

Overexpression of the Anti-Adhesin Podocalyxin Is an Independent Predictor of Breast Cancer Progression

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Abstract

Podocalyxin is a CD34-related cell surface molecule with anti-adhesive qualities. We probed a tissue microarray ($n = 272$) linked to long-term outcome data and found that podocalyxin was highly overexpressed in a distinct subset of invasive breast carcinomas ($n = 15$; 6%). Univariate disease-specific ($P < 0.01$) and multivariate regression ($P < 0.0005$) analyses indicated that this overexpression is an independent indicator of poor outcome. Forced podocalyxin expression perturbed cell junctions between MCF-7 breast carcinoma cells, and it caused cell shedding from confluent monolayers. Therefore, podocalyxin overexpression is a novel predictor of breast cancer progression that may contribute to the process by perturbing tumor cell adhesion.

Introduction

The expression of specific cell junction proteins is often suppressed in breast tumors and the resulting perturbation of cell-cell interactions is thought to facilitate the emergence of the metastatic phenotype (1). As the degree and the specificity of junctional disruption varies widely with stage, grade, and tumor subtype, we reasoned that an up-regulation of anti-adhesive molecules might also be involved in breast cancer progression. Podocalyxin, which is normally expressed on hematopoietic progenitors, vascular endothelia, and kidney podocytes is a heavily sialated and sulfated member of the CD34 family of integral membrane proteins that is one such candidate anti-adhesin (2–4). Podocalyxin's anti-adhesive characteristics are evident when it is overexpressed in kidney epithelial cells where it subtly perturbs cell junction protein localization and decreases tight junction-dependent transepithelial resistance (5). We found the same to be the case when podocalyxin was overexpressed in MCF-7 breast carcinoma cells. Using a tissue microarray (TMA) linked to long-term outcome data characterized previously (6), we assessed changes in podocalyxin expression in 272 invasive human breast carcinomas. The results

indicate that podocalyxin overexpression is tightly correlated with poor outcome in a distinct subset of tumors. Taken together, the data indicate that podocalyxin overexpression may have both prognostic and functional significance in breast cancer progression.

Materials and Methods

TMA Construction. Formalin-fixed, paraffin-embedded primary invasive breast cancer tissue blocks (outcome-linked archival cases from 1974–1995) graded according to the Nottingham method were used to construct a TMA as described previously (7) with institutional review board approval (Vancouver General Hospital, Vancouver, Canada). This TMA contains many tumors in terms of stage, grade, and nodal status. In addition, estrogen receptor, p53, and Her-2/*neu* status was determined previously (6).

TMA Immunohistochemistry, Scoring, and Analysis. Deparaffinized, citrate buffer-treated normal tissue and TMA sections were blocked with 3% hydrogen peroxide and incubated with mouse monoclonal antibodies against human podocalyxin (clone “3D3”; 1:80 dilution; Ref. 8) and CD34 (clone “8G12”; BD Biosciences, San Diego, CA) followed by detection with the Envision system (Dako, Carpinteria, CA) and hematoxylin counterstaining. Podocalyxin levels were scored by staining intensity, and the proportion of cells were stained (see Results below for description of groupings) without knowledge of patient outcome. Scores were then processed using the TMA-Deconvolter 1.06, Cluster and TreeView programs (9). Differences among podocalyxin groupings in terms of disease-free survival were assessed by univariate Kaplan-Meier analysis (log-rank test). Multivariate analysis of disease-specific survival was performed using the multi-step Cox regression proportional hazard model (confidence interval, 99%).

Cell Culture, Transfection, and Junctional Analysis. T47D, MCF-7, and MDA-231 human breast carcinoma cell lines were routinely maintained in DMEM/F12 medium supplemented with 5% fetal bovine serum and insulin (5 μ g/ml). Endogenous podocalyxin levels were determined by Western blotting of whole cell lysates from subconfluent cultures using the 3D3 antibody.

MCF-7 cells, which expressed low levels of endogenous human podocalyxin (see Fig. 3A), were transfected with a control pIRES-enhanced green fluorescent protein (EGFP) expression vector (BD Biosciences) or with the same vector containing a full-length mouse podocalyxin cDNA (10). We chose to express the mouse gene in the human cells for two reasons. First, we had a mouse-isoform-specific antibody on hand (10), and it has not yet been established if epitope tagging can be successfully carried out without affecting podocalyxin function/localization (see below). Second, although there are differences in the extracellular domain of podocalyxin across species, these are largely restricted to the mucin domains whereas the cytoplasmic domains are >90% identical (11). Within the mucin domains, although these molecules lack linear sequence homology, they contain a very similar frequency of serine, threonine, and proline residues which is the key prerequisite for the O-linked glycosylation that allows these molecules to block adhesion (5, 10). Pooled, stable transfectant populations were generated under G418 (400 μ g/ml) selection.

EGFP and coincident podocalyxin transgene expression was confirmed by dual fluorescence excitation for EGFP and immunofluorescence using a rat monoclonal antibody against mouse podocalyxin (1:100; Ref 10). Ectopic podocalyxin localization was determined by Z-series confocal microscopy in

Received 1/23/04; revised 5/27/04; accepted 6/11/04.

Grant support: Grants from the Canadian Breast Cancer Research Alliance (C. D. Roskelley, D. G. Huntsman) and the Canadian Institutes of Health Research (K. M. McNagny) as well as a grant-in-aid from the Heart and Stroke Foundation of British Columbia and the Yukon (K. M. McNagny). A. Somasiri was a recipient of predoctoral awards from the National Cancer Institute of Canada and the Michael Smith Foundation for Health Research; J. S. Nielsen is a recipient of a National Sciences and Engineering Research Council of Canada predoctoral award; N. Makretsov was supported in part by an educational grant from Aventis; M. L. McCoy is a recipient of a RM Babicki Fellowship; and K. L. McNagny is a scholar of the Canadian Institutes for Health Research and the Michael Smith Foundation for Health Research, as is D. G. Huntsman.

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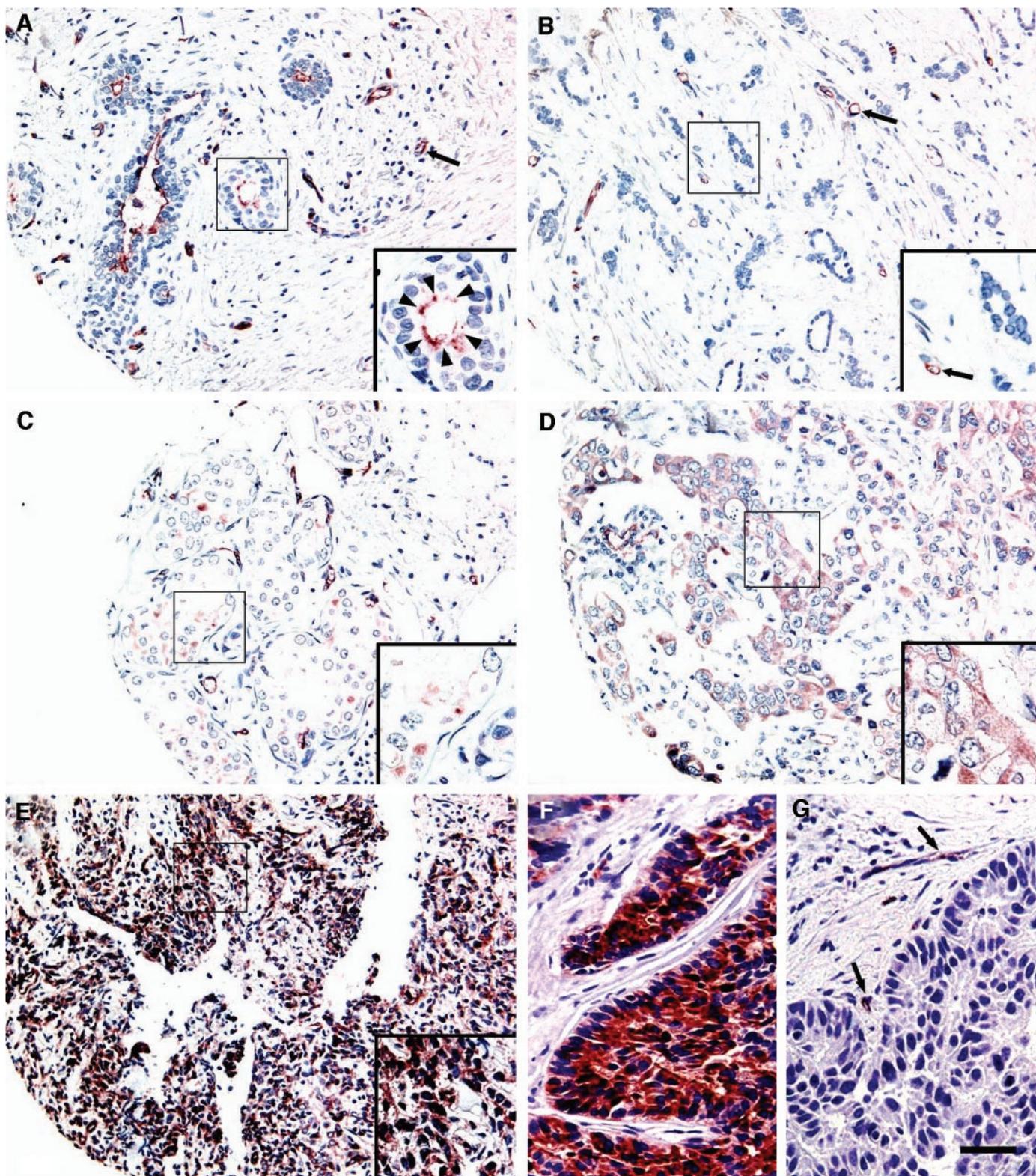


Fig. 1. Podocalyxin is highly expressed in a subset of invasive breast tumors. Normal breast tissue sections (A) and sections from an invasive breast carcinoma TMA (B to E) were immunostained for podocalyxin. In normal breast, positive staining was observed in the apical regions of luminal breast epithelial cells (A inset; arrowheads) and the vascular endothelium (A arrow). Invasive breast carcinomas were scored as follows: "0" (i.e., B) if there was no discernible staining on the carcinoma cells (positive staining is on endothelial cells, arrows); "1" (i.e., C) if <10% of the cells stained positively; "2" (i.e., D) if there was a mixture of diffuse staining in >10% of the cells and/or intense staining in <50% of the cells; or "3" (i.e., E) if there was intense staining in >50% of the cells. In F and G, sections from the same group 3 tumor were immunostained for podocalyxin (F, positive staining prominent in tumor cells) and CD34 (G, positive staining in endothelial cells only, arrows; A-E, bar = 60 μ m, 30 μ m in insets; F and G, bar = 30 μ m).

Table 1 Clinicopathological characteristics of high podocalyxin-expressing tumors

	Tumor histology	Tumor size	Lymph node metastasis	Tumor grade
High podocalyxin group 3; <i>n</i> = 15 (6%)	IDC-13 (87%); ILC-1 (7%); medullary-1 (7%)	≤20 mm-6 (40%); >20 mm-7 (46.6%); unknown-2 (13.4%)	Present-3 (20%); absent-9 (60%); unknown-3 (20%)	I-none; II-6 (40%); III-9 (60%)
Low or no podocalyxin groups 0, 1, 2; <i>n</i> = 257 (94%)	IDC-214 (83%); ILC-25 (10%); mucinous-6 (2%); other-12 (5%)	<20 mm-122 (47.6%); >20 mm-94 (36.6%); unknown-20 (7.8%)	Present-77 (30%); absent-151 (59%); unknown-29 (11%)	I-54 (21%); II-141 (55%); III-62 (24%)
Significance (<i>P</i>)	NS *	NS *	NS *	0.001 *

NOTE. Detailed clinico-pathological characteristics of a subset of tumors with high level of Podocalyxin expression compared to the remainder of the cases (low or no Podocalyxin groups) on the invasive breast tumor microarray.

Abbreviations: IDC, infiltrative ductal carcinoma (not otherwise specified category); ILC, infiltrative lobular carcinoma; NS, non-significant (*P* > 0.05).

* Spearman's non-parametric test was used.

† Log-rank test, disease specific survival.

conjunction with dual staining for E-cadherin (rabbit polyclonal, 1:200; Santa Cruz Biotechnology Biotech, Santa Cruz, CA) or ZO-1 (rabbit polyclonal, 1:200; Zymed, San Francisco, CA).

Transepithelial resistance was assessed on confluent monolayers maintained on Transwell filters with a 3-μm pore size (Costar) using a Millicell electrical resistance system (Millipore, Bedford, MA) according to the manufacturer's instructions. Each transfectant was assessed in duplicate cultures; variation was <10% of the mean, and the data presented are representative of two independent experiments.

Results

Podocalyxin is Highly Expressed in a Subset of Invasive Breast Carcinomas. Podocalyxin was present on podocytes, but not tubular cells, in normal kidney sections (data not shown) confirming that the appropriate conditions were being used for the immunostaining (8). Podocalyxin was also present in normal breast epithelia, but its expression was limited, and its localization was spatially restricted. Specifically, podocalyxin was localized to the apical region of luminal epithelial cells (Fig. 1A; inset, arrowheads). As expected, it was also present on vascular endothelial cells in both normal breast and breast tumor tissue (Fig. 1, A and B, arrows).

Sixty percent (163 of 272) of the invasive breast cancer cases on the TMA did not exhibit detectable podocalyxin in the tumor cells, and they were assigned a score of "0" (Fig. 1B); 23% (62 of 272),

exhibited staining in <10% of the tumor cells, and were assigned a score of "1" (Fig. 1C); 12% (32 of 272) exhibited diffuse staining in >10% of the cells and/or intense staining in <50% of the cells and were assigned a score of "2" (Fig. 1D). The remaining 6% (15 of 272) of the cases on the TMA exhibited intense podocalyxin staining in the majority of the tumor cells, and they were assigned a score of "3" (Fig. 1E). There was no evidence that the intense podocalyxin staining observed in group 3 tumor cells colocalized with CD34 (Fig. 1F and G). Thus, the podocalyxin staining observed on the TMA was very likely specific for this particular member of the CD34 family.

Univariate Kaplan-Meier analysis of disease-specific survival (log-rank test) indicated that the high podocalyxin expression group (*i.e.*, group 3) was associated with poor outcome (Fig. 2). This was most clearly evident when the extremes were compared (*i.e.*, group 0 versus group 3, *P* = 0.007; and 1 versus 3, *P* = 0.04). Although there was a trend in a similar direction, a statistically significant difference was no longer evident when the moderate (group 2) and high overexpressors were compared (2 versus 3, *P* = 0.1). Comparisons between all other individual groups showed no trends or statistical significance (*P* = 0.6–0.8). Thus, for further analysis we pooled groups 0, 1, and 2 (*i.e.*, low/no podocalyxin) and compared them to group 3 only (*i.e.*, high podocalyxin).

There were statistically significant differences in both cumulative disease-free (*P* = 0.01) and overall (*P* = 0.025) survival rates between the pooled low/no podocalyxin and high podocalyxin groups. As a result, the high podocalyxin group had a much lower mean survival time of 9.0 ± 1.8 years compared with 15 ± 0.5 years for the combined low/no podocalyxin pool. The mean survival time of the entire TMA population was 14.9 years.

High Podocalyxin Expression Is an Independent Marker of Poor Outcome. A comparison of clinicopathological characteristics indicated that there were no significant differences in histological subtype, tumor size, lymph node status, or Her-2/*neu* staining between the low/no podocalyxin pool and the high podocalyxin expressors (Table 1). In contrast, there were proportionally more high grade, estrogen receptor negative and abnormal p53 tumors in the high podocalyxin expression group (Table 1).

We next performed a multi-step, Cox regression proportional hazard analysis (Table 2). As expected, regional lymph node involvement was an independent indicator of poor outcome (*P* = 0.012). Strikingly, high podocalyxin expression, on its own, was also a highly significant independent predictor of poor outcome (*P* = 0.0005). Furthermore, the increased relative risk associated with high podocalyxin expression (8.4-fold), although broad in terms of confidence interval, was as great, or greater, than either regional lymph node involvement or Her-2/*neu* overexpression. Thus, podocalyxin overexpression identifies a unique subpopulation of invasive breast tumors with an increased potential to undergo progression.

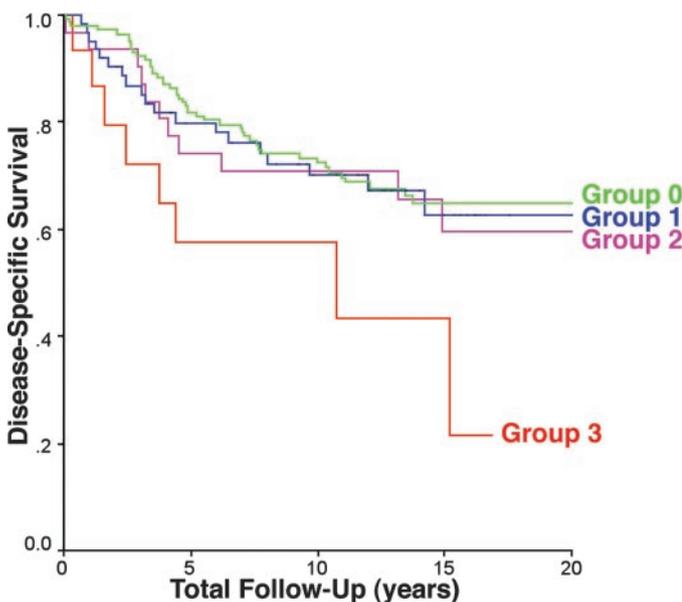


Fig. 2. High podocalyxin expression is associated with decreased disease-specific survival. Long-term disease-specific survival was assessed for all podocalyxin expression groups on the tissue microarray. Note the differential decrease in cumulative survival of the high podocalyxin expressing group (group 3).

Table 1 Continued

ER status	P53 staining	Her-2 staining	Vital status
Positive-5 (33%); negative-9 (60%); unknown-1 (7%)	Positive-5 (33%); negative-8 (53%); unknown-2 (13%)	Strong-2 (13%); weak/neg-13 (87%)	Breast cancer death-8 (53%); other cause death-3 (20%); alive-4 (29%)
Positive-167 (65%); negative-61 (23.7%); unknown-29 (11.3%)	Positive-29 (11%); negative-194 (75%); % unknown-24 (9%)	Strong-22 (9%); weak/neg-225 (88%); unknown-10 (4%)	Breast cancer death-69 (27%); other cause death-58 (22.6%); alive-130 (50.6%)
0.003 *	0.01 *	NS *	0.01 †

Ectopic Podocalyxin Expression Perturbs MCF-7 Breast Carcinoma Cell Junctions. Previously, ectopic expression of human podocalyxin in canine kidney epithelial cells was shown to disrupt junctional complexes between the cells (5). Before determining if the same occurs in breast carcinoma cells, we first examined endogenous podocalyxin levels in human breast tumor lines by Western blotting (Fig. 3A). T47D and MCF-7 breast carcinoma cells, which are weakly metastatic and estrogen receptor positive, contained considerably less podocalyxin than MDA-231 breast carcinoma cells, which are highly metastatic and estrogen receptor negative.

MCF-7 cells form cohesive, junction-containing monolayers, and were thus chosen for ectopic overexpression studies. Cells stably transfected with a control EGFP-expressing vector formed flat epithelial cobblestone monolayers that were indistinguishable from those observed in the parental MCF-7 line (Fig. 3B, top panel; data not shown). In contrast, cells stably transfected with the same vector encoding EGFP and a full-length mouse podocalyxin cDNA formed monolayers that contained areas where the cells bulged apically. When these podocalyxin-transfected cultures reached confluence the bulging cells delaminated from the monolayer and were shed into the medium (Fig. 3B, middle panel). On the basis of triple-labeling for the EGFP marker, transgenic mouse podocalyxin, and nuclear DNA, it was clear that the ectopically expressed podocalyxin was appropriately targeted to the cell surface and that the cells expressing this protein were often apically displaced in the monolayer (Fig. 3B, bottom panel).

In this initial study, we chose to analyze cell junction protein localization in pooled transfected populations as this afforded us internal controls in which ectopic podocalyxin was expressed at levels below the limits of detection. Additionally, those cells that did express ectopic mouse podocalyxin in the pooled population did so at levels that were similar to endogenously expressed podocalyxin in mouse endothelial cells (Fig. 3C).

As expected, the adherens junction protein E-cadherin was localized basolaterally in vector control-transfected MCF-7 cells (Fig. 3D, top panel). In contrast, E-cadherin was often localized around the entire cell circumference in the podocalyxin expressing transfectants.

The tight junction protein ZO-1, which was apically localized in discrete terminal bars in the control cells, was found at multiple levels in the podocalyxin transfectants (Fig. 3D, bottom panel). In addition, transepithelial resistance, which is a functional measure of tight junction patency (5), was significantly reduced in the podocalyxin-transfected cultures (210 ohms cm²) compared with the vector alone-transfected cells (497 ohms cm²). Therefore, we conclude that podocalyxin was capable of perturbing cell junctions between MCF-7 cells.

Discussion

Locally invasive breast cancers, particularly those in which regional lymph node involvement has not been detected at the time of diagnosis, can have markedly different outcomes. Thus, it is extremely difficult to predict which patients with these lesions will most benefit, or not benefit, from adjuvant therapy. Large scale expression profiling has had some impact on this problem, particularly in terms of identifying those tumors that will not progress, which constitutes the majority of the diagnosed lesions of this type (12). Despite these advances, the identification of novel independent indicators of poor outcome continues to be useful as it increases the resolving power of all prognostic strategies. Our initial study with a moderately sized (n = 272) tissue array indicates that high podocalyxin overexpression is very likely such an independent prognostic indicator. However, we are currently screening a much larger TMA (n = 3000) to confirm this conclusion. We are also expanding our screening efforts to include other members of the CD34 family, including endoglycan (10, 13).

In addition to invasive breast cancers, podocalyxin is also overexpressed in high-grade ovarian carcinomas,⁶ and it is dysregulated in human embryonal carcinomas (14). The human podocalyxin gene (*PODXL*) has been assigned to chromosome 7q32-q33 (15). This places *PODXL* between two regions, 7q21-q22 and 7q35ter, that have been identified as chromosomal gain sites by comparative genomic hybridization in ductal breast carcinoma *in situ*, infiltrating ductal carcinoma, and in breast tumor lines (16, 17). Because 7q32-33 has not specifically been implicated as a frequent region of chromosomal gain, it is not surprising that we were only able to identify one *PODXL* amplification event, using fluorescent *in situ* hybridization, among the 15 overexpressors on the TMA.⁷

The paucity of amplification events suggests that podocalyxin gene

Table 2 Podocalyxin overexpression is an independent predictor of poor outcome

Marker	Degrees of freedom	Significance (P) *	Relative risk (RR) †	95% confidence interval for RR ‡	
				Lower	Upper
High podocalyxin (group 3)	1	0.0005	8.446	2.982	23.917
P53 immunoreactivity	1	0.581	1.329	0.485	3.643
Estrogen receptor status	1	0.498	0.716	0.273	1.881
HER-2 overexpression	1	0.136	1.913	0.814	4.494
Lymph node involvement	1	0.012	3.688	1.581	8.601
Tumor grade, high	2	0.663	1.253	0.454	3.545
Tumor size, >2 cm	1	0.369	1.364	0.692	2.689

NOTE. Cox expression multivariate analysis of disease-specific survival.

* Considered a significant independent indicator of poor outcome at P < 0.05.

† Mean-fold increase in relative risk of the group compared to the entire population used to generate the tissue microarray.

‡ Upper and lower margins of relative risk using two standard deviations of variation about the mean.

⁶ M. L. McCoy, C. B. Gilks and C. D. Roskelley, unpublished observations.

⁷ L. Prentice and D. G. Huntsman, unpublished observations.

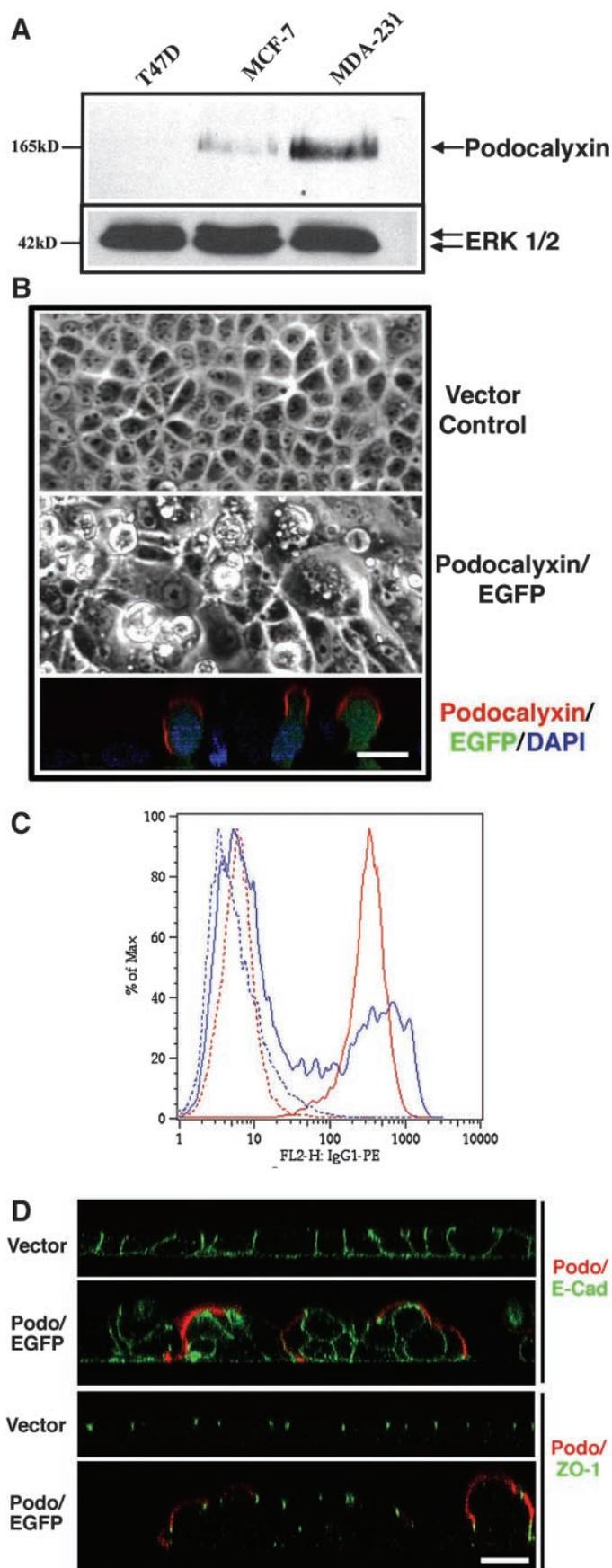


Fig. 3. Podocalyxin overexpression perturbs breast carcinoma cell junctions. A, endogenous podocalyxin levels in three human breast carcinoma lines, T47D, MCF-7, and MDA-231, were assessed by Western blotting (bottom panel), extracellular signal-regulated kinase (ERK)1/2 loading control). B, MCF-7 cells were stably transfected with

expression and/or protein stability are dysregulated in the group 3, high expressing tumors. Although a detailed dissection of the podocalyxin promoter has not yet been performed, the expression of the gene is altered, either directly or indirectly, by estrogen signaling during normal mammary gland development (18). Steady-state levels and the localization of podocalyxin, both of which were altered in the high expressing tumors, might also be regulated by functionally important binding proteins. One class of binding proteins worth considering in this regard are the sodium-hydrogen exchanger regulatory factors that interact with the cytoplasmic domain of podocalyxin and link it to the actin cytoskeleton (19). Interestingly, sodium-hydrogen exchanger regulatory factor-1 is often down-regulated in estrogen receptor-negative breast tumors (20), and the majority of the group 3 high podocalyxin expressors on the TMA were estrogen receptor negative.

It has been proposed that the disruption of epithelial cell junctions contributes to invasive breast carcinoma progression (1). During normal kidney development, podocalyxin, which is the major glomerular anion, disrupts epithelial cell junctions between differentiating podocytes during the formation of the primary urinary filter (10). Additionally, forced overexpression of human podocalyxin perturbs cell junctions in cultured canine MDCK cells (5). We found that forced overexpression of podocalyxin initiated similar changes in human MCF-7 breast carcinoma cells. We are now carrying out combinatorial oncogene/podocalyxin expression experiments to definitively determine whether this anti-adhesive ability contributes to the metastatic progression *in vivo*. If this turns out to be the case, in addition to its prognostic significance, podocalyxin could become a new therapeutic target in the fight against breast cancer progression.

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vectors expressing EGFP alone or coexpressing EGFP and full-length mouse podocalyxin. EGFP/vector-control-transfected cells formed classical MCF-7 cobblestone epithelial monolayers (top panel) whereas bulging cells were shed from the monolayers of EGFP/podocalyxin transfectants (middle panel). EGFP (green) and mouse podocalyxin (red) were coordinately expressed in cells transfected with the EGFP/podocalyxin vector (bottom panel). Ectopic mouse podocalyxin protein was targeted to the cell surface, and it was consistently expressed by cells that bulged apically as demonstrated by the upward migration of the 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei (blue). Top two panels, live phase microscopy (bar = 50 μm); bottom panel, Z-series confocal microscopy (bar = 15 μm). C, pooled EGFP/podocalyxin transfectants were stained for mouse podocalyxin (solid blue line) or the rat IgG1 isotype control (dotted blue line) followed by biotinylated antirat IgG1 and streptavidin-conjugated to phycoerythrin. The entire heterogeneous transfectant population was then subjected to fluorescence activated cell-sorting analysis for phycoerythrin on the X-axis. The red lines represent the mouse endothelial cell line BEND3 that expresses endogenous podocalyxin (Ref. 10; solid red line, with antipodocalyxin; dotted red line with antirat IgG1 control). Note that those transfectants that expressed the mouse podocalyxin transgene did so at levels similar to that observed in the BEND cells (i.e., solid blue and red line peaks between 100 and 1,000 arbitrary fluorescent units). D, vector control and pooled EGFP/podocalyxin transfectants were double-stained for mouse podocalyxin (red) and either the adherens junction protein E-cadherin (top panel, green) or the tight junction protein ZO-1 (bottom panel, green) and then subjected to Z-series confocal microscopy. Note that in the podocalyxin transfectants, E-cadherin was often localized around the entire cell circumference instead of just along basolateral domains, as was the case in the vector controls. In addition, ZO-1 puncta were located at numerous levels in podocalyxin transfectants rather than being exclusively confined to the discrete apical terminal bar, as was the case in the controls (bar = 15 μm).

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The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

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Cancer Res 2004;64:5068-5073.

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