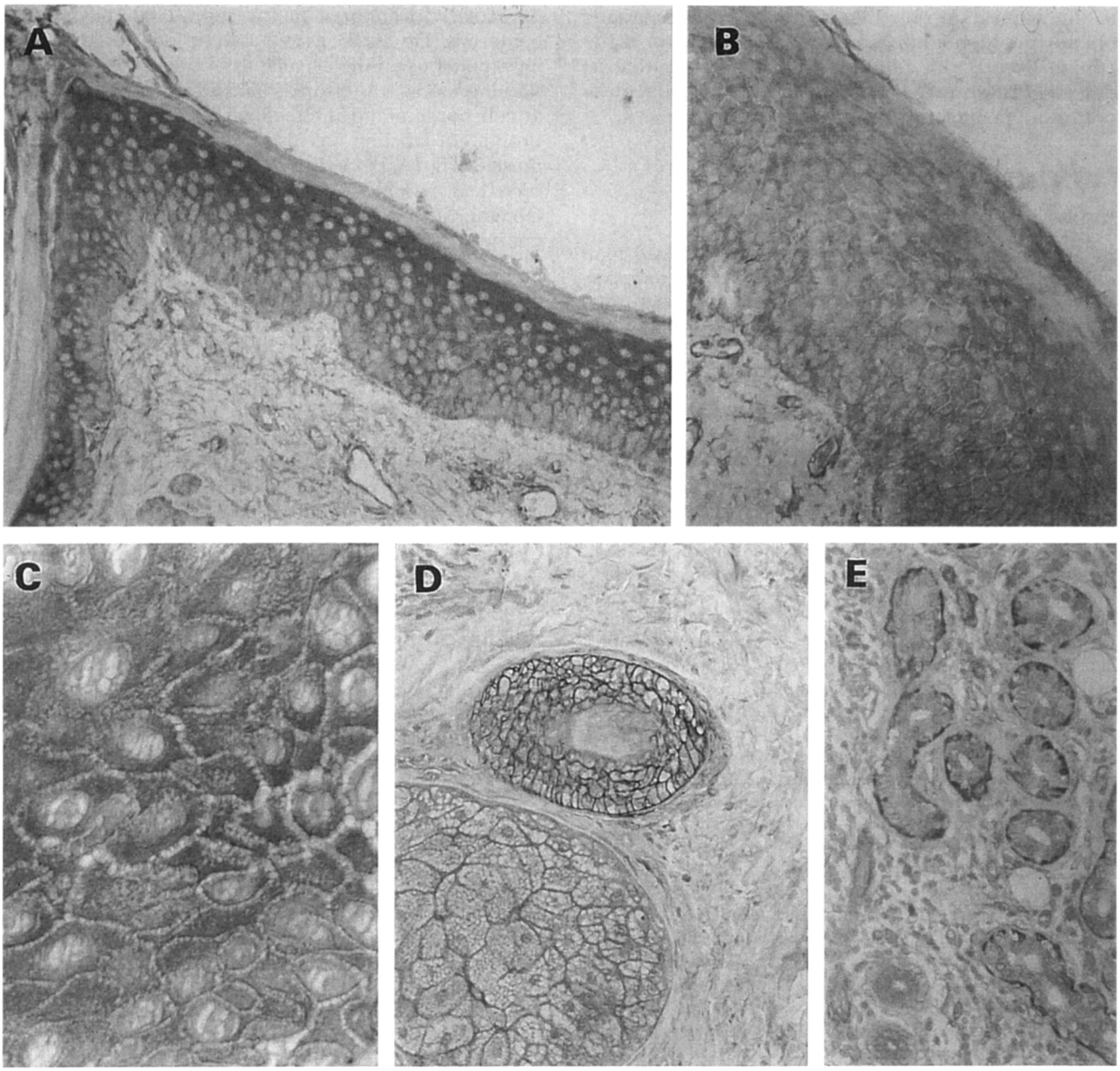

FIGURE 1. Immunohistochemical staining with monoclonal anti-vinculin antibody of normal skin. There is uniform labeling of the epidermis (A-C), which is particularly prominent in the suprabasal cells (A). Staining is cytoplasmic but often enriched at cell borders and intercellular bridges (C). Hair follicle epithelium stains strongly and sebaceous glands weakly at cell borders (D). Myoepithelial cells of sweat glands are prominently labeled (E). Note strong staining of dermal blood vessels (A,B). (Original magnification: A, ×75; B, D, E, ×150; C, ×300.)

cate that the level of expression of vinculin is inversely related to the degree of tumor-metastasizing potential. Thus, most BCCs, keratoacanthomas, and in situ SCC were uniformly positive, whereas most invasive and metastatic SCC were either negative or highly nonuniform with extensive negative regions, or showed uniform low levels of labeling. No correlation was found between vinculin staining and grade of tumor differentiation. However, in focal areas of invasive SCC in which horn pearls were present, the latter stained strongly for vinculin on the background of a largely vinculin-negative tumor. This seems to indicate that even in invasive tumors with metastasizing potential, highly differentiated elements retain significant vinculin expression, which is characteristic of differentiated squamous epithelia.

The data described above suggest that the level of vinculin expression may be more indicative of metastatic potential than the apparent degree of squamous differentiation of the neoplasm.

The focus in this study, on vinculin expression in human tumors, was motivated by the general indication that malignant transformation is commonly associated with reduced adhesiveness and by data pointing to the

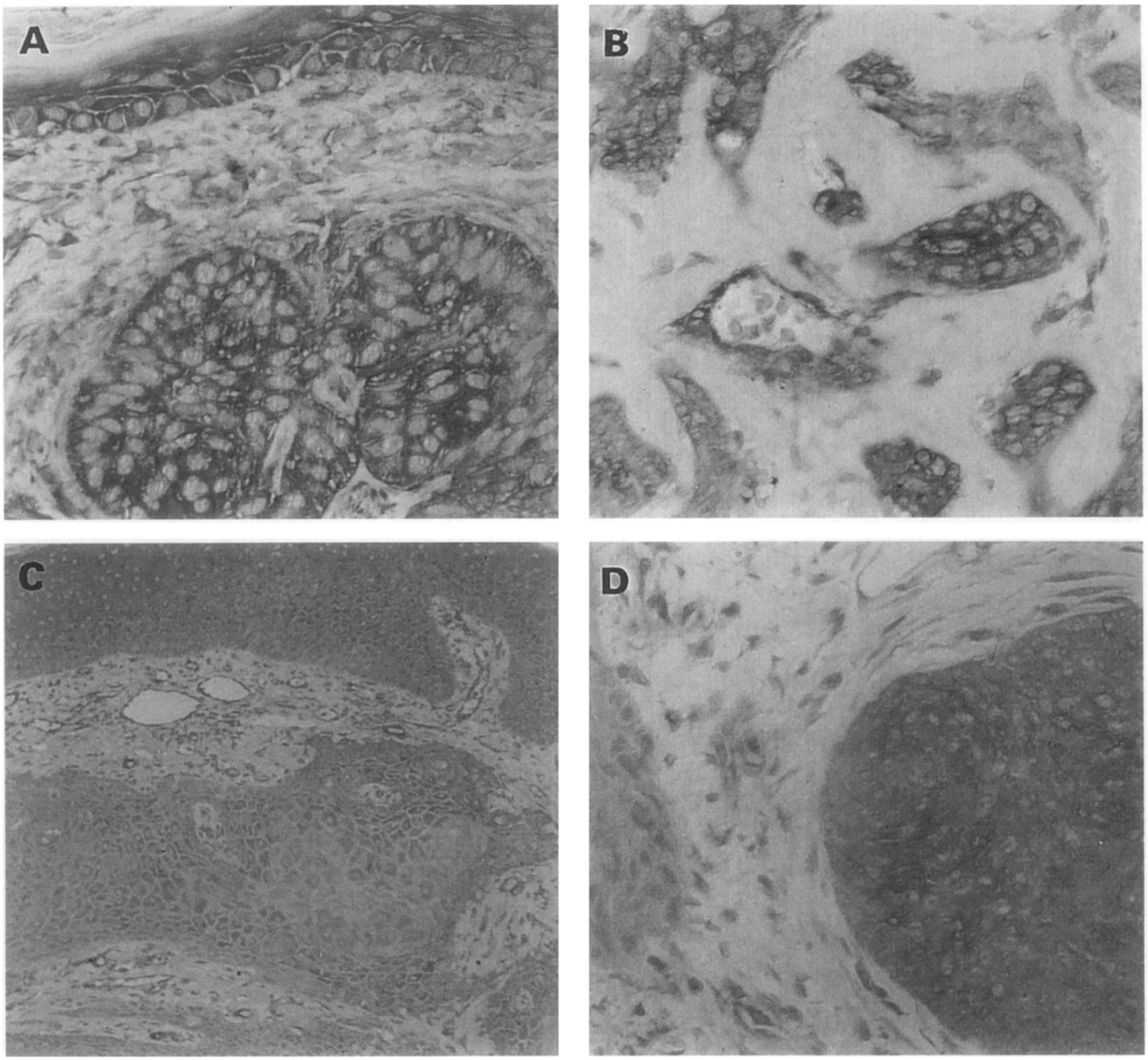

FIGURE 2. Immunohistochemical staining of various skin lesions with monoclonal anti-vinculin antibodies. There is strong, uniform labeling with vinculin in nests of BCC and similar staining of overlying epidermis, as well as positively stained stromal cells surrounding tumor nests (A). Morphea-type BCC showing typical invasive pattern also shows prominent vinculin labeling. Note vinculin-stained blood vessels (B). Keratoacanthoma displays uniform labeling with vinculin (C, D). (Original magnification: A-D, ×150.)

central role of vinculin in the formation of cell-matrix and cell-cell adhesion.[17]

The association of transformation and, in particular, the acquisition of an invasive or metastatic phenotype, with reduced cellular adhesiveness has been extensively documented over the last years, as discussed.[1-7] Recently, it was shown that tumors often display a marked deficiency in the expression of specific ad-

TABLE 1. Vinculin Expression in Skin Tumors

Staining Level	BCC (n = 15)	Keratoacanthoma (n = 5)	In Situ SCC (n = 6)	Invasive SCC (n = 15)
UD	—	—	—	5
+	3 (uniform)	—	1 (focal)	6 (uniform)
++	12 (4 focal, 8 uniform)	5 (uniform)	5 (3 focal, 2 uniform)	4 (3 focal, 1 uniform)

Abbreviations: +, weak staining; ++, strong staining; UD, undetectable; BCC, basal cell carcinoma; SCC, squamous cell carcinoma.

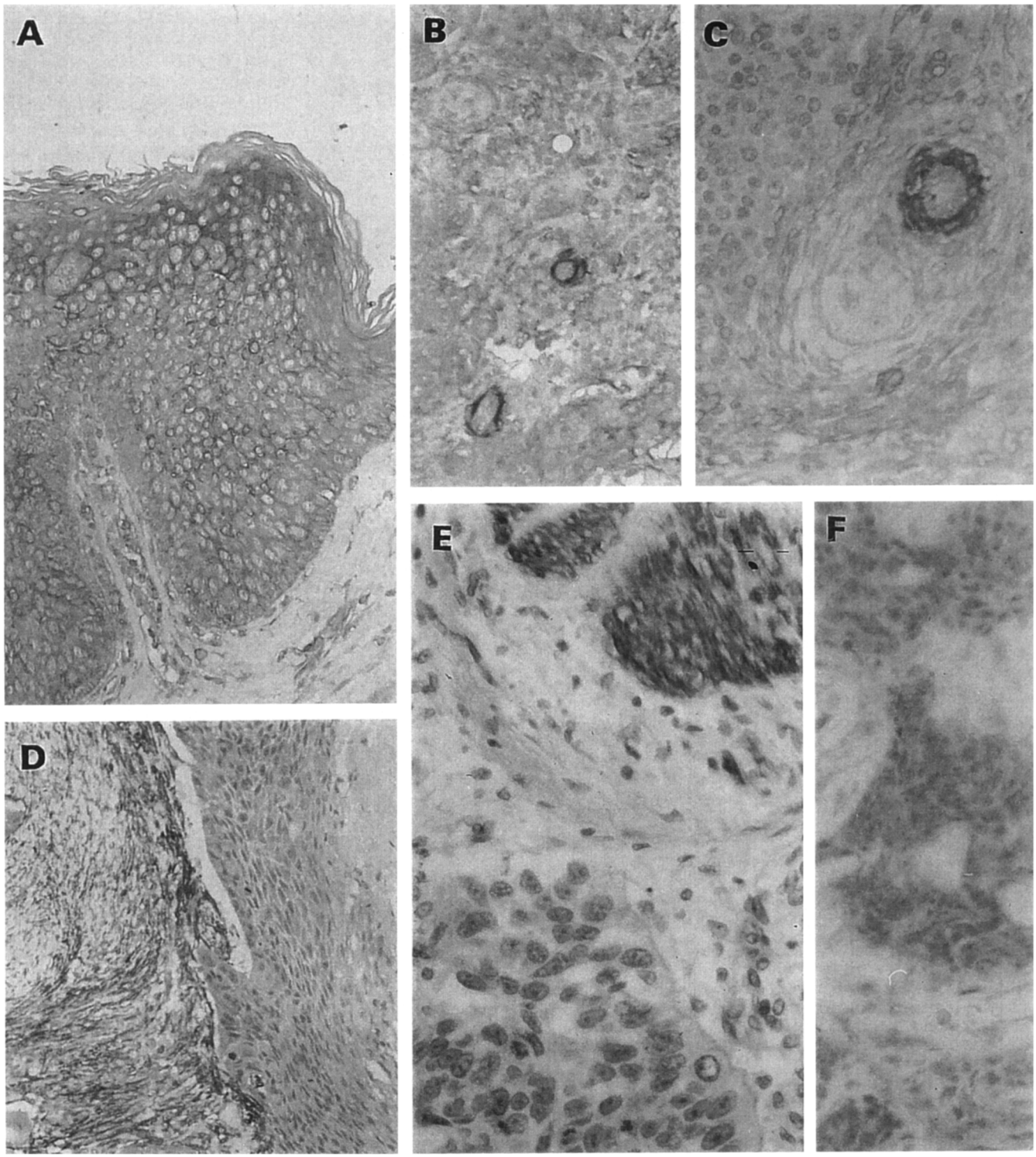

FIGURE 3. Immunohistochemical staining with monoclonal anti-vinculin antibody of SCC of the skin, esophagus, and lymph node metastasis. SCC in situ of the skin (Bowen's disease) shows uniform, prominent labeling for vinculin throughout (A). Invasive SCC of the skin shows undetectable vinculin (B, C, D), with the exception of positive staining of horn pearls (C). Note in B prominently stained blood vessels and in D strong labeling of stromal cells surrounding tissue nests. Staining for vinculin is also negative in esophageal carcinoma (E, left lower corner) with strong staining of smooth muscle fibers (E, right upper corner). Metastases in lymph node from primary esophageal SCC (F) is entirely negative for vinculin. (Original magnification: A, B, ×150; C, E, F, ×300; D, ×75; E, F, ×300.)

hesion-related molecules. In fact, changes in the expression of most of the major families of adhesion molecules have been implicated in malignant transformation, tumor invasiveness, and metastasis. These include differences in the expression of integrins,[3,18,19] cadherins,[14,15,20] and adhesion molecules of the immunoglobulin superfamily.[21] Moreover, it has been shown that elevated posttranslational modifications, such as

TABLE 2. Vinculin Expression in Squamous Cell Carcinomas of Larynx, Nasopharynx, Upper Esophagus, and Lymph Node Metastases

Staining Level	Invasive SCC (n = 15)	Lymph Node Metastases From SCC (n = 9)
UD	9	6
+	4 (3 uniform, 1 focal)	2 (diffuse)
++	2 (focal)	1 (focal)

Abbreviations: +, weak staining; ++, strong stimulus; UD, undetectable; SCC, squamous cell carcinoma.

tyrosine-specific protein phosphorylation, which is commonly found in different types of transformed cells, exert a major detrimental effect on cell adhesion.[9,10,22-24] The primary subcellular targets of these kinases are cell-cell and cell-matrix adhesions and the elevated phosphorylation apparently affected the interaction of the cytoskeleton with these junctions.[10,11,17]

In view of the central role of the microfilament system and its attachment to the membrane, in cell adhesion,[17] we have examined the expression and subcellular distribution of different components of the submembrane junctional "plaque" in transformed cells. These surveys indicated that the levels of several of these proteins, such as vinculin, α-actinin, and plakoglobulin, are indeed reduced in cultured cells derived from a variety of tumors.[1,25-28] Moreover, it was shown that the "forced expression" of the missing proteins, achieved by transfection of the tumor cells with the relevant cDNA, had a marked tumor suppressive effect.[25-29] It remained, however, unclear how common and prominent is the deficiency of these junctional plaque proteins in human cancers, and how its modulation correlates the malignant behavior of the cells.

The results presented here strongly suggest that the metastatic and invasive phenotype of human SCC is associated with markedly reduced expression of vinculin. In contrast, nonmetastasizing squamous tumors, such as BCCs or keratoacanthomas, were usually strongly positive. It is noteworthy that even BCC with a definite invasive pattern, such as the morphea variant, was vinculin positive, indicating that low levels of vinculin are primarily related to the metastatic potential of the tumor, rather than to its local invasive potential. The reduction in vinculin levels was manifested either by a uniform absence of labeling from the entire tumor tissue or by variable expression displaying negative areas and positively labeled foci. Furthermore, it is conceivable that suppression of vinculin levels below a certain threshold renders it essentially ineffective in supporting cell adhesion, in line with our previous results.[28,29] Similarly, in cases in which the primary tumor showed nonuniform expression of vinculin, the vinculin-negative subpopulation of cells was, most likely, the primary contributor to the metastatic process, as suggested by the fact that most lymph node metastases, including those derived from primary tumors with focal expression of vinculin, were either negative or poorly labeled.

The significance of these results should be discussed in two contexts. The first is related to specific stages in the malignant process that may be affected by the modulation of vinculin levels. The data presented here suggest that the loss of normal growth control is not the primary target for vinculin, because both basal cell carcinomas and keratoacanthomas, which display deregulated growth, contained high levels of vinculin. Conversely, the loss of vinculin expression closely correlated with altered cytodynamic properties of the cells, particularly their metastasizing potential, which requires active penetration into surrounding tissues, including blood vessels or lymphatis, extravasation, proliferation at new sites, and the formation of metastases. It is conceivable that reduction in vinculin levels and hence in cell adhesiveness directly promotes these processes. It is noteworthy that previous studies confirmed that metastatic properties are associated with low adhesiveness and poor cytoskeletal organization.[1-8,12]

In conclusion, our results indicate that vinculin immunostaining of tumors originating in stratified squamous epithelium distinguishes between those with low and high metastatic potential and may thus be of value in determining the biological behavior and clinical properties of such neoplasms.

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REFERENCES

1. Ben-Ze'ev A, Rodriguez-Fernandez YL, Glück U, et al: Changes in adhesion plaque protein levels regulate cell motility and tumorigenicity, in Estes LE, Higgins PY (eds): Actin Biophysiology Biochemistry and Cell Biology. New York, NY, Plenum Press, 1994, pp 147-157

2. Ben-Ze'ev A: The cytoskeleton of cancer cells. Biochim Biophys Acta 780:197-212, 1985

3. Giancotti FG, Mainiero F: Integrin-mediated adhesion and signaling in tumorigenesis. Biochim Biophys Acta 1198:47-64, 1994

4. Kinch MS, Burridge K: Altered adhesions in ras-transformed breast epithelial cells. Cancer Metastasis Rev 14:173-189, 1995

5. Kemperman H, Driessens MH, La Riviere GAM, et al: Adhesion mechanisms in liver metastasis formation. Cancer Surv 24:67-79, 1995

6. Birchmeier W, Hulsken J, Behrens J: Adherens junction proteins in tumour progression. Cancer Surv 24:129-140, 1995

7. Lafrenie RM, Buchanan MR, Orr FW: Adhesion molecules and their role in cancer metastasis. Cell Biophys 23:3-89, 1993

8. Shin SI, Freedman VH, Isser P, et al: Tumorigenicity of virus-transformed nude mice is correlated specifically with anchorage independent growth in vitro. Proc Natl Acad Sci U S A 72:4435-4439, 1975

9. Volberg T, Geiger B, Dror R, et al: Modulation of intercellular adherens-type junctions and tyrosine phosphorylation of their components in RSV-transformed cultured chick lens cells. Cell Regul 2:105-120, 1991

10. Pasquale EB, Maher PA, Singer SJ: Talin is phosphorylated on tyrosine in chicken embryo fibroblasts transformed by Rous Sarcome Virus. Proc Natl Acad Sci 83:5507-5511, 1986

11. Volberg T, Zick Y, Dror R, et al: The effect of tyrosine-specific protein phosphorylation on the assembly of adherens-type junctions. EMBO J 11:1733-1742, 1991

12. Ben-Ze'ev A, Rodriguez-Fernandez JL, Glück U, et al: Regula-

tion of adherens junction protein levels: Its role in cell motility and tumorigenicity. Adv Mol Cell Biol 12:143-163, 1995

13. Stetler-Stevenson WG, Aznavoorian S, Liotta LA: Tumor cell interactions with the extracellular matrix during invasion and metastases. Annu Rev Cell Biol 9:541-574, 1993

14. Takeichi M: Cadherins in cancer: Implications for invasion and metastasis. Curr Opin Cell Biol 5:806-811, 1993

15. Birchmeier W, Weidner KM, Hulsken J, et al: Molecular mechanisms leading to cell junction (cadherin) deficiency in invasive carcinomas. Semin Cancer Biol 4:231-239, 1992

16. Hsu SM, Raire I, Fanger H: Use of avidin-biotin peroxidase complex (ABC) in immunoproxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC and unlabelled antibody (PAP) procedure. J Histochem Cytochem 29:577-580, 1981

17. Geiger B, Yehuda-Levenberg S, Bershadsky AD: Molecular interactions in the submembrane plaque of cell-cell and cell-matrix adhesions. Acta Anat 154:46-62, 1995

18. Giancotti FG, Rouslahti E: Elevated levels of the $\alpha 5\beta 1$ fibronectin receptor suppress the transformed phenotype of chinese hamster ovary cells. Cell 69:849-859, 1990

19. Akiyama SK, Olden K, Yamada KM: Fibronectin and integrins in invasion and metastasis. Cancer Metastasis Rev 14:173-189, 1995

20. Behrens J, Mareel MM, Van Roy FM, et al: Dissecting tumor cell invasion: Epithelial cells acquire invasive properties after loss of uvomorulin-mediated cell-cell adhesion. J Cell Biol 108:2435-2447, 1989

21. Zandwijk N, Mooi W: Neural cell adhesion molecule expression, neuroendocrine differentiation and prognosis in lung carcinoma. Eur J Cancer 27:4312-4435, 1991

22. Hamaguchi M, Matsuyoshi N, Ohnishi Y, et al: $p60^{v\text{-}src}$ causes phosphorylation and inactivation of the N-cadherin-catenin cell adhesion system. EMBO J 12:307-314, 1993

23. Guan JL, Shalloway D: Regulation of focal adhesion associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. Nature 358:690-692, 1992

24. Behrens J, Vakaet L, Friis R, et al: Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin/β-catenin complex in cells transformed with a temperature sensitive v-src gene. J Cell Biol 120:757-766, 1993

25. Glück U, Ben-Ze'ev A: Modulation of alpha-actinin levels affects cell motility and confers tumorigenicity on 3T3 cells. J Cell Sci 107:1773-1782, 1994

26. Glück U, Kwiatkowski DJ, Ben-Ze'ev A: Suppression of tumorigenicity in simian virus 40-transformed 3T3 cells transfected with alpha-actinin cDNA. Proc Natl Acad Sci U S A 90:383-387, 1993

27. Rodriguez Fernandez JL, Geiger B, Salomon D, et al: Suppression of tumorigenicity in transformed cells after transfection with vinculin cDNA. J Cell Biol 119:427-438, 1992

28. Simcha I, Geiger B, Yehuda-Levenberg S, et al: Suppression of tumorigenicity by plakoglobin: An augmenting effect of N-cadherin. J Cell Biol 133:199-209, 1996

29. Rodriguez Fernandez JL, Geiger B, Salomon D, et al: Suppression of vinculin expression by antisense transfection confers changes in cell morphology, motility and anchorage-dependent growth of 3T3 cells. J Cell Biol 122:1285-1294, 1993