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Advances in Brief

Decreased E-Cadherin Immunoreactivity Correlates with Poor Survival in Patients with Bladder Tumors[1]

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Abstract

E-cadherin, an intercellular adhesion molecule, has been shown to behave like an invasion suppressor gene *in vitro*. This may explain the inverse relation between expression of E-cadherin and tumor grade that was found in certain cancers. We therefore examined E-cadherin expression in bladder cancer samples from patients with known clinical follow-up. Forty-nine snap-frozen specimens (24 superficial and 25 invasive tumors) and 4 samples of normal urothelium were retrospectively analyzed with anti-E-cadherin monoclonal antibodies. In normal urothelium E-cadherin is expressed homogeneously with a typical membranous staining at cell-cell borders. Decreased expression is found in 5 of 24 superficial tumors and in 19 of 25 invasive cancers. Completely negative tumors are infrequent (4 cases). Most of the time a heterogeneous staining, which may correspond to an unstable E-cadherin expression during tumor development, is seen. Decreased E-cadherin expression correlates with both increased grade and stage ($\chi^2 = 9.5$, $P < 0.01$, and $\chi^2 = 14.9$, $P < 0.005$, respectively). More importantly, abnormal E-cadherin expression correlates with shorter survival (log rank test: $\chi^2 = 16.5$, $P < 0.001$). In keeping with its *in vitro* invasion suppressor function, decreased E-cadherin expression correlates with the clinical aggressiveness of bladder tumors. This is the first report of E-cadherin as a marker with prognostic value. This parameter must now be tested in a large prospective study to assess its precise clinical relevance.

Introduction

Bladder cancer is characterized by a diverse biological behavior, in which acquisition of the metastatic capacity is clinically most relevant. Despite intra- and interobserver inconsistencies, tumor grade and stage are to date the best prognostic factors. Several molecular parameters (*e.g.*, blood group antigen expression, DNA content) have been tested for prognostic value but none of these proved to be superior to classical pathological parameters. Thus there is still a paucity of markers for bladder tumor aggressiveness (1). Clearly, identification of the molecular steps associated with the acquisition of metastatic ability is important and should enable the design of diagnostic methods with predictive value.

To metastasize, cancer cells must be released in the blood or lymphatic stream. Decreased intercellular adhesiveness favors detachment of tumor cells and may play a role in the early steps of the metastatic process. Although cell-cell adhesion is a complex mechanism involving at least four families of adhesion molecules (integrin, immunoglobulin, selectin, and cadherin families), several lines of evidence indicate that the Ca^{2+}-dependent, E-cadherin-mediated adhesiveness is critically important for epithelial integrity (for review see Ref. 2). Indeed, anti-E-cadherin antibodies (also called anti-uvomorulin) profoundly disturb the epithelial junctional complex (3–5) and prevent mouse morula compaction (6). Conversely, inactivation of other systems (N-cell adhesion molecule or β_1-integrin) has little effect on cell-cell cohesion when cadherin adhesiveness is not impaired (7, 8). The causal relationship between decreased E-cadherin expression and acquisition of invasive capacity has recently received strong support; modulation of E-cadherin function of several tumor cell lines either by blocking antibodies or by transfection experiments revealed an invasion suppressor role *in vitro* for E-cadherin-mediated cell-cell adhesion (9–11).

As far as bladder cancers are concerned, 3 cell lines have been tested for both E-cadherin expression and *in vitro* invasiveness. The RT4 and RT112 cell lines express E-cadherin and are not invasive, whereas the EJ24 cell line does not express E-cadherin and is invasive (10). However, data on tumor specimens are almost completely missing [Edelman *et al.* (12) found a normal staining pattern in 2 transitional cell carcinomas]. In a search for more accurate prognostic factors, we investigated E-cadherin expression in bladder tumors. We report here results of E-cadherin immunostaining and compare them with histopathological and survival data.

Materials and Methods

Twenty-four snap-frozen superficial carcinomas (pTa-pT1) and 25 invasive carcinomas (pT ≥ 2) have been analyzed. Superficial tumors comprised 2 recurrences of previously analyzed tumors. Among the invasive tumors, 2 were squamous cell carcinomas. All the other tumors were transitional cell carcinomas. Pathological and clinical data for the patients are summarized in Table 1. As normal control 2 cystectomy specimens from patients with nonurothelial tumors and 2 ureters from renal cell carcinoma patients were used.

Sections were fixed with 3% paraformaldehyde and permeabilized with 0.2% Triton X-100. E-cadherin immunohistological detection was performed using the HECD-1 monoclonal antibody (British Biotech Products, Ltd., Abindon, United Kingdom) at the concentration of 10μg/ml. Furthermore, 43 tumors were also stained with the L-CAM monoclonal antibody (Eurodiagnostic, Apeldoorn, the Netherlands) used at 1/10 dilution. As described previously (13), a classical immunoperoxidase technique was used to reveal E-cadherin immunoreactivity.

Evaluation of E-cadherin expression used a classification derived from the work of Shiozaki *et al.* (14), in which tumors were classified as normal if the staining was similar to that of normal urothelium. Abnormal tumors were negative (*i.e.*, complete absence of immunoreactivity) or heterogeneous (*i.e.*, when the tumor is composed of positive and negative areas). We also noticed cytoplasmic immunoreactivity in some cases. When it was associated with negative areas the tumor was classified as heterogeneous. E-cadherin expression has been scored in blind by 3 persons.

For statistical analysis, a χ^2 test was used for the correlations with pathological data. The log rank test has been used for survival analysis.

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Table 1 *E-cadherin staining, clinical, and pathological data for each patient*

Case	Age (yr)	Grade	Stage	E-cadherin expression	Survival (mo)	Treatment
1	77	1–2	a	Normal	31	TURT[a]
2	75	1	a	Normal	>41	TURT
3	72	1	a	Normal	>38	TURT
4	68	1	a	Normal	>32	TURT, C
5	66	3	a	Normal	23	TURT, Ct, R, Ch
6	66	2	a	Normal	>57	TURT, Ct,
7	49	1	a	Normal	>45	TURT, BCG, Ct
7 rec.		1	a-1	Normal		
8	62	1	a	Cytoplasmic	>64	TURT
9	53	1	a	Heterogeneous	>61	TURT, BCG
10	42	1	a	Heterogeneous	>65	TURT, Ch, BCG
11	88	2	a	Heterogeneous	52	TURT, Ch
12	44	2	a-1	Normal	>39	TURT
13	65	1	a-1	Normal	>19	TURT
14	68	3	1	Normal	>55	TURT, BCG
15	47	1	1	Normal	>43	TURT, Ch
16	86	2	1	Normal	>58	TURT, Ch
17	76	2	1	Heterogeneous	9	TURT, Ch
18	53	2	1	Normal	>37	TURT, BCG
19	64	1	1	Normal	>55	TURT
20	85	2	1	Normal	>30	TURT, Ch, ureterostomy
20 rec.		3	a	Normal		
21	71	2	1	Normal	>36	TURT, Ch
22	68	2	1	Normal	>25	TURT, Ch, BCG
23	81	2	2	Normal	9	TURT
24	73	2	2	Normal	8	TURT, Ch, Ct
25	49	3	2	Normal	>27	TURT, R, iridium
26	79	3	2	Negative	4	Ct
27	81	2	2	Heterogeneous	6	TURT
28	53	3	2	Heterogeneous	7	TURT, Ch
29	70	3	2	Heterogeneous	3	TURT, Ch
30	47	3	2	Heterogeneous	8	TURT, Ch
31	62	3	≥ 2	Normal	>27	TURT, Ch, R
32	65	3	≥ 2	Negative	>66	TURT, Ct
33	83	3	≥ 2	Heterogeneous	21	TURT, R
34	79	3	≥ 2	Heterogeneous	10	TURT, Ct
35	61	3	≥ 2	Heterogeneous	15	TURT, Ch, R
36	59	3	≥ 2	Heterogeneous	3	TURT, R
37	73	3	2–3	Negative	2	TURT, R
38	73	3	2–3	Heterogeneous	9	TURT
39	76	2, SCC	2–3	Heterogeneous	32	TURT, Ct, R
40	68	3	2–3	Heterogeneous	2	TURT
41	63	3	3	Negative	5	TURT, R
42	74	3	3	Heterogeneous	10	Ct, Ch
43	50	2, SCC	3	Heterogeneous	5	TURT
44	70	2	3b	Normal	>21	Ct
45	75	3	3b	Heterogeneous	>29	TURT, R, Ct
46	44	3	4	Normal	>25	TURT, Ct
47	82	2	4	Heterogeneous	5	TURT

[a] Ch, chemotherapy; Ct, cystectomy; R, radiotherapy; TURT, transureteral resection of the tumor; SCC, squamous cell carcinoma; rec., recurrence.

Results

In normal urothelium, only the cell-cell borders are stained as described for other epithelial tissues (*i.e.*, the luminal membrane (Fig. 1, *arrowheads*) and the part of the cells in contact with the basement membrane (Fig. 2*a*, *arrows*) do not react with the anti-E-cadherin antibody). The *arrows* in Fig. 1 indicate a focal stronger reactivity at the apical part of the lateral membrane, probably reflecting the E-cadherin enrichment at the junctional complex (zonula adherens). Twenty-six tumors show a similar staining pattern referred as normal staining (Fig. 2*a*) whereas 23 specimens present an abnormal E-cadherin expression (Table 1). Only 4 tumors are completely negative (Fig. 2*c*). The prevailing abnormal pattern is heterogeneous, certain parts of the tumor being positive and others negative. In most of those cases, multiple foci of negative carcinomatous cells are found (Fig. 2*b*). In certain tumors with heterogeneous immunoreactivity, a capping of E-cadherin can be seen in some cells (Fig. 2*b*, *arrows*). In two cases, immunoreactivity is found rather in the cytoplasm than at the membrane. One of these tumors (tumor 47) showed also negative areas and was therefore classified as heterogeneous.

Confrontation of the E-cadherin expression results with the classical histopathological data (Table 2) reveals that abnormal E-cadherin expression is correlated with both grade and stage ($\chi^2 = 9.5$, $P < 0.01$ and $\chi^2 = 14.9$, $P < 0.005$, respectively). The mean age of patients, 65.3 ± 2.6 for patients with a normal staining *versus* 68 ± 2.6 for those with an abnormal one, is not significantly different. Analysis of

Fig. 1. Normal urothelium showing a staining at the cell-cell borders. The luminal membranes of the superficial (24) cells (*arrowheads*) are devoid of staining. *Arrows*, reinforced stainings probably corresponding to junctional complexes. Due to folding two luminal surfaces are facing each other. × 400.

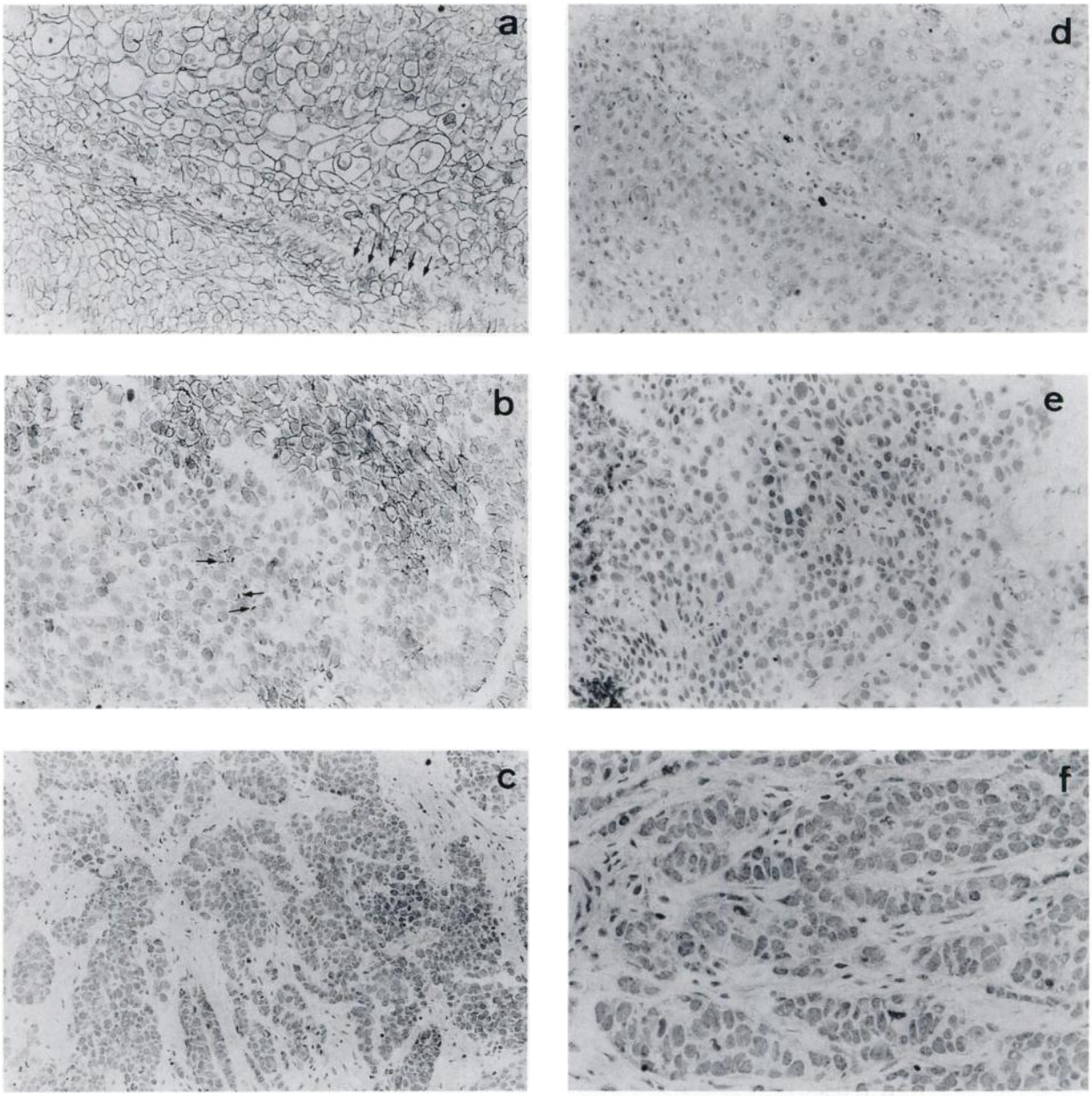

Fig. 2. *a*, transitional cell carcinoma with a conserved staining at the cell-cell border. As in normal urothelium, a lack of immunoreactivity is seen along the cell surface in contact with the basement membrane (*arrows*). *d*, negative control. × 200. *b*, heterogeneous staining. Some cells show a clear staining at the cell-cell border whereas other areas are negative. *Arrows*, capping observed in some cells. *e*, negative control. × 200. *c* and *f*, negative staining. All the cells are completely negative. *c*, × 200; *f*, × 400.

the survival during 3 years after tumor resection shows that abnormal immunoreactivity is strongly correlated with poor prognosis [$\chi^2 = 16.5$, $P < 0.001$ (Fig. 3*a*)]. This correlation seems to persist when only invasive tumors are analyzed [$\chi^2 = 3.7$, $P < 0.06$ (Fig. 3*b*)]. For superficial tumors, too few events (3 deceases and 1 progressive disease) have been observed to enable any analysis of this subgroup.

Table 2 *E-cadherin staining according to histopathological data.*

The frequency of abnormal staining increase with both grade and stage ($\chi^2 = 9.5$, $P < 0.01$ and $\chi^2 = 14.9$, $P < 0.005$, respectively).

	E-cadherin staining	
	Normal	Abnormal
Grade		
1	9	3
2	11	6
3	5	15
Stage		
Superficial	19	5
Invasive	6	19

Discussion

In this study, we have investigated E-cadherin expression in normal and transformed urothelium. Normal urothelial cells express E-cadherin homogeneously at all cell-cell borders. Reasoning that, according to the theory of the clonal evolution of tumors, the more aggressive clone within a cancer will determine the tumor behavior (15), we based our grading system on the presence of a negative subpopulation. Since E-cadherin is thought to function as an invasion suppressor gene, tumors with only a minor fraction of negative cells should be able to invade and thus must be regarded as abnormal with respect to E-cadherin expression. We show that abnormal staining pattern (negative, cytoplasmic or heterogeneous) are found in 21% (5 of 24) of the superficial tumors, and in 76% (19 of 23) of the invasive tumors. Taken together with the experimental demonstration of a causal relationship between impaired E-cadherin function and invasiveness (9–11), this strong correlation with tumor stage seems to indicate that

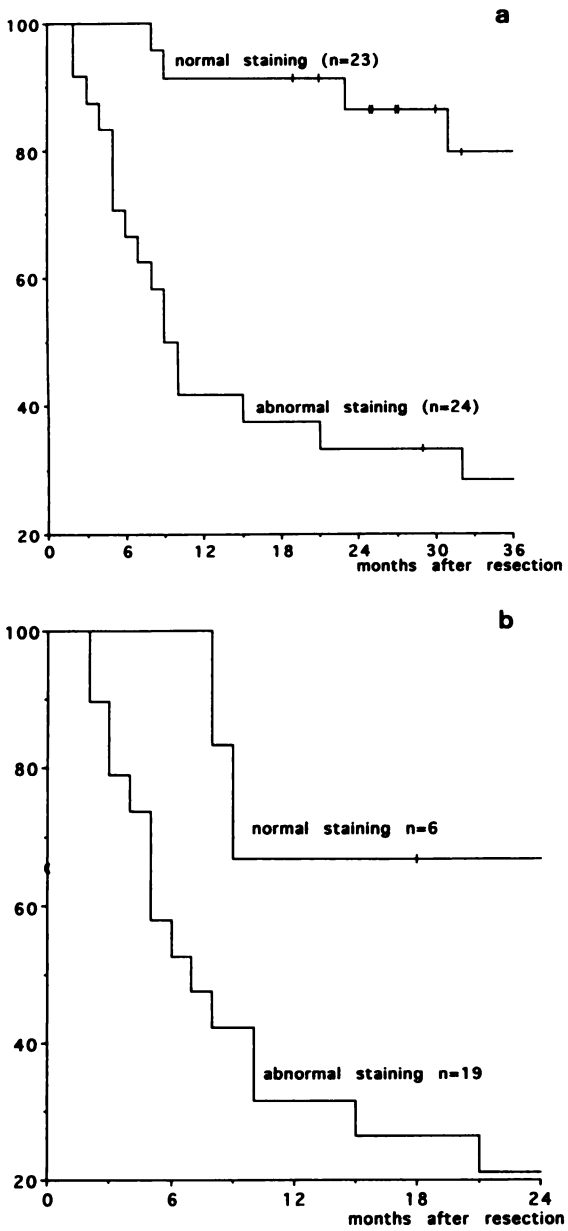

Fig. 3. Kaplan-Meyer survival curves. *Bars,* censured data. *a,* 3-year survival according to E-cadherin staining: $\chi^2 = 16.5$, $P < 0.001$. *b,* 2-year survival of patients with infiltrative tumors according to E-cadherin staining: $\chi^2 = 3.7$, $P < 0.06$.

indeed disturbance of E-cadherin expression plays a role in bladder tumors invasiveness.

It is striking to see that in most cases (19 of 23), tumors are not completely negative but are composed of positive and negative areas. Similar findings are reported by Shiozaki for esophagus, stomach, and breast cancers. The proportion of heterogeneous tumors is less in the study of Schipper *et al.* (16) on squamous cell carcinomas of the head and neck, possibly due to the use of immunofluorescence which does not allow simultaneous assessment of both morphology and staining. Heterogeneous E-cadherin expression has also been found in the metastatic variant of a murine ovarian cell line. Since this heterogeneity persists upon subcloning it is thought to reflect unstable E-cadherin expression (17). Thus, this staining pattern may indicate that unstable E-cadherin expression occurs during tumor development. Using H-*ras* transformed Madin-Darby canine kidney cells, Mareel *et al.* (18) have obtained evidence that host factors play a role in this unstable E-cadherin expression. Indeed, these cells homogeneously express E-cadherin *in vitro* whereas tumors obtained after injection in nude mice are heterogeneous; this E-cadherin down-modulation is completely reversible within 8 *in vitro* passages of cells derived from heterogeneous metastasis.

However, other mechanisms may also lead to impaired E-cadherin function. It has been shown that the cytoplasmic tail of E-cadherin interacts with 3 molecules (α-, β-, and γ-catenins) which bridge E-cadherin to the cytoskeleton and that E-cadherin function is dependent on its proper anchorage to the cytoskeleton (19–21). Interestingly, Shimoyama recently reported that α-catenin is lacking in 45.8% of gastric carcinoma of the scatter type (22) with an apparently conserved normal E-cadherin staining (23). The cytoplasmic immunoreactivity found in two bladder tumors might be caused by disturbance of E-cadherin-cytoskeleton interaction. Conversely, those cases can be a mere artifact due to cell retraction during freezing. However, cytoplasmic immunoreactivity has also been noticed by Shiozaki *et al.* (14) in their study on esophageal, stomach, and breast cancer and by Inoue *et al.* (24) in gynecological tumors. Another evidence for the possible involvement of the cytoskeleton is the clear capping of E-cadherin seen in some bladder tumors (Fig. 2*b*).

Based on the correlation between 16q deletion and abnormal staining patterns, inactivating mutations of the E-cadherin gene have been proposed (13, 16, 25). E-cadherin would thus behave as a classical suppressor gene. As far as we know, 16q deletions have not been documented in bladder tumors.

Up to now, the correlation found in certain cancers, between decreased E-cadherin immunoreactivity and classical prognostic parameters was the only indication for a potential use of E-cadherin as prognostic factor (13, 14, 16). Besides showing such a correlation in bladder tumors, our study reveals a strong correlation between abnormal E-cadherin staining and survival, showing that immunohistochemical determination of E-cadherin expression can be a useful prognostic factor for bladder tumors. Furthermore, the fact that decreased E-cadherin expression correlates with shorter survival within the group of patients with invasive disease seems to indicate that the prognostic value of E-cadherin staining does not merely reflect the correlation with stage. However, this retrospective study, on a limited number of cases, does not allow definite assessment of the clinical usefulness of this assay. Particularly, no conclusion can be drawn for patients with superficial tumors while early detection of aggressive tumors in those patients would improve disease management. For patients with invasive tumors, our results seem to indicate that conservation of normal E-cadherin staining is of relatively good prognosis even if the tumor is at an advanced stage as in case 46. Given the rate of progression of superficial bladder tumors (about 4% in our series), the accurate evaluation of the clinical usefulness of E-cadherin immunostaining will require a large scale prospective study.

References

1. Raghavan, D., Shipley, W. U., Garnick, M. C., Russell, P. J., and Richie J. P. Biology and management of bladder cancer. N. Engl. J. Med., *322:* 1129–1138, 1990.
2. Takeichi, M. Cadherin cell adhesion receptors as a morphogenetic regulator. Science (Washington DC), *251:* 1451–1455, 1991.
3. Behrens, J., Birchmeier, W., Goodman, S. L., and Imhof, B. A. Dissociation of Madin-Darby canine kidney epithelial cells by the monoclonal antibody anti-arc-1: mechanistic aspects and identification of the antigen as a component related to uvomorulin. J. Cell Biol., *101:* 1307–1315, 1985.
4. Gumbiner, B., Stevenson, B., and Grimaldi, A. The role of the cell adhesion molecule uvomorulin in the formation and maintenance of the epithelial junctional complex. J. Cell Biol., *107:* 1575–1587, 1988.
5. Sommers, C. L., Thompson, E. W., Torri, J. A., Kemler, R., Gelmann, E. P., and Byers, S. W. Cell adhesion molecule uvomorulin expression in human breast cancer cell lines: relationship to morphology and invasive capacity. Cell Growth & Differ., *2:* 365–372, 1991.
6. Hyafil, F., Morello, D., Babinet C., and Jacob, F. Cell-cell interactions in early embryogenesis: a molecular approach to the role of calcium. Cell, *26:* 447–454, 1981.
7. Duband, J. L., Dufour, S., Hatta, K., Takeichi, M., Edelman, G. M., and Thiery, J. P. Adhesion molecules during somitogenesis in the avian embryo. J. Cell Biol., *104:* 1361–1374, 1987.

8. Larjava, H., Peltonen, J., Akiyama, S. K., Yamada, S. S., Gralnick, H. R., Uitto, J., and Yamada, K. M. Novel function for β_1 integrins in keratinocyte cell-cell interactions. J. Cell Biol., *110:* 803–815, 1990.
9. Behrens, J., Mareel, M. M., Van Roy, F. M., and Birchmeier, W. Dissecting tumor cell invasion: epithelial cells acquire invasive properties after the loss of uvomorulin-mediated cell-cell adhesion. J. Cell Biol., *108:* 2435–2447, 1989.
10. Frixen, U. H,. Behrens, J., Sachs, M., Eberle, G., Voss, B., Warda, A., Lochner, D., and Birchmeier, W. E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. J. Cell Biol., *113:* 173–185, 1991.
11. Vleminckx, K., Vakaet, J. L., Mareel, M., Fiers, W., and Van Roy, F. Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. Cell, *66:* 107–119, 1991.
12. Eidelman, S., Damsky, C. H., Wheelock, M. J., and Damjanof, I. Expression of the cell-cell adhesion glycoprotein cell-CAM 120/80 in normal human tissues and tumors. Am. J. Pathol., *135:* 101–110, 1989.
13. Umbas, R., Isaacs, W. B., Aalders, T. W., Carter, B. S., Karthaus, H. F. M., Schaafsma, H. E., and Debruyne, F. M. J. Decreased expression of E-cadherin in high grade prostate cancer. Cancer Res., *52:* 5104–5108, 1992.
14. Shiozaki, H., Tahara, H., Oka H., Miyata, M., Kobayashi, K., Tamura, S., Lihara, K., Doki, Y., Hirano, S., Takeichi, M., and Mori, T. Expression of immunoreactive E cadherin adhesion molecule in human cancers. Am. J. Pathol., *139:* 17–23, 1991.
15. Nowell, P. C. Mechanisms of tumor progression. Cancer Res., *46:* 2203–2207, 1986.
16. Schipper, J. H., Frixen, U. H., Behrens, J., Unger, A., Jahnke, K., and Birchmeier, W. E-cadherin expression in squamous cell carcinomas of head and neck: inverse correlation with tumor dedifferentiation and lymph node metastasis. Cancer Res., *51:* 6328–6337, 1991.
17. Hashimoto, M., Niwa, O., Nitta, Y., Takeichi, M., and Yokoro, K. A. F. Unstable expression of E-cadherin adhesion molecules in metastatic ovarian tumor cells. Jpn. J. Cancer Res., *80:* 459–463, 1989.
18. Mareel, M. M., Behrens, J., Birchmeier, W., De Bruyne, G. K., Vleminckx, K., Hoogewijs, A., Fiers, W., and Van Roy, F. Down-regulation of E-cadherin expression in Madin Darby canine kidney (MDCK) cells inside tumors of nude mice. Int. J. Cancer, *47:* 922–928, 1991.
19. Ozawa, M., Ringwald, M., and Kemler, R. Uvomorulin-catenin complex formation is regulated by a specific domain in the cytoplasmic region of the cell adhesion molecule. Proc. Natl. Acad. Sci. USA, *87:* 4246–4250, 1990.
20. Herrenknecht, K., Ozawa, M., Eckerskorn, C., Lottspeich, F., Lenter, M., and Kemler, R. The uvomorulin-anchorage protein α catenin is a vinculin homologue. Proc. Natl. Acad. Sci. USA, *88:* 9156–9160, 1991.
21. Nagafuchi, A., and Takeichi, M. Cell binding function of E-cadherin is regulated by the cytoplasmic domain. EMBO J., *7:* 3679–3684, 1988.
22. Shimoyama, Y, Nagafuchi, A., Fujita, S., Gotoh, M., Takeichi, M., Tsukita, S., and Hirohashi, S. Cadherin dysfunction in a human cancer cell line: possible involvement of loss of α-catenin expression in reduced cell-cell adhesiveness. Cancer Res., *52:* 5770–5774, 1992.
23. Shimoyama, Y., and Hirohashi, S. Expresssion of E and P cadherin in gastric carcinomas. Cancer Res., *51:* 2185–2192, 1991.
24. Inoue, M., Ogawa, H., Miyata, M., Shiozaki, H., and Tanizawa, O. Expression of E-cadherin in normal, benign, and malignant tissues of female genital organs. Am. J. Clin. Pathol., *98:* 76–80, 1992.
25. Giroldi, L. A., and Schalken, J. Decreased expression of the intercellular adhesion molecule E-cadherin in prostate cancer: biological significance and clinical implication. Cancer Metastasis Rev., *12:* 29–37, 1992.

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