Progression of Cervical Carcinomas Is Associated with Down-Regulation of CD9 But Strong Local Re-expression at Sites of Transendothelial Invasion

Georg Sauer, Jasmin Windisch, Christian Kurzeder, Volker Heilmann, Rolf Kreienberg, and Helmut Deissler

Department of Obstetrics and Gynecology, University of Ulm Medical School, Ulm, Germany

ABSTRACT

Purpose and Experimental Design: **Lymphovascular space invasion plays a critical role in the progression of cervical cancer and is an indicator of an unfavorable prognosis, even in patients with early-stage disease. Identification and functional characterization of molecules that are predominantly expressed in tumors able to penetrate lymphatic vessels may therefore help to improve the clinical assessment of cervical neoplasias with unclear prognosis. We used immunohistochemical staining to assess expression of the tetraspanin adapter protein CD9 in cervical tumors because inverse correlations with tumor invasiveness, ability to form metastases, and poor clinical outcome have been described for several other tumor types.**

Results and Conclusion: **We found that CD9, strongly expressed by cells forming the basal layer of normal squamous epithelium of the cervix, is down-regulated in most invasive cervical carcinomas (correlation with stage, $P = 0.015$) but apparently re-expressed at distinct regions during tumor progression. Tumor sites with pronounced localized expression of CD9 (CD9 hotspots) include cones growing into blood or lymphatic vessels, pointing to a functional role of CD9 in transendothelial migration as a crucial step in the formation of lymph node metastases. Remarkably, CD9 hotspots were found to be a highly significant ($P < 10^{-5}$) indicator of lymphangiosis: they were observed in 15 of 18 cases with histopathologically confirmed lymphangiosis compared with 4 of 26 other cervical carcinomas. We postulate, therefore, that clusters of tumor cells characterized by strong expression of CD9 may be useful as an indicator of high risk of recurrence in early-stage cervical cancer, providing a basis for clinical decisions in favor of additional treatment.**

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Requests for reprints: Helmut Deissler, Department of Gynecology and Obstetrics, University of Ulm Medical School, Frauensteige 14, D-89075 Ulm, Germany. Phone: 49-731-500-27660; Fax: 49-731-500-26683; E-mail: helmut.deissler@medizin.uni-ulm.de.

INTRODUCTION

Cervical cancer is one of the most common malignant diseases in females worldwide. It is frequently diagnosed at early stages by cytological screening. Despite the generally good prognosis for stage I cervical cancer, approximately one-third of these patients are expected to eventually die from that disease. To improve the assessment of an individual patient's risk of disease progression, several prognostic parameters, including lymph node status, tumor size, paracervical involvement, depth of stromal invasion, and infiltration of the lymphovascular space, have been identified. However, clinical staging remains the most important and informative prognostic factor for patients suffering from invasive cervical cancer. The invasion of lymphatic or blood vessels by cancer cells is considered a critical step in the formation of lymphatic or distant metastases. During progression of cervical cancer, the malignant cells usually spread by direct local invasion to the surrounding tissue and the lymphatic system to form metastases in the pelvic and para-aortal lymph nodes. Involvement of lymphatic vessels clearly is an indicator of an unfavorable prognosis, even in patients with early-stage disease. The identification and functional characterization of molecules involved in lymphovascular space invasion may therefore reveal targets for new diagnostic and therapeutic approaches beneficial to clinically defined subgroups of patients.

On the molecular level, adhesion, motility, and the capacity of tumor cells to invade surrounding tissue is determined by diverse groups of cell surface glycoproteins that influence a wide variety of biological processes by linking cell–cell and cell–matrix interactions to signaling pathways. Among those, tetraspanins play a special role as transmembrane adapter proteins in functional complexes with adhesion molecules and other proteins that are implicated in various cell types in the regulation of cell differentiation, proliferation, motility, and invasion (1). Modulation of adhesive properties by tetraspanins is likely to result from their association with integrins, but E-cadherin and other tetraspanin-binding proteins may also play important roles in certain cell types (2). For several malignant diseases, *e.g.*, breast, colon, lung, and ovarian cancer, the level of expression of the best-studied tetraspanin, CD9, was found to correlate with the stage of tumor progression or appearance of metastases (3–8). These results point to a high prognostic value of CD9 expression that may be helpful in the clinical assessment of these tumors.

We analyzed CD9 expression of normal and neoplastic cervical tissue and demonstrated a clear inverse correlation with the tumor stage. Moreover, hotspots of CD9 expression were found to be indicative of lymphovascular space invasion and poor prognosis.

MATERIALS AND METHODS

Tissue Samples. We examined formalin-fixed, paraffin-embedded surgical specimens from 44 consecutive patients with invasive cervical cancer: 11 without lymph node metastases, 15 with metastases to pelvic lymph nodes, and 18 with both lymphatic space invasion and lymph node metastases.

Before immunohistochemical staining, H&E-stained slides of all samples were re-examined, and the original histopathological diagnoses submitted by the attending pathologists were confirmed. Under approved internal review board protocols (no. 134/2001; University of Ulm), additional samples of normal cervical tissue were obtained from women who underwent hysterectomy for reasons other than neoplasias of either the cervix or endometrium.

Immunohistochemistry. Immunohistochemical staining was performed with the monoclonal mouse IgG1 antibodies NCL-CD9 (Novo Castra Laboratories, Newcastle, United Kingdom), which binds to the major extracellular loop of human CD9, and for double staining of vessels, MCAP547 (Serotec, Oxford, United Kingdom), which recognizes the class II epitope on CD34.

From paraffin-embedded specimens fixed in 4% buffered-formalin, 4-μm sections were prepared and deparaffinized with xylol and ethanol. After sections were heated in 0.01 M citrate buffer for 15 min in a microwave oven, they were allowed to cool to room temperature and rinsed thoroughly with PBS. Endogenous alkaline phosphatases were blocked by subsequent incubations for 10 min with 20% acetic acid and 2.3% sodium periodate; endogenous biotin was blocked by treatment with avidin for 15 min with a Biotin/Avidin Blocking Kit (BioGenex, San Ramon, CA) according to the manufacturer's instructions. Tissue samples were then blocked with a buffered solution of casein (Power Block; BioGenex) and incubated with the diluted (1:20) anti-CD9 antibody at 4°C overnight. Bound primary antibodies were detected with a Super Sensitive Immunostaining Kit (DakoCytomation, Hamburg, Germany) containing a biotinylated link antibody and alkaline phosphatase-conjugated streptavidin and the recommended Fast Red Substrate Pack (BioGenex) containing the substrates Fast Red and naphthol phosphate.

For double immunohistochemical staining with anti-CD9 and anti-CD34 antibodies, the EnVision Doublestain System (DakoCytomation) was used according to the protocol supplied by the manufacturer. Briefly, endogenous peroxidases were inactivated and specimens were incubated with the anti-CD9 antibody for 30 min. Bound primary antibody was detected by subsequent incubations with a horseradish peroxidase-labeled polymer coupled to antimouse immunoglobulins (for 30 min) and the substrate/chromogen solution (Liquid DAB+) containing hydrogen peroxide and 3,3′diaminobenzidine. Before incubation with the diluted (1:100) anti-CD34 antibody for 30 min, all soluble components were washed off, and tissues were blocked with the provided doublestain block solution. Anti-CD34 immunoreactivity was detected with an alkaline phosphatase-labeled polymer conjugated with antimouse immunoglobulins (for 30 min) and a solution containing the substrate naphthol phosphate and the chromogen Fast Red. In contrast to monostaining, which always ended with precipitated red dye indicating CD9 expression, doublestaining produced CD34 stained red and CD9 stained brown.

All sections were counterstained with hematoxylin. For all sections immunostained with specific antibodies, parallel sections were processed with isotype-matched control antibodies to confirm specificity.

Evaluation of Immunohistochemical Staining. Immunohistochemical staining of plasma membranes, considered indicative of expression of CD9, was examined independently by two investigators (G. S and J. W.) who had no previous knowledge of the histopathological classification. At least four representative fields were examined twice by light microscopy, which was sufficient in view of the low variability of CD9 staining throughout the tumor mass. The overall intensities of CD9-specific staining of the tumor tissues were indicated by scores that were assigned according to the following scale: (−), no staining; (+), <30%; (++), 30–70%; (+++), >70% of the intensity observed in the basal layers of squamous epithelium in normal cervix. In addition, small areas consisting of intensely (intensity >70%) stained CD9-positive cells at a high density (CD9 hotspots), which were observed even in mainly CD9-negative tumors, were counted. The cervical carcinomas were classified according to the presence of CD9 hotspots (see Fig. 2*E*) in certain regions of the tumor sections. These tumor cell clusters with strong CD9 immunoreactivity were observed at a typical density of three to five in at least three microscopic fields, but were never detected in tumors classified as hot spot-negative. Correlations between CD9 expression or the presence of CD9 hotspots and tumor characteristics or clinical data were tested for statistical significance by analyses of contingency tables with Fisher's exact test.

RESULTS

Immunohistochemical Detection of CD9 in Normal Cervix and Intraepithelial Neoplasias. The cells forming the basal layer of normal squamous epithelium of the cervix showed strong CD9-specific immunoreactivity at cell–cell and cell–basal membrane adhesive sites, which was pronounced in regions adjacent to stromal tissue. In the direction of the cervical surface, expression of CD9 decreased and could not be detected in the outer cell layers (Fig. 1). Neoplastic cell growth within the squamous epithelium (similar to cervical intraepithelial neoplasia lesions), which was observed in regions of analyzed invasive cervical carcinomas with a still intact basal membrane, was not associated with a decrease in CD9 expression.

Expression of CD9 in Cervical Carcinomas. Expression of CD9 was assessed by immunohistochemical staining of 44 cervical carcinomas classified as stage I tumors (18 of 44) or stage II–IV malignancies. The strong CD9 expression on cells forming the basal layers of the squamous epithelium from which the tumors arose was not conserved in most tumors. An interesting example is shown in Fig. 2: CD9 was found to be down-regulated in the main tumor mass (Fig. 2, *C* and *D*) surrounded by stromal tissue, whereas it was still expressed by tumor cells in other regions immediately after invasion through the basement membrane (Fig. 2, *A* and *B*). Most of the analyzed invasive cervical carcinomas were CD9-negative or showed only weak or moderate CD9-specific immunoreactivity (Table

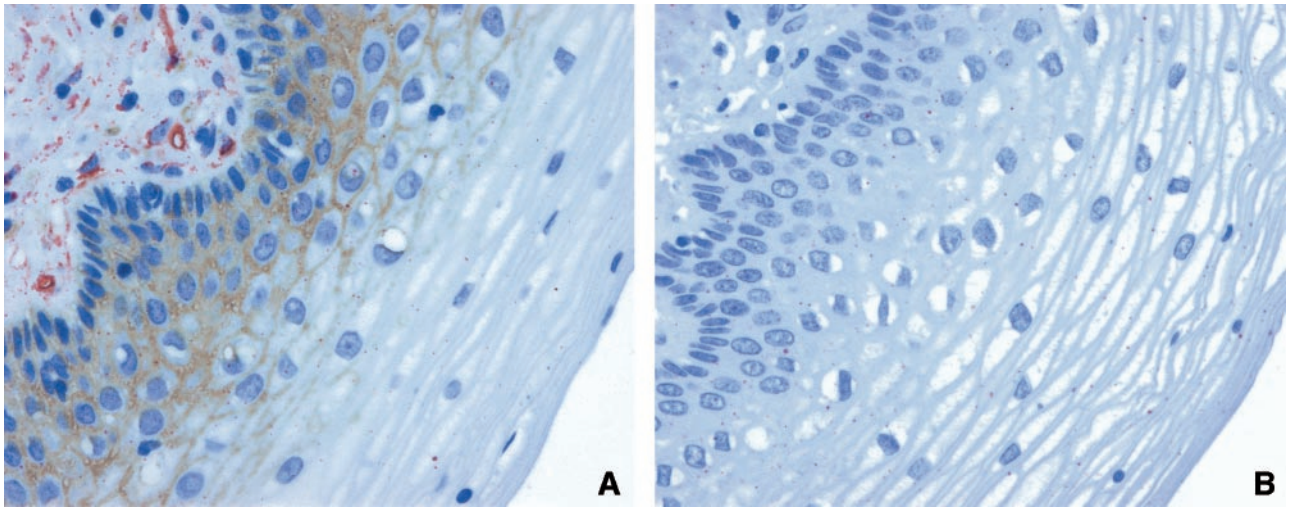

Fig. 1 *A*, double CD9- and CD34-specific immunohistochemical staining of normal squamous epithelium of the cervix. *B*, staining with isotype-matched control antibodies. *Brown staining* is indicative of CD9 expression; *red precipitates* indicate binding of the anti-CD34 antibody.

1). Complete loss of CD9 was observed in 77% stage II–IV tumors but in only 39% of the tumors classified as stage I, which was a significant (Fisher's $P = 0.015$) correlation. Similar analyses revealed that CD9 expression was not associated with other clinical features, *e.g.*, lymphangiosis ($P = 0.346$) and involvement of lymph nodes ($P = 0.716$).

Interstingly, several of the carcinomas contained small clusters of tumor cells that strongly expressed CD9 even in weakly stained or negative tumor sections (Fig. 2*E*). Immunohistochemical doublestaining with antibodies binding to CD9 and CD34 revealed that these CD9 hotspots, in which CD9 appeared to be re-expressed, were predominately located at sites of invasion into blood or lymphatic vessels (Fig. 2*F*). CD9 hotspots were detected at a density of at least three per microscopic field in $\sim$ 40% of both stage I and stage II–IV cervical carcinomas. They were observed in only 1 of 10 tumors from patients with tumor-free lymph nodes, but in 18 of 34 (53%) tissues derived from N1 tumors (Fisher's $P = 0.271$). Most remarkable, CD9 hotspots were a highly significant ($P < 10^{-5}$) indicator of lymphangiosis: they were observed in 15 of 18 (83%) cases with histopathologically confirmed lymphangiosis compared with 4 of 26 (15%) tumors without this characteristic associated with poor prognosis (Fig. 3).

DISCUSSION

In the present study, we observed altered expression of CD9 in cervical carcinoma cells with distinct regions of strong immunoreactivity at sites of vessel penetration. These investigations were based on numerous reports pointing to a role of CD9 and other members of the tetraspanin family of transmembrane adapter proteins in the regulation of cellular processes considered relevant for malignant conversion, *e.g.*, cell adhesion, migration, and invasion (1, 9, 10). Accordingly, the level of CD9 expression was demonstrated to correlate with invasiveness and ability to form metastases in various types of human malignant diseases. Miyake *et al.* (3) described an inverse correlation between CD9 expression in invasive ductal breast carcinomas and nodal status of the patients and showed that CD9 is often down-regulated in the corresponding metastases. Further investigations revealed that weak expression of CD9 is an indicator of disease-free and overall survival for breast cancer patients (4, 11). However, these results were not confirmed by a recent report on routine immunohistochemical staining of biopsies from 80 malignant breast tumors (12), suggesting the need for methodological refinement and additional studies before routine staining of CD9 can be used to aid in decisions on therapeutic options in the management of breast cancer. Other human malignancies for which down-regulation of CD9 expression during tumor progression and a prognostic value of this tetraspanin have been reported include lung, pancreatic, endometrial, ovarian, and oral squamous cell carcinomas (6, 8, 13–15). Among neuroepithelial tumors, however, high expression of CD9 appears to be a characteristic of high-grade rather than low-grade astrocytic tumors (16).

The role of CD9 in the development of cervical carcinomas to stages with more malignant phenotypes of tumor cells, which are responsible for the clinically highly relevant local invasion and infiltration of nearby lymph nodes, has not been investigated. In accordance with analyses of other tumor types, we report a clear inverse correlation of overall CD9 expression of these tumors with their clinical stage. Moreover, our results invite the hypothesis that during tumor progression, not only is CD9 expression down-regulated to promote the expansion of malignant cells but that CD9 is locally re-expressed to adjust to microenvironmental requirements. In a model based on these observations, constant high or even elevated expression of CD9 is needed for the transition of tumor cells through the basement membrane. Tumor growth is then driven by cells possessing low amounts of cell surface CD9, but only cells able to up-regulate CD9 can enter blood or lymphatic vessels during further progression. The assumption that progression of cervical cancer is associated with continuous remodeling of adhesion and adapter

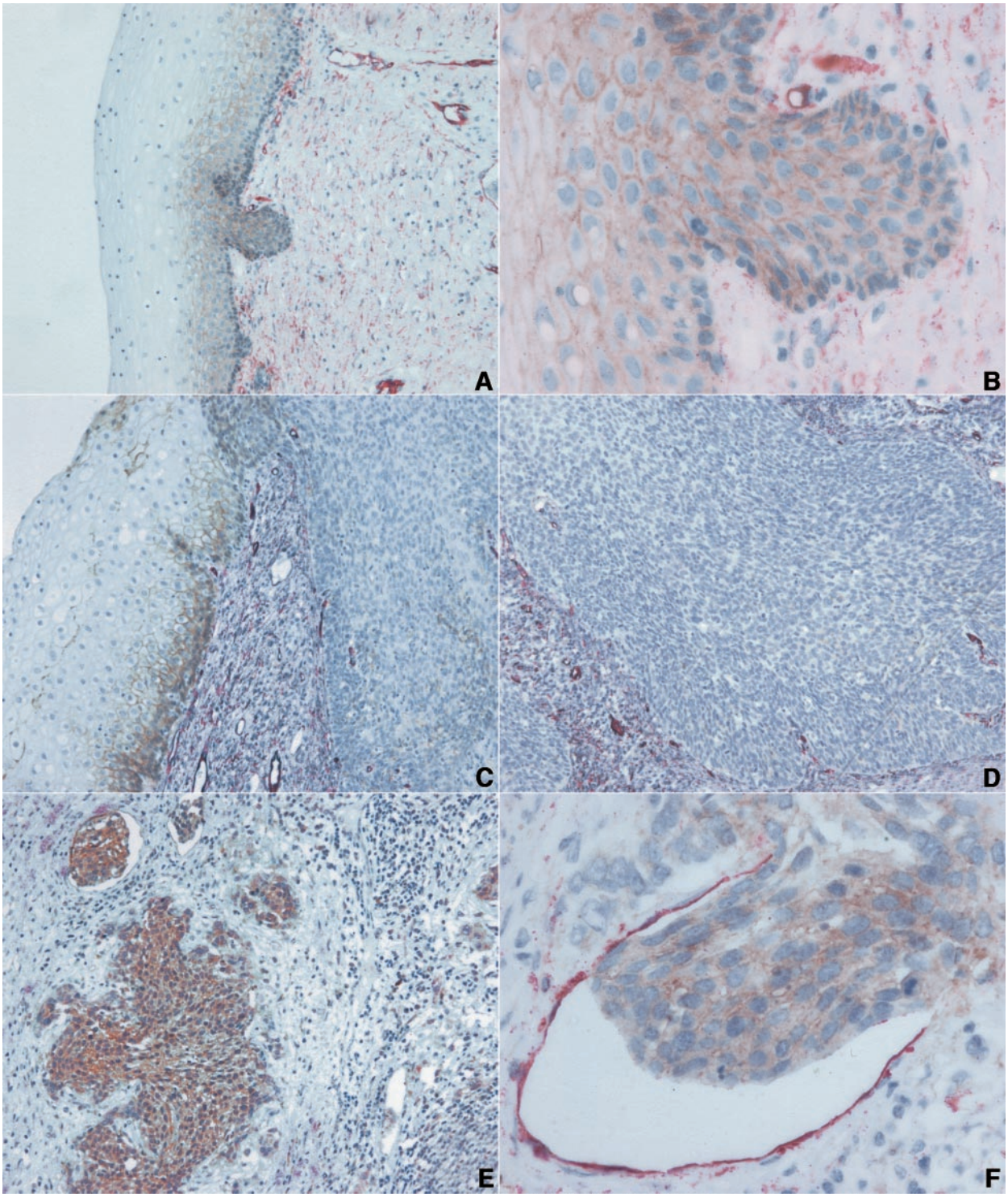

Fig. 2 Double CD9- and CD34-specific immunohistochemical staining of cervical carcinomas. *Brown staining* is indicative of CD9 expression; *red precipitates* indicate binding of the anti-CD34 antibody. *A* and *B*, CD9-expressing tumor cells immediately after invasion through the basement membrane. *C* and *D*, typical CD9-negative main tumor mass of a high-stage tumor, showing contrasting CD9-expressing basal layers of the squamous epithelium. *E*, intensely stained CD9 hotspots detected adjacent to the CD9-negative main tumor mass. *F*, cluster of CD9-expressing cells, present in a CD9-negative tumor, entering a CD34-positive vessel.

molecules, which may also be up-regulated in complex *in vivo* processes contributing to malignancy, has recently been supported by Wollscheid *et al.* (17), who described strong expression of the tetraspanin Tspan-1 (NET-1) in highly malignant carcinomas, whereas normal epithelial cells, early cervical intraepithelial neoplasia lesions, and well-differentiated squamous cell carcinomas never showed Tspan-1-specific immunoreactivity. Thus, analyses of the expression levels of the tetraspanins CD9 and Tspan-1 may be combined to yield information helpful in the clinical management of cervical cancer. More tumors will have to be analyzed, however, to confirm the prognostic value of Tspan-1.

In addition to these histopathological observations, direct evidence for an important role of CD9 in tumor–endothelial cell interaction and vascular dissemination of malignant cells came recently from *in vitro* studies of transendothelial migration of melanoma cells. Longo *et al.* (18) found that CD9, as well as the tetraspanins CD81 and CD151, was localized at sites of contact between A375 melanoma and endothelial cells. CD9 expressed on the surface of endothelial cells appeared to be redistributed during transendothelial transition and focused adjacent to the inserted tumor cells. Furthermore, transendothelial migration was shown to be inhibited by treatment with anti-CD9 antibodies. These results support a specific function of CD9 necessary for vascular dissemination during late tumor progression and are, therefore, in accordance with our results pointing to the existence of CD9 re-expressing cell clusters that mediate transendothelial invasion of cervical carcinoma cells. However, the underlying mechanism may be different from that postulated by Longo *et al.* (18) because they found that CD9 was provided by endothelial rather than melanoma cells, for which, similar to other tumor types, increased malignancy seems to be associated with low expression of this tetraspanin (19). It can be concluded from these and numerous other studies, *e.g.,* on the formation of heterologous gap junctions in the B16 mouse melanoma model (20), that specific interactions between tumor and endothelial cells are crucial for the formation of new blood vessels and vascular dissemination of malignant cells by transendothelial migration.

Table 1 Patient characteristics and CD9-specific immunoreactivity of cervical carcinomas

	No. of tumors with CD9-specific immunoreactivity				
	−	+	++	+++	Hotspots
Tumor stage					
I (18/44)	7	4	4	3	8
II (22/44)	17	2	1	2	9
III (3/44)	3	0	0	0	2
IV (1/44)	0	1	0	0	0
Tumor grading					
G_1 (1/44)	1	0	0	0	0
G_2 (24/44)	13	4	4	3	10
G_3 (15/44)	10	2	1	2	8
G_4 (1/44)	1	0	0	0	0
G_x (3/44)	2	1	0	0	1
Lymph node involvement					
Positive nodes (34/44)	20	7	3	4	18
Negative nodes (10/44)	7	0	2	1	1
Lymphatic vessel invasion					
With invasion (18/44)	13	2	2	1	15
Without invasion (26/44)	14	5	3	4	4

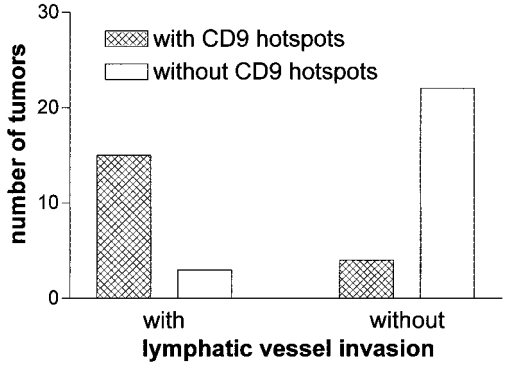

Fig. 3 CD9 expression of tumors with (left bars) and without (right bars) lymphangiosis.

Our observation that CD9 is predominately expressed by clusters of cervical carcinoma cells close to vessels and in the process of transendothelial transition evidently supports a key function of tumor cell CD9 at heterologous cell–cell contacts indicative of increased malignancy. We demonstrated that the presence of CD9 hotspots is associated with invasion and infiltration of lymphatic vessels. Their localization at sites of transendothelial invasion suggests an important role of these cell clusters in tumor progression. Detection of such small regions of functional relevance may, therefore, be more informative than overall assessment of expression and may be used as an indicator of poor prognosis in cases with unclear or unknown histopathological characteristics. This is reminiscent of the well-established concept of microvessel density as a surrogate marker of neoangiogenesis (21). Furthermore, these clusters with strong CD9-specific immunoreactivity in mainly CD9-negative tumors may define a subgroup of patients with tumors that contain highly malignant cells before the involvement of lymph nodes, lymphangiosis, or other negative prognostic factors can be detected at later stages of tumor progression. The prognostic value of CD9 expression, particularly of CD9 hotspots, will have to be confirmed by future studies with more cervical tumors and analyses of patients' clinical courses. Moreover, this study may initiate further investigations of other types of tumors for which down-regulation during tumor progression has been reported, with the aim of detecting small CD9-positive cell clusters associated with transendothelial transition.

REFERENCES

1. Boucheix, C., and Rubinstein, E. Tetraspanins. Cell. Mol. Life Sci., *58:* 1189–1205, 2001.

2. Yanez-Mo, M., Tejedor, R., Rousselle, P., and Madrid, F. Tetraspanins in intercellular adhesion of polarized epithelial cells: spatial and functional relationship to integrins and cadherins. J. Cell Sci., *114:* 577–587, 2001.

3. Miyake, M., Nakano, K., Ieki, Y., Adachi, M., Huang, C. L., Itoi, S., Koh, T., and Taki, T. Motility related protein 1 (MRP-1/CD9) expression: inverse correlation with metastases in breast cancer. Cancer Res., *55:* 4127–4131, 1995.

4. Miyake, M., Nakano, K., Itoi, S. I., Koh, T., and Taki, T. Motility-related protein-1 (MRP-1/CD9) reduction as a factor of poor prognosis in breast cancer. Cancer Res., *56:* 1244–1249, 1996.

5. Mori, M., Mimori, K., Shiraishi, T., Haraguchi, M., Ueo, H., Barnard, G. F., and Akiyoshi, T. Motility related protein 1 (MRP1/CD9) expression in colon cancer. Clin. Cancer Res., *4:* 1507–1510, 1998.

6. Higashiyama, M., Taki, T., Ieki, Y., Adachi, M., Huang, C. L., Koh, T., Kodama, K., Doi, O., and Miyake, M. Reduced motility related protein-1 (MRP-1/CD9) gene expression as a factor of poor prognosis in non-small cell lung cancer. Cancer Res., *55:* 6040–6044, 1995.

7. Hashida, H., Takabayashi, A., Tokuhara, T., Taki, T., Kondo, K., Kohno, N., Yamaoka, Y., and Miyake, M. Integrin alpha3 expression as a prognostic factor in colon cancer: association with MRP-1/CD9 and KAI1/CD82. Int. J. Cancer, *97:* 518–525, 2002.

8. Houle, C. D., Ding, X. Y., Foley, J. F., Afshari, C. A., Barrett, J. C., and Davis, B. J. Loss of expression and altered localization of KAI1 and CD9 protein are associated with epithelial ovarian cancer progression. Gynecol. Oncol., *86:* 69–78, 2002.

9. Hemler, M. E. Specific tetraspanin functions. J. Cell Biol., *155:* 1103–1107, 2001.

10. Maecker, H. T., Todd, S. C., and Levy, S. The tetraspanin superfamily: molecular facilitators. FASEB J., *11:* 428–442, 1997.

11. Huang, C. I., Kohno, N., Ogawa, E., Adachi, M., Taki, T., and Miyake, M. Correlation of reduction in MRP-1/CD9 and KAI1/CD82 expression with recurrences in breast cancer patients. Am. J. Pathol., *153:* 973–983, 1998.

12. Jamil, F., Peston, D., and Shousha, S. CD9 immunohistochemical staining of breast carcinoma: unlikely to provide useful prognostic information for routine use. Histopathology, *39:* 572–577, 2001.

13. Sho, M., Adachi, M., Taki, T., Hashida, H., Konishi, T., Huang, C. L., Ikeda, N., Nakajima, Y., Kanehiro, H., Hisanaga, M., Nakano, H., and Miyake, M. Transmembrane 4 superfamily as a prognostic factor in pancreatic cancer. Int. J. Cancer, *79:* 509–516, 1998.

14. Miyamoto, S., Maruyama, A., Okugawa, K., Akazawa, K., Baba, H., Maehara, Y., and Mekada, E. Loss of motility-related protein 1 (MRP1/CD9) and integrin alpha3 expression in endometrial cancers. Cancer (Phila.), *92:* 542–548, 2001.

15. Kusukawa, J., Ryu, F., Kameyama, T., and Mekada, E. Reduced expression of CD9 in oral squamous cell carcinoma: CD9 expression inversely related to high prevalence of lymph node metastasis. J. Oral Pathol. Med., *30:* 73–79, 2001.

16. Kawashima, M., Doh-ura, K., Mekada, E., Fukui, M., and Iwaki, T. CD9 expression in solid non-neuroepithelial tumors and infiltrative astrocytic tumors. J. Histochem. Cytochem., *50:* 1195–1203, 2002.

17. Wollscheid, V., Kuhne-Heid, R., Stein, I., Jansen, L., Kollner, S., Schneider, A., and Durst, M. Identification of a new proliferation-associated protein NET-1/C4.8 characteristic for a subset of high-grade cervical intraepithelial neoplasia and cervical carcinomas. Int. J. Cancer, *99:* 771–775, 2002.

18. Longo, N., Yanez-Mo, M., Mittelbrunn, M., de la Rosa, G., Munoz, M. L., Sanchez-Madrid, F., and Sanchez-Mateos, P. Regulatory role of tetraspanin CD9 in tumor-endothelial cell interaction during transendothelial invasion of melanoma cells. Blood, *98:* 3717–3726, 2001.

19. Kondoh, M., Ueda, M., Ichihashi, M., and Mishima, Y. Decreased expression of human melanoma-associated antigen ME491 along the progression of melanoma pre-canceroses to invasive and metastatic melanomas. Melanoma Res., *3:* 241–245, 1993.

20. Ito, A., Katoh, F., Kataoka, T. R., Okada, M., Tsubota, N., Asada, H., Yoshikawa, K., Maeda, S., Kitamura, Y., Yamasaki, H., and Nojima, H. A role for heterologous gap junctions between melanoma and endothelial cells in metastasis. J. Clin. Investig., *105:* 1189–1197, 2000.

21. Weidner, N., Semple, J. P., Welch, W. R., and Folkman, J. Tumor angiogenesis and metastasis—correlation in invasive breast carcinoma. N. Engl. J. Med., *324:* 1–8, 1991.