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Prognostic Value of the Immunocytochemical Detection of Extramural Venous Invasion in Dukes' C Colorectal Adenocarcinomas

A Preliminary Study

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In postsurgical staging of colorectal adenocarcinomas, it is sometimes difficult to determine the range of possible venous spread. Distinguishing between the extramural veins (especially when the neoplastic embolus takes up the whole lumen and the endothelium cannot be identified) and the smallest extramural lymph nodes (when they are completely replaced by metastatic carcinoma, leaving the capsule alone) is also difficult. This work proposes a more precise definition of true venous invasion to improve histopathologic staging. Immunohistochemical techniques employing commercial antibodies against Factor VIII RAG, with and without enzymatic digestion, and UEA I lectin for residual endothelium detection, were applied, as well as antibodies against vimentin, desmin, and alpha sm-1 actin to detect wall components. The immunohistochemical evaluation of colorectal adenocarcinomas, in particular by anti-alpha sm-1 actin antibodies, permitted a reliable morphologic distinction of the true venous invasion. This factor proved to be relevant for survival rate prediction. (Am J Pathol 1989, 135:939–945)

Permeation of extramural veins by malignant cells in invasive colorectal adenocarcinoma is associated with poor prognosis.[1,2] Furthermore, distant metastases are more likely when large veins rather than small venules are invaded.

Many investigators studied the relationships among venous invasion, visceral metastases, and survival. Their inconsistent results, reviewed by Talbot and coworkers,[3] are probably due to the lack of reliable identification of venous spread. It is thus important to find accurate histopathologic criteria for this purpose.

It is also difficult to determine the real nature of a small caliber vessel by conventional histochemical staining, as reported for tumors in other sites, such as carcinoma of the breast,[4–9] bladder,[10] thyroid,[11,12] and uterine cervix.[13]

All the above-mentioned researchers focused their attention on endothelium or basement membranes immunohistochemically detected by means of specific antibodies or ligands. In blood vessel identification, relating to tumor staging, however, conventional histochemical methods are usually employed. These sometimes give doubtful results and leave out a precise definition of wall components.

The original size of veins completely distended by tumors is sometimes difficult to determine on histologic section. Invaded veins are thus classified by their walls: "thin-walled" veins or venules with little or no muscle and "thick-walled" veins with prominent muscle.[3] They cannot always be distinguished by using conventional stainings, because muscle cells are sometimes masked by fibrosis and tissue reaction. In addition, recognition of true venous invasion may be difficult, especially when the wall is partially damaged by neoplastic emboli or completely destroyed or replaced by fibrous tissue (the so-called "possible" venous invasion). Furthermore, differential diagnosis in possible invasion[14] may be difficult due either to the smallest extramural lymph node being completely replaced by metastatic carcinoma, leaving only the capsule, or to a nest of neoplastic cells surrounded by reactive desmoplastic tissue.

Therefore, we examined a series of Dukes' C colorectum adenocarcinomas, with neoplastic nests in pericolic fibrous tissues, that had doubtful vessel invasion or lymph node involvement. We hoped to 1) find more available identification of definite extramural vein invasion (also in

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Table 1. *Immunoperoxidase (ABC) Procedure*

Steps	Time
1. Dewax tissue sections (collected on gelatinized slides) through xylene, alcohols, to water	
2. Blocking of endogenous peroxidase activity[20]	
Wash with phosphate buffered saline	30 minutes
solution (PBS)	5 minutes
3. Background blocking with normal nonimmune serum	
Remove excess serum	30 minutes
4. Incubate with primary specific antiserum in PBS-azide; wash with PBS (three	overnight
changes)	15 minutes
5. Incubate with biotinylated antibodies (Vector)*	30 minutes
Wash with PBS (three changes)	15 minutes
6. Incubate with avidin-biotin-peroxidase complex (ABC, Vector)	45 minutes
Wash with PBS (three changes)	15 minutes
7. Incubate in the dark in diaminobenzidine-H_2O_2 solution in PBS[21]	2 to 5 minutes
Wash with PBS	5 minutes
8. Counterstain in Mayer's hemalum; blue in litium carbonate; dehydrate, clear, mount in balsam	

* Not for biotinylated lectin UEA I.

relationship to vessel size), applying immunohistochemical techniques on routinely fixed material, and 2) establish the prognostic value of true extramural venous invasion.

Materials and Methods

Thirty-four Dukes' C[15] adenocarcinomas from 20 men and 14 women aged 32 to 85 obtained from the routine histopathology files of the Institute of Pathological Anatomy and Histology of the University of Genoa were examined with representative sections of subserosal connective tissue, fat, and blood vessels consecutively cut around the tumor (nine right colon, four left colon, and 21 sigmoid-rectum; six well-differentiated G1, 19 moderately differentiated G2, and nine poorly differentiated G3).[16] These tumors presented true venous invasion (three cases), no venous invasion (18 cases), and neoplastic nests in pericolic fibrous tissues with doubtful vessel invasion or lymph node involvement (13 cases).

All tissues were fixed in 10% formol-saline[17] and paraffin wax embedded. Serial sections from each block were studied by routine histochemical techniques, including hematoxylin and eosin (H&E) and Weigert's resorcin-fuchsin and Gomori's and Van Gieson's stains, to demonstrate elastic, reticulin, and collagen fibres.[18]

The immunoperoxidase technique, avidin-biotin-peroxidase complex (ABP) (Table 1)[19-21] was also applied with the following antisera at different dilutions (Table 2): anti-human Factor VIII related antigen (Factor VIII-RAG),[22,23] with or without enzymatic (pronase and trypsin) digestion[24]; vimentin[25]; actin[26]; desmin[27]; laminin[28]; and type IV collagen.[29] We also used biotinylated lectin Ulex Europaeus Agglutinin I (UEA I, Vector Laboratories, Burlingame, California).[30]

Results

UEA I lectin and antibodies against FVIII-RAG were immunocytochemically used to visualize endothelial cells; antibodies against alpha sm-1 actin, desmin, vimentin, laminin, and type IV collagen were used to visualize vascular wall components (Table 3).

Of 34 Dukes' C adenocarcinomas, three presented true venous invasion with H&E examination and 18 no signs of venous invasion; these findings were confirmed by immunohistochemical investigations. Of the remaining 13 Dukes' C adenocarcinomas with doubtful venous invasion, seven cases showed true venous invasion and six showed lymph node involvement or nests of neoplastic cells surrounded by fibrous tissue; nevertheless, some tumors presented, either separately or in combination, true venous invasion, lymph node involvement, and neoplastic nests in pericolic fibrous tissue.

In cases with possible venous invasion, endothelial cells were detected by both UEA I and Factor VIII-RAG antibodies. It was thus possible to distinguish an artifact, due to connective tissue shrinkage around a nest of neoplastic cells, from a distended vein with a neoplastic embolus in the lumen. It was also possible to find evidence of some residual endothelial cells when a cap of thrombus adhered to the venous wall. Nevertheless, both antibodies were inefficient when the vein lumen was completely occluded by tumor embolus; UEA I also stained neoplastic epithelial cells, resulting in uncertain interpretation. Factor VIII-RAG might also be negative, because the endothelial cells can lose their antigenic properties when the lumen is completely occluded by neoplastic cells. Therefore, we employed antibodies against alpha sm-1 actin, desmin, and vimentin, which are typically well represented in the vascular wall.

Large amounts of actin, mainly the alpha-isoform,[31] were present in the smooth muscle cells of the wall of extramural arteries and veins (Figure 1a, b). Desmin was

Table 2. *Antisera Employed*

Antigen	Source	Dilution
Factor VIII RA	Ortho, Raritan, NJ	1:30*
Vimentin	Dakopatts, Copenhagen, Denmark	1:100
Alpha sm-1 actin	Prof. G. Gabbiani, Geneva, Switzerland†	1:100
Desmin	Immunonuclear, Stillwater, MN	1:10*
Laminin	Miles Scientific, Naperville, IL	1:10
Type IV collagen	Euro Diagnostics, Holland	1:300

* The antisera were prediluted by the manufacturers.
† Anti-alpha sm-1 actin monoclonal antibody is currently commercially available and sold by Sclavo, Siena Italy.

Table 3. *Immunocytochemical Parameters*

Marker	Endothelium	Venous wall		Lymph node capsule	Fibrous pseudocapsule
		SCV	LCV		
UEA I	++	−	−	−	−
F VIII	++	−	−	−	−
Actin	−	++	++	±	±
Desmin	−	±	++	±	−
Vimentin	−	++	+	−	±
Laminin	−	+	+	+	+
Type IV collagen	−	+	+	+	+

SCV, small caliber vessels; LCV, large caliber vessels.

almost absent in small caliber vessels, whereas it was abundant in large caliber and extramural vessels. A large amount of vimentin was always present in small caliber, submucosal, and extramural vessels, although less abundantly than desmin in the latter ones.

Alpha sm-1 actin showed a characteristic pattern formed of dense and intertwined actin-rich cells in thick vascular walls. Conversely, in thin-walled veins the pattern was formed of elongated and linear actin-rich cells. It was thus possible to distinguish between thin- and thick-walled veins in some doubtful cases (Figure 2a, b). For example, when the wall was distended by neoplastic endolumina emboli, the presence of intertwined actin-rich cells in its less dilated part showed a thick-walled vein.

By contrast, the presence of rare, elongated, and linear actin-rich cells suggested that wall thickness, visualized with H&E, was due to a reactive fibrous tissue. Occasional, elongated actin-rich cells[32] (Figure 2c), likely to be confused with a thin-walled vein, were also present in the reactive lymph node capsule[33,34] and in the residual capsule of the completely metastasized lymph nodes. The capsule contained occasional elongated desmin-positive cells (Figure 2d), but it was always negative to vimentin. The latter, on the contrary, was always present in extramural veins, although in very small amounts. In the most doubtful cases, therefore, vimentin negativity provided the best differentiation of the vascular wall from a lymph node capsule.

The use of antibodies against type IV collagen (component of vascular wall matrix) and laminin (which particularly lines the peripheral wall) did not give good results, because both markers were present in the vessel wall as well as in the lymph node capsule.

Laminin and type IV collagen were equally ineffective in distinguishing an extramural vein completely occluded by neoplastic cells from a fully metastasized lymph node and neoplastic nest surrounded by fibrous tissue. The peritumoral stroma, in fact, also contained laminin and type IV collagen[35,36] and occasional elongated cells, which were positive to alpha sm-1 actin (smooth muscle cells) and vimentin, but negative to desmin. Desmin negativity, therefore, was the most satisfactory parameter for the identification of fibrous peritumoral stroma.

A 10-year follow-up was performed. All patients who died of surgical complications, accidents, other causes independent of colorectal cancer, or uncertain causes were not eligible for our study. The survival rate of the 34 Dukes' C adenocarcinomas examined, according to the Kaplan-Meier method, showed that the curve referring to the ten cases with true venous invasion (three diagnosed by H&E and seven by immunohistochemical methods) had a peak mortality between 6 and 12 months (group A), with all patients dead within 3 years; conversely, the curve concerning the 24 cases without venous invasion (18 diagnosed by H&E and six by immunohistochemical methods) had a peak mortality between 1 and 5 years (group B), with five patients surviving longer than 5 years. By comparing the two curves, according to Logrank test or Mantel-Haensel method,[37,38] a high significance value appeared ($\chi^2 = 21.05$; $P < 0.001$) (Figure 3). For group B, the 18 cases diagnosed by H&E showed a survival curve similar to that of the six immunohistochemically defined cases, without significant difference ($\chi^2 = 0.51$). Furthermore, of 34 cases studied, eight patients showed metastatic involvement of the liver at the time of surgery (Table 4); five belonged to the group of ten patients with venous

Table 4. *Venous Invasion and Metastatic Diseases*

	H&E	Immunohistochemical study	Total	Liver Metastasis at time of surgery	Later Metastasis
Venous invasion	3	3	10	5	3 { peritoneum, diaphragm, bone/brain
		7			
Doubtful	13				
		6			
No venous invasion	18	18	24	3	1 brain

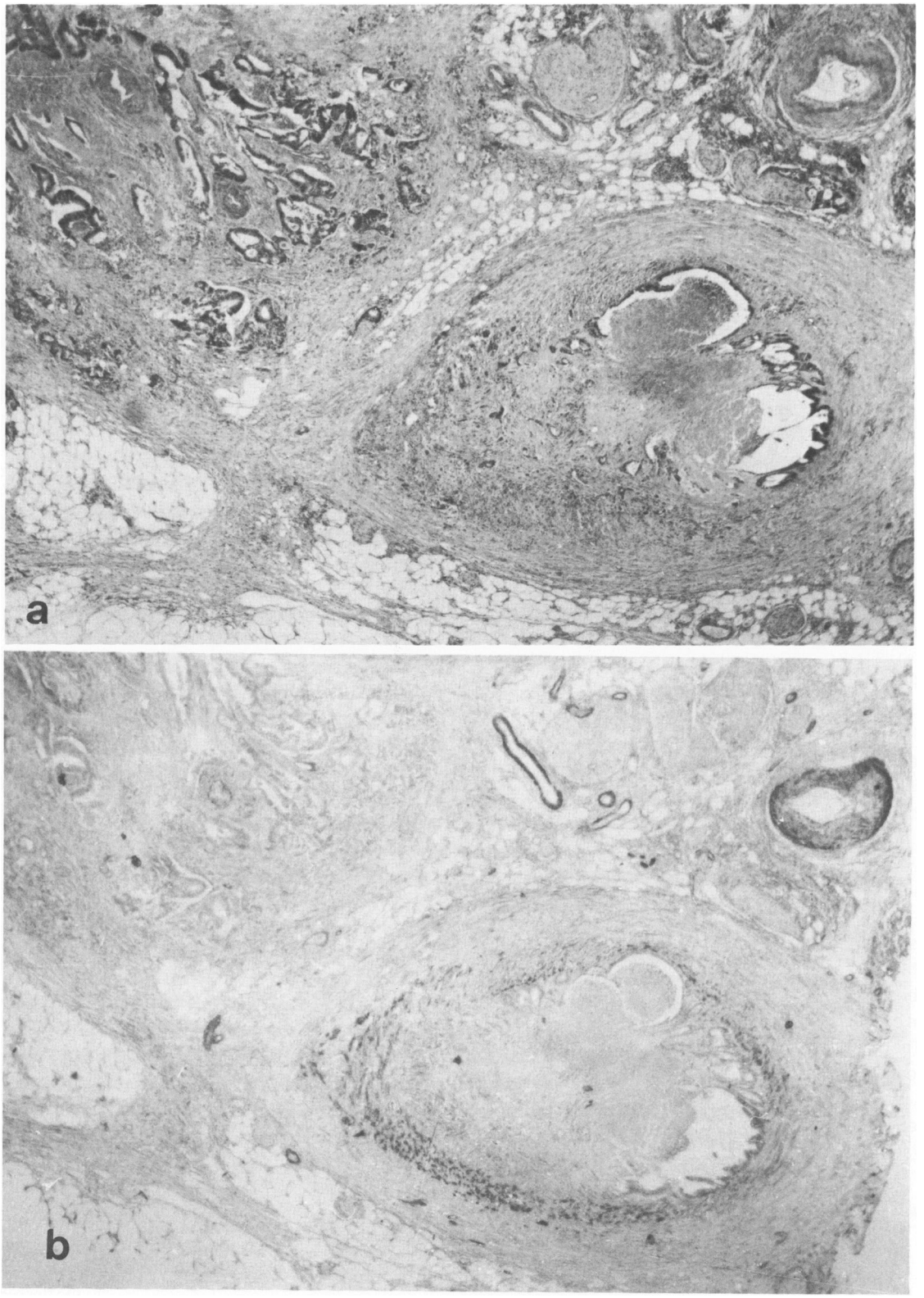

Figure 1. *Dukes' C adenocarcinoma with neoplastic nest in the pericolic tissue.* **a:** *Venous invasion shown with H&E.* **b:** *The presence of actin-rich cells confirms true venous invasion (ABC anti-alpha sm-1 actin ×25).*

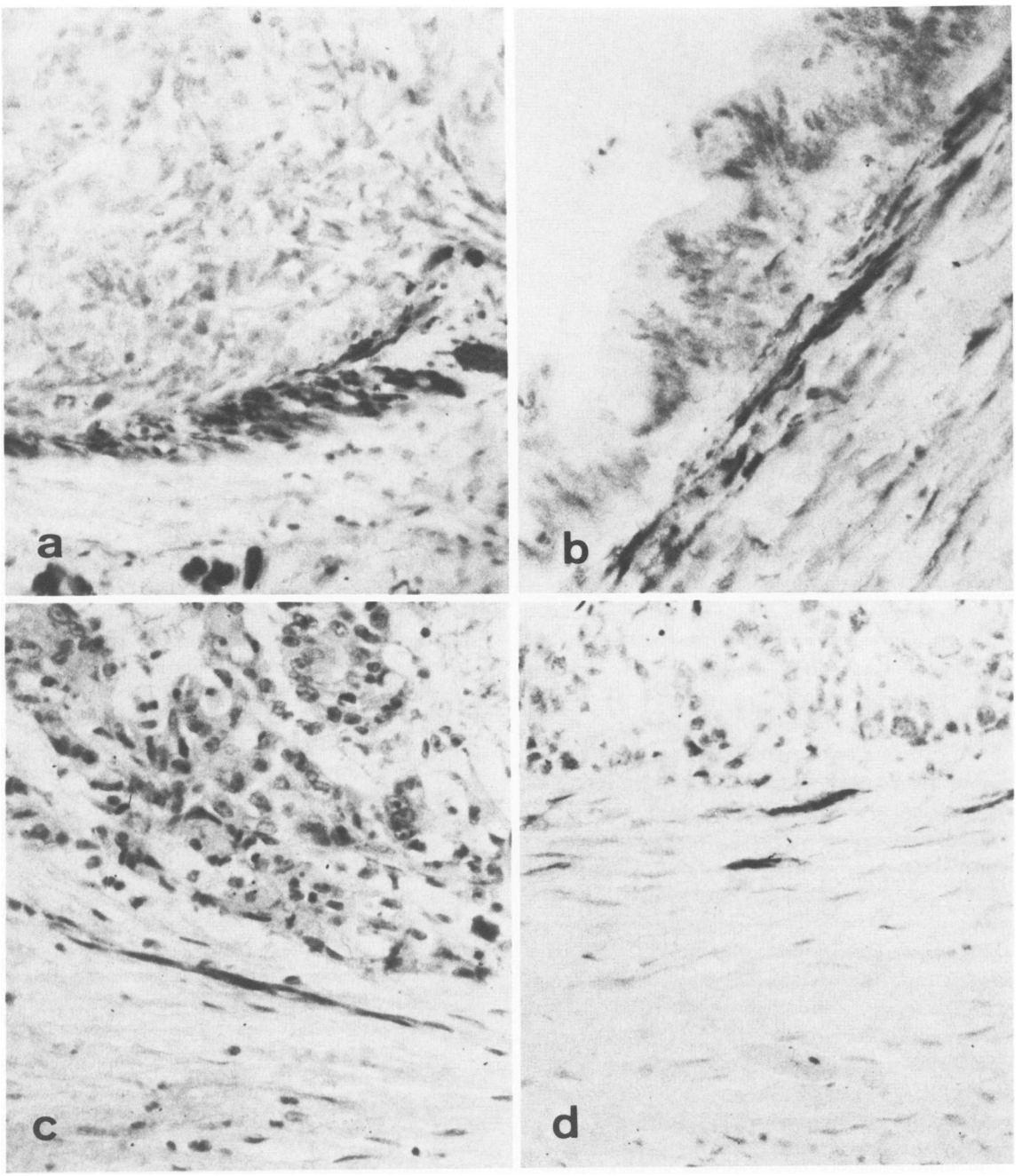

Figure 2. a: *Dense and intertwined actin-rich cells in a thick-walled vein.* b: *Elongated and linear actin-rich cells in a thin-walled vein.* c: *Rim of elongated actin-rich cells in the residual capsule of a lymph node completely replaced by metastatic carcinoma (ABC anti-alpha sm-1 actin).* d: *Rare and elongated desmin-positive cells in the residual capsule of a lymph node completely replaced by metastatic carcinoma (ABC anti-desmin, ×250).*

invasion (50%), and the remaining three (12.5%) belonged to the group of 24 patients without proven venous invasion. All patients with metastatic involvement of the liver died. Moreover, three of the ten patients with venous invasion and metastatic involvement of the liver later developed distant metastases in the peritoneum, diaphragm, bone, and brain. Only one of the 24 patients without venous invasion and with metastatic involvement of the liver later developed brain metastasis. No survivors had evidence of metastatic diseases. Minor complications caused by this disease could not be considered.

Discussion

Prognosis for colorectal adenocarcinomas is poor when extramural veins are invaded by neoplastic emboli, with the 5-year survival rate reduced from 55% to about 30%.[39]

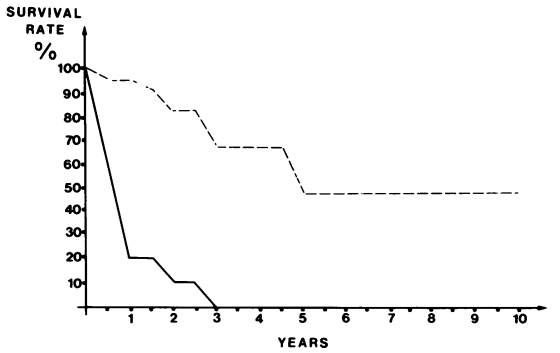

Figure 3. *Survival curves. The continuous line indicates patients with Dukes' C carcinoma and true venous invasion (group A); the dot line indicates patients with Dukes' C carcinoma without venous invasion (group B).*

Moreover, considerable variation in the incidence of vein invasion (11% and 62%) have been reported.[3]

In a recent article, Jass and Coworkers[40] identified some histopathologic parameters in rectal cancer grading (lymphocytic infiltration, node involvement, and spread through the bowel wall). Moreover, confirming Talbot's report, the same article showed that venous involvement was not required for the predictive model, although its role is recognized as important to this condition, especially in Dukes' A stage.

We believe that the difficulty in identifying true venous invasion is likely to be the reason for not considering this parameter in a well-reproducible prognostic model. Therefore, in histopathologic staging, it is important to consider pitfalls in the interpretation of venous invasion, which can be mistaken for lymph node involvement or for nest of neoplastic cells. Poor morphologic criteria and unreliable histochemical methods for elastic fibres may thus lead to vessel invasion overdiagnosis. Furthermore, the presence of blood cells between neoplastic cells and the wall is not always an indication of an invaded vein. In some cases, the presence of occasional actin-positive and vimentin-positive cells and the absence of desmin-positive cells reveal a nest of neoplastic tissue surrounded by fibrous tissue with little peritumoral hemorrhage.

The immunohistochemical methods employing desmin, vimentin, and in particular alpha-actin, therefore, may permit a better identification of histopathologically equivocal venous invasion. The usefulness of a more detailed evaluation of venous invasion is further confirmed by comparing the survival curves of the two groups (A and B), with and without venous invasion. Moreover, the development of metastatic disease in our patients seems to prove the correlation between hepatic metastases and venous invasion.

Although the prognosis may have been affected by the small number of cases and the heterogeneity of tumors studied (according to site, grade of differentiation, and patient age), we believe that the detection of true venous invasion by anti-alpha actin antibodies could become one of several useful parameters,[2,40] in the prognosis and survival of colorectal cancer.

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