

Prognostic Values of Galectin-3 and the Macrophage Migration Inhibitory Factor (MIF) in Human Colorectal Cancers

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This study aims to investigate whether the immunohistochemical levels of expression of galectin-3 and the macrophage migration inhibitory factor (MIF) are associated with prognostic values in human colorectal tumors. This was performed on 99 specimens including 69 colorectal tumors (17 Dukes A, 19 Dukes B, 15 Dukes C and 18 metastatic tumors that we labeled as D), 10 hepatic metastases from colorectal cancers and 20 normal specimens (biopsies). The immunohistochemical levels of expression of MIF and galectin-3 were quantified on routine histological slides by means of computer-assisted microscopy. Separate analyses were performed on epithelial and connective tissue. The levels of expression of both MIF and galectin-3 were very significantly higher in epithelial tumor tissue when compared with normal epithelial specimens. A positive and significant correlation between MIF and galectin-3 expression was evidenced in connective tumor tissue, and in particular in the cases associated with short survival periods (less than 5 years). In the case of the Dukes A or B tumors, we established two new prognostic groups (labeled I and II) on the basis of the levels of galectin-3 expression measured in the tumor epithelium. In the case of the Dukes C or D tumors, we established two other prognostic groups (labeled III and IV) on the basis of the levels of MIF expression measured in

the connective tissue. Kaplan-Meyer analyses confirmed the additional prognostic values (as compared with conventional clinical staging) given by this new classification (groups I to IV). They show that the Dukes A or B tumors characterized by low levels of galectin-3 expression in the tumor epithelium are associated with significantly better prognoses than those characterized by high levels. In addition, the Dukes C or D tumors characterized by high levels of MIF expression in the connective tumor tissue are associated with significantly better prognoses than those characterized by low levels. In conclusions, MIF and galectin-3 expression levels in colorectal tumors are related to their levels of biological aggressiveness. These markers could be used to identify patients at risk, for whom more aggressive adjuvant therapy seems to be indicated.

KEY WORDS: Colorectal cancer, Connective tissue, Dukes classification, Epithelium, Galectin-3, MIF, Prognosis.

Mod Pathol 2003;16(5):491-504

In the industrial world colorectal cancer is the second most common form of cancer, with some 140,000 new cases diagnosed annually (1-4). Forty percent of these patients die due to the progression of their colorectal cancers, even if about 75% of these patients have undergone surgery for therapeutic purposes (1-4). Adjuvant therapies (chemotherapy, radiotherapy) can be performed in relation with tumor prognosis in addition to surgery (5). Prognostic evaluations are established based on distinct criteria including the macroscopic appearance of the colon (the presence or absence, for example, of occlusion or perforation, etc.), the histological tumor type, the degree of tumor cell differentiation and the presence of vascular and/or

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VOL. 16, NO. 5, P. 491, 2003 Printed in the U.S.A.

Date of acceptance: February 21, 2003.

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DOI: 10.1097/01.MP.0000068235.45178.C1

lymphatic emboli (6–8). The first system of predicting the progression of colorectal cancer was proposed by Dukes in 1932 (9). This classification has proven its value and remains in use today either in its original or in one of its modified forms. The different ways (clinical staging, histopathological grading, measures of biological markers in the sera, etc.) of classifying human colorectal cancers in terms of potential biological aggressiveness in fact enable prognostic assessments to be provided for particular clinical groups of patients, but not for individual patients (10, 11). Thus, in addition to conventional methods of classification, the search for biological factors as new prognosticators remains of major interest. The Vogelstein group specified the sequence of genomic mutations leading to the transformation of normal colorectal mucosa into adenomas and, subsequently, into cancers (12–14). This means that when colorectal tissue accumulated an increasing number of different mutations, it can be assigned to a certain position in the normal—adenoma—cancer sequence. However, this pinpointing will not enable precise prognoses to be established at individual level. The identification of certain gene products (proteins including their posttranscriptional modifications) whose expression levels are significantly down- or up-regulated in cancers as compared with benign tissue can be used for such purposes. In this context, the analysis of protein-carbohydrate interactions is receiving increasing attention. Because it is known that malignancy is associated with characteristic changes in glycosylation, the ensuing alterations in this system (sugar coding) can well have a bearing on establishing the tumor phenotype (15–19). One family of proteins of interest in this respect are the galectins, an evolutionarily ancient group of lectins that are found in species ranging from sponges and nematodes to humans (20–24). These β -galactoside-specific lectins are able to mediate cell adhesion, to regulate cell growth, and to trigger or inhibit apoptosis: they are therefore suspected of playing a number of important roles in tumor development (20–24). Galectin-3, one of the 14 types of mammalian galectins identified to date, has already been shown to be associated with colon cancer development: it is frequently expressed by various colon cancer cell lines (25, 26) and seems to be more highly expressed in colon cancer cells with increased metastatic capacity (27). Galectin-3 ligands include, for example, carcinoembryonic antigen (25) and colon cancer mucin (27), which are already known to be involved in colon cancer progression (25, 27). However, as emphasized by Itzkowitz (28), an inconsistent picture had emerged from the literature with respect to the prognostic value of galectin-3 in human colon cancer. Whereas some studies report decreasing levels of galectin-3 ex-

pression in colon carcinomas (29, 30), others describe increasing galectin-3 expression during colon cancer progression (31–33). In the present study we therefore investigated whether galectin-3 expression differs in normal colon tissue, primary colorectal cancers and colorectal cancer metastases. We then investigated whether galectin-3 expression differs in primary colorectal cancers depending on their Dukes' clinical stages. Finally, we investigated whether galectin-3 expression differs in a particular Dukes' clinical stage in relation to patients' survival. Immunohistochemical galectin-3 expression was quantitatively monitored separately in epithelial and connective tissue.

In addition to galectin-3, we also investigated the potential prognostic value of the macrophage migration inhibitory factor (MIF) for the following reasons. Firstly, we had observed that MIF expression correlates with the expression of galectin-3 in relation to recurrences in human cholesteatomas (34). Secondly, recent studies suggest that MIF, first identified nearly 40 years ago as a potent pro-inflammatory cytokine, may also contribute to the multiple aspects of tumor progression and neoplasia including impairment of p53 activity (35–37). Thirdly, MIF activity has already been shown to differ in normal and neoplastic colon tissue (38, 39). We thus characterized the MIF expression (using a polyclonal antibody) in the present study exactly as we did for galectin-3.

MATERIALS AND METHODS

Clinical Data

We collected for immunohistochemical analysis 69 colorectal tumors, 10 hepatic metastases from colorectal cancers and 20 normal specimens. The normal cases came from routine biopsies made on colorectal areas without any sign of clinical aberrations and carried out on patients subjected to total colonoscopy for screening purposes. The cancer series includes a retrospective, continuous, unselected cohort of patients who underwent a primary resection with curative intent. Cancer staging was performed according to the three categories of the Dukes' classification (Dukes A, B and C) (9). At least 12 lymph nodes were analyzed per case to assess the Dukes stage. We added to this series a set of tumors resected from patients with distant metastases at the time of diagnosis. These tumors were classified in category "D." Finally, the 69 cancers included 17 Dukes A, 19 Dukes B, 15 Dukes C and 18 D. Of these, 19 were rectal tumors (8 Dukes A, 6 B, 4 C and 1 D). The ages of the 79 patients (with colorectal tumors or hepatic metastases) ranged from 29 to 88 years (mean = 64) and did not significantly vary between the groups of patients as-

sociated with the different tumor stages. There were 40 men and 39 women. All the patients were operated on by two surgeons (HL and J-CP) at the Institut Bordet (Brussels, Belgium). All those suffering from Dukes C tumors received adjuvant chemotherapy with levamisole and fluorouracil following the so-called "Mayo Clinic regimen" (40). Patients with distant metastasis at the time of diagnosis (stage D) were treated following protocols undergoing at the time of diagnosis (the association of FU and CPT11 or oxaliplatin). The clinical follow-ups of all the colorectal cancer patients under study are illustrated in the Results. All the specimens were first fixed in buffered formalin before further processing for paraffin embedding. Tissue samples from 4 cases were also kept in liquid nitrogen for Western blotting and PCR analysis.

MIF and Galectin-3 Immunohistochemistry

All the histochemical procedures were carried out as detailed previously (34, 41). Briefly, 5 μm -thick sections were taken from each of the 99 clinical samples (*i.e.*, 20 normal cases, 69 colorectal tumors and 10 hepatic metastases). Incubation with the antibody-containing solutions was carried out at $25 \pm 1^\circ\text{C}$ for 60 minutes at a concentration of 10 $\mu\text{g}/\text{mL}$. The extent of the specifically bound antibodies was visualized by avidin-biotin-peroxidase complex (ABC) kit reagents (Vector Labs, Burlingame, CA), with diaminobenzidine/ H_2O_2 as the chromogenic substrates.

The anti-MIF antibody is a polyclonal rabbit antibody raised against MIF purified from bovine lenses following chromatographical steps (42) and high-resolution preparative gel electrophoresis. The antibody was also assessed for specificity by Western blotting, as described below. The polyclonal antibody against galectin-3 produced by recombinant expression with the expression vector prCBP35, kindly provided by Dr. J.L. Wang (East Lansing, MI) was raised as described previously (41). To exclude cross-reactivity to galectin family members present in colorectal cancer tissue, Western blotting with purified galectins-1, -4, -7, and -8 were performed in addition to routine quality controls. These controls included the omission of the incubation step with polyclonal antibody to exclude any kit-reagent-independent staining (*e.g.*, by binding of the *N*-glycan chains of the kit reagent glycoproteins avidin and/or horseradish peroxidase to glycan-binding sites in the tissue or of avidin to endogenous biotin), the incubation with serum containing no specific antibody to exclude antigen-dependent binding via Fc receptors, the immunohistochemical analysis of cases shown to be negative/positive in analysis by Western blotting (for technical details, see below) and the preincubation of the polyclonal antibodies with

an excess of antigen to saturate their antigen-dependent binding *in situ*.

Computer-Assisted Microscopy

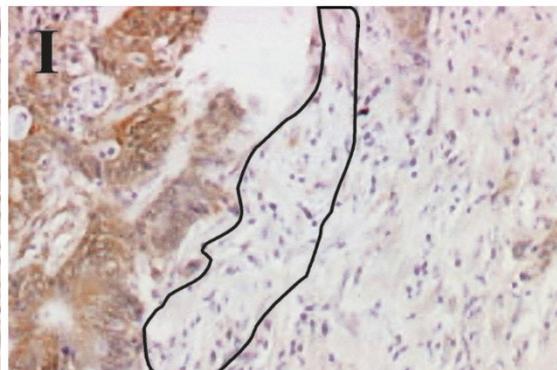
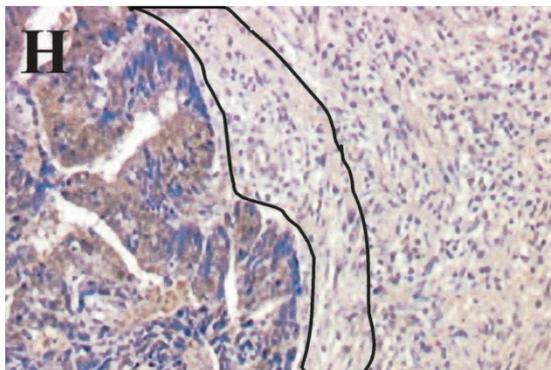
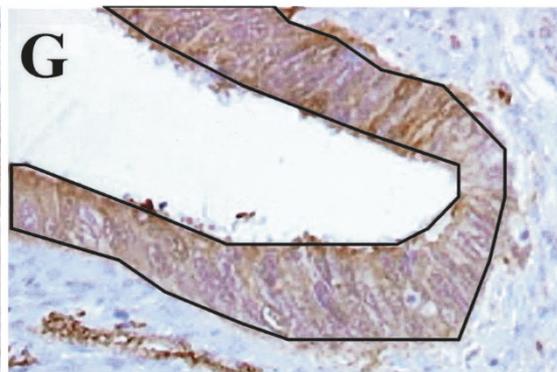
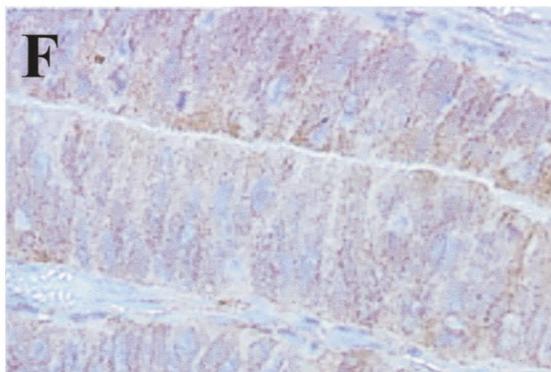
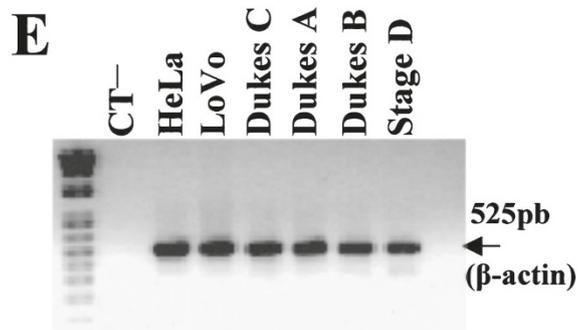
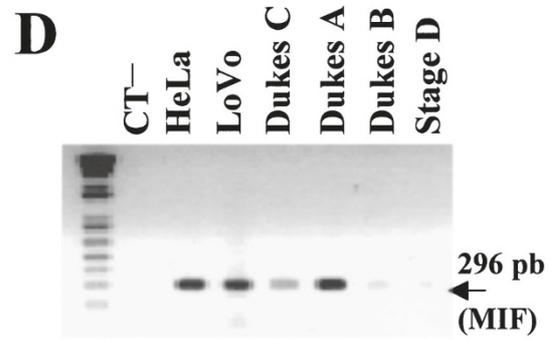
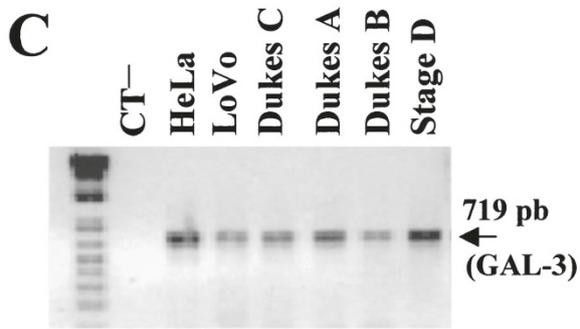
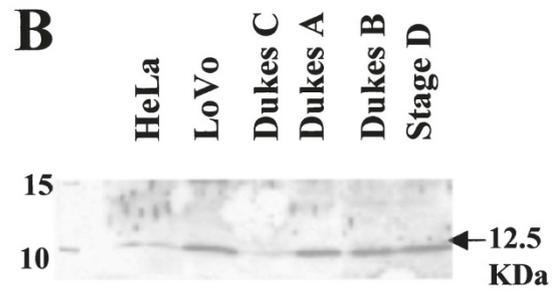
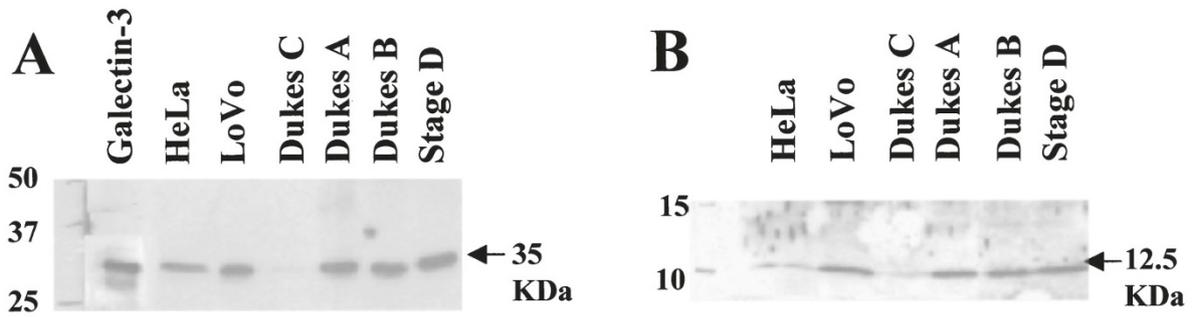
The immunohistochemical galectin-3 and MIF stainings were quantitatively characterized in two different ways by a SAMBA 2005 computer-assisted microscope system (Samba Technologies, Grenoble, France) with a 20x (aperture 0.50) magnification lens. While the Labeling Index (LI) refers to the percentage of tissue area specifically stained by a given histochemical marker, the Mean Optical Density (MOD) denotes the mean staining intensity computed in immunopositive cells only. The way in which we used the computer-assisted system to quantify the histochemical staining is detailed elsewhere, as are all the standardization procedures dealing with the manner in which we used the computer-assisted microscopy and ensuring measure reproducibility (34, 41, 43). A negative histological control slide (from which either the primary anti-MIF or the anti-galectin-3 antibody was omitted, as mentioned above) was analyzed for each case under study. The software used in the computer-assisted microscope automatically subtracted the LI and MOD values of the negative control sample from each corresponding positive one.

Two types of histological structure were analyzed for each of the cases under study. The first of these structures was the epithelial component while the second was the adjacent peritumoral connective tissue. The software that we set up (34, 41, 43) enabled these two tissues to be analyzed separately in each case by means of a computer-mouse linked to this software. This mouse enabled the epithelial component and the connective tissue to be delineated on a control video screen, so leading to a separate quantitative analysis of each of the biological markers under study (as illustrated in Fig. 1, G-I). Ten fields of between 60,000 and 120,000 μm^2 each were scanned for epithelial and connective tissue in each of the 99 cases studied.

Western Blotting Analyses

The check for anti-galectin-3 and anti-MIF antibody specificities was carried out on four human colon cancers (one for each Dukes stage, with the addition of "Dukes D") and two positive controls. These positive controls included two human cell lines (HeLa [a human cervical carcinoma cell line] and LoVo [a human colon cancer cell line] obtained from the American Type Culture Collection; Manassas, VA).

Western blotting analyses were carried out as detailed previously (44). Briefly, after the colon tumor tissue and the cell lines had been homogenized, the tissue extracts were prepared by sonica-



tion in PBS containing 1 mM PMSF and 10 $\mu\text{g}/\text{mL}$ aprotinin. The protein concentration was determined by the BCA protein assay (Pierce, Polylabo, Antwerp, Belgium). The samples in each lane (10 μg for galectin-3 and 10 μg for MIF) were loaded onto a 15% polyacrylamide gel under denaturing and reductive conditions. After electrophoresis, the proteins were transferred onto a Polyscreen®PVDF membrane (NEN™ Life Science Products, Boston, MA) by tank blotting. The target proteins (galectin-3 and MIF) were immunodetected by the affinity-purified antibodies (0.05 $\mu\text{g}/\text{mL}$ anti-galectin-3 and 0.05 $\mu\text{g}/\text{mL}$ anti-MIF) in T-TBS containing 5% of milk powder, in conjunction with a secondary antibody conjugated with horseradish peroxidase (0.2 $\mu\text{g}/\text{mL}$; NEN). The visualization of the immunoreactive proteins was carried out with reagents from a chemiluminescence kit (NEL103, NEN). The control experiments included the omission of the incubation step with the specific anti-protein antibody (negative control). Fig. 1A and B illustrates the Western blots obtained for the anti-galectin-3 and anti-MIF antibodies.

Analysis of Galectin-3 and MIF RNA Expression in Human Colon Cancer Tissue

Specimens from the same four human colon cancers and the two cell lines employed for Western blotting were lysed by adding Tripure™ Isolation Reagent (Roche, Mannheim, Germany). Total RNA was prepared according to the manufacturer's recommendation. Before cDNA synthesis, the RNA was incubated with DnaseI (1,71 IU/ μL) (Roche Diagnostics) for 15 min at 37°C. The RNA was purified by phenol/chloroform extraction. One μg of total RNA was used as template for cDNA synthesis: reverse transcription was performed for 50 min at 42°C in RT buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM MgCl₂, 10 mM DTT, Gibco), oligodT (12-18) primers (25 ng/ μL , Gibco), 500 nM dNTP, 8 IU RNAsin Ribonuclease Inhibitor (Promega, Leiden, The Netherlands) and 200 IU Superscript™ Rnase H Reverse Transcriptase (Gibco). The reaction was terminated by incubation for 15 minutes at 70°C.

The integrity of the cDNA was confirmed by β -actin specific PCR analysis.

The primers for the galectin-3 and MIF were designed using the HYBsimulator software (Advanced

Gene Computing Technologies, Irvine, CA) (45) and purchased from Biosource Europe S.A. (Nivelles, Belgium). They were:

Galectin-3:

atggcagacaattttcgctcc (sense), and
atgtczzcagaaattcccagtt (anti-sense);

MIF:

gatgttcacgtaaacaccaa (sense), and
cgtaatagttgatgtagaccctg (anti-sense).

The PCR products were analyzed by electrophoresis on 1% agarose gel. Fig. 1, C-E illustrates the data obtained with the present PCR analyses.

Data Analysis

To compare independent groups of numerical data we used the non-parametric Mann-Whitney test (for independent samples) or the Wilcoxon matched pair tests (for paired data) because the conditions for applying the parametric *t* tests were not satisfied in general. This is why data presentation uses the medians and the 25% and 75% percentiles. The rank correlation test involved the non-parametric Kendall test. Survival time analyses were performed using Kaplan-Meier curves and the log-rank test. As previously detailed (46, 47), we used a decision tree-based technique to determine threshold values (for the quantitative variables analyzed) able to discriminate between two different groups of patients. Briefly, this technique investigates all possible splits (for each variable) to find the split producing the largest improvement in discriminating groups of cases a priori defined. Finally, standard Cox regression analysis was used to fit to the survival data the explanatory models generated on the basis of different variables analyzed in the study. This enabled the possible simultaneous influence of several variables on the survival period to be tested. All the statistical analyses were carried out using Statistica (Statsoft, Tulsa, OK, USA).

RESULTS

Morphological Illustrations of Immunohistochemical Detection of Galectin-3 and MIF Expression in Human Colorectal Tissue

While Fig. 1, A-B illustrates the specificity check for anti-galectin-3 and anti-MIF antibodies per-

FIGURE 1. Panels A and B illustrate the specificity check for anti-galectin-3 (A) and anti-MIF (B) antibodies performed by means of Western blotting. Panels C-E show the check on the presence of galectin-3 (C) and MIF mRNAs (D), and cDNA quality illustrated on the β -actin mRNAs (E). These data are given for two cell lines (Hela and LoVo) used as positive controls and four human colon cancers (with different stages). Panels F-G ($\times 200$) illustrate the immunostaining pattern for galectin-3 in a Dukes A colon cancer associated with a favorable prognosis (more than 8 years of survival postdiagnosis) and in a Dukes B colon cancer associated with a dismal prognosis (2 years of survival postdiagnosis), respectively. Panels H-I ($\times 100$) show the immunostaining pattern for MIF in a Dukes C colon cancer associated with a good prognosis (more than 8 years of survival postdiagnosis) and in a Dukes C colon cancer associated with a dismal prognosis (1 year of survival postdiagnosis), respectively. Panels G-I also illustrated different tissue areas selected for immunohistochemical analysis (in G: epithelial tumor tissue area, in H-I: adjacent peritumoral connective tissue).

formed by means of Western blotting, Fig. 1, C to E illustrates the check on the presence of galectin-3 and MIF mRNAs, and cDNA quality in the four human colon cancers and the two cell lines used as positive controls.

Fig. 1, F-G shows the immunostaining pattern for galectin-3 in a Dukes A colon cancer associated with a favorable prognosis (more than 8 years of survival postdiagnosis) and in a Dukes B colon cancer associated with a dismal prognosis (2 years of survival postdiagnosis), respectively. In the same way, Fig. 1, H-I shows the immunostaining pattern for MIF in a Dukes C colon cancer associated with a good prognosis (more than 8 years of survival postdiagnosis) and in a Dukes C colon cancer associated with a dismal prognosis (1 year of survival postdiagnosis), respectively.

MIF and Galectin-3 Expression in Normal Colon Tissue, Primary Colorectal Tumors, and Hepatic Metastases from Colorectal Cancers

All the significant variations observed in terms of MIF and galectin-3 expression across the groups of samples analyzed in the Results section concerned the immunostaining intensity in immunopositive tissue areas, which was quantified by means of the Mean Optical Density variable (MOD). In contrast, the Labeling Index (LI, *i.e.*, the percentage of immunopositive tissue area) of the MIF and galectin-3 expression was uniformly high and did not vary significantly across the groups of samples. This variable thus will not be taken into consideration in the data reported in the Results section. (In the following, the terms “galectin-3—or MIF—expression” thus mean “immunostaining intensity.”)

With respect to the epithelial tissue (Fig. 2A), the levels of expression in terms of staining intensity (the MOD variable) of both the MIF ($P = 0.0009$) and the galectin-3 ($P = 0.00003$) significantly increased both in the tumors and the metastases (with less significant *p*-values because of the smaller number of cases in the latter tissue group), as compared with the normal samples. With respect to the connective tissue (Fig. 2B), a slight increase in the level of galectin-3 expression was observed in the tumors ($P = 0.03$) while the metastases exhibited a decrease in both galectin-3 ($P = 0.01$) and MIF expression ($P = 0.04$), when compared with the normal samples. A comparison of Fig. 2A and B shows that, in each category of specimens (*i.e.*, normal, tumor or metastasis), compared with its connective counterpart, epithelial tissue exhibited much higher levels of MIF and galectin-3 expression. Wilcoxon tests (for paired samples) confirmed these observations ($P < 0.001$ for normal samples, $P < 10^{-6}$ for tumor samples and $P = 0.005$ for metastases). We also investigated whether the tumor

location (rectum *versus* colon) influenced galectin-3 and MIF expression. This analysis was performed on the samples classified as Dukes A or B only because these included a large majority of the rectal tumors analyzed (*i.e.*, 15 cases of 19). The results evidenced no significant variations between rectal and colon tumors for the different markers and tissues investigated (data not shown). Finally, a survival analysis confirmed that the survival periods did not significantly vary when patients suffering of (Dukes A and B) rectal tumors were compared with those with (Dukes A and B) colon tumors (data not shown). This is why rectal and colon tumors were not distinguished afterward.

Correlation between MIF and Galectin-3 Expression (Staining Intensity) in Colorectal Tumor Specimens

Fig. 2, C-D shows the levels of galectin-3 expression (vertical axis) as function of the levels of MIF expression (horizontal axis) measured in epithelial and connective tumor tissue, respectively. Kendall rank correlation tests showed that while no statistically significant correlation could be evidenced between MIF and galectin-3 staining intensities measured in epithelial tissue (Fig. 2C), a positive and significant correlation (Kendal tau = 0.28, $P = 0.001$) was present between these two staining intensities in connective tissue (Fig. 2D). Furthermore, we established that the cases that contributed the most to this correlation were those associated with short survival periods (*i.e.*, fewer than 5 years, all the Dukes stages taken together). Indeed, whereas the cases associated with long survival periods (*i.e.*, exceeding 6 years) did not exhibit any significant correlation (Fig. 2E), those associated with short survival periods (Fig. 2F) revealed a clearly positive correlation (Kendal tau = 0.49, $P = 0.0002$).

Prognostic Value of MIF and Galectin-3 Staining Intensities

To investigate whether prognostic groups could be determined, we analyzed the distribution of the patients' survival periods as function of the levels of galectin-3 and MIF expression in epithelial and connective tissue (Fig. 3). We separately analyzed the tumors classified as Dukes A or B and those classified as Dukes C or D. In Fig. 3, the living patients are represented as open dots and the dead ones as black dots; the vertical lines establish two thresholds, which enabled prognostic groups to be determined as demonstrated below. These thresholds were determined (by means of a decision tree technique) to discriminate at best the dead patients from the patients associated with a long survival

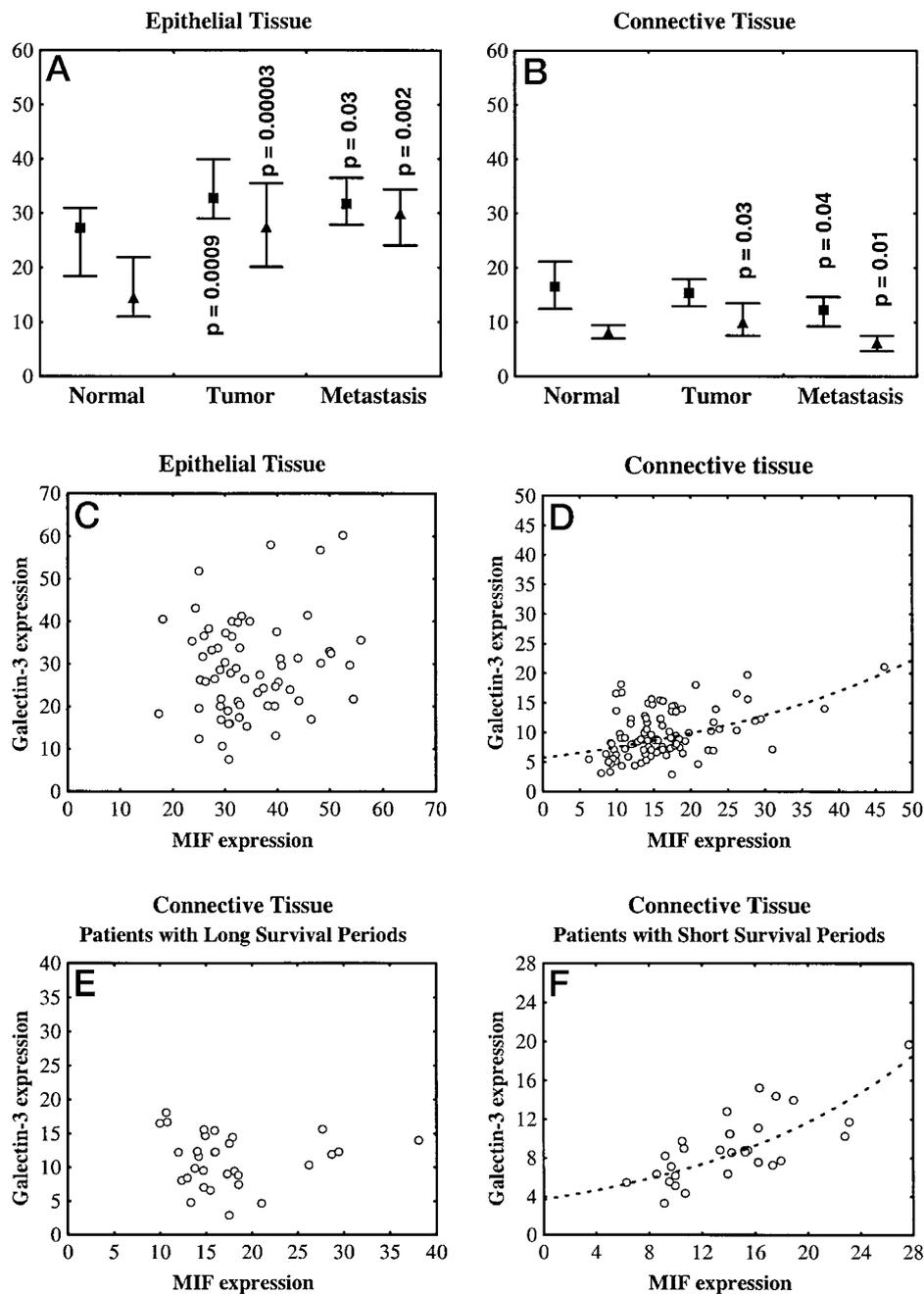


FIGURE 2. Illustration of the variations and correlations of MIF and galectin-3 expression concerning the Mean Optical Density variable (quantifying the immunostaining intensity in arbitrary units). Frames A-B report the variations observed in the epithelial (A) and the connective (B) compartments between normal, tumor and metastasis specimens annotated by the significant p-values (Mann-Whitney tests taking the expression levels in the normal samples as references). The MIF (squares) and galectin-3 (triangles) data are reported as medians (squares or triangles) and the 25% and 75% percentiles (bars). Frames C-F illustrate the distribution of galectin-3 expression levels (Y-axis) as function of the MIF expression levels (X-axis) in the tumor samples. While frame C shows the expression levels measured in each tumor epithelium, frame D reports those measured in each connective tumor tissue. Frames E-F detail frame D data by representing the cases associated with long survival periods (more than 6 years; frame E), and those with short survival periods (less than 5 years; frame F). The dashed curves indicate a significant correlation between the two variables (detailed in the text).

period (see Materials and Methods). In the case of the Dukes A or B tumors we evidenced a prognostic value for the level of galectin-3 expression in epithelial tissue (Fig. 3A). In contrast, no prognostic information was found for either galectin-3 expression in connective tissue (Fig. 3B), or MIF expression (in both epithelial and connective tissue, data

not shown). Inversely, in the cases of the Dukes C or D tumors a prognostic value was associated with MIF expression in connective tissue (Fig. 3D) but not with MIF expression in epithelial tissue (Fig. 3C) or galectin-3 expression (data not shown).

Four prognostic groups were so determined and were labeled I, II, III and IV, as shown in Fig. 3A and

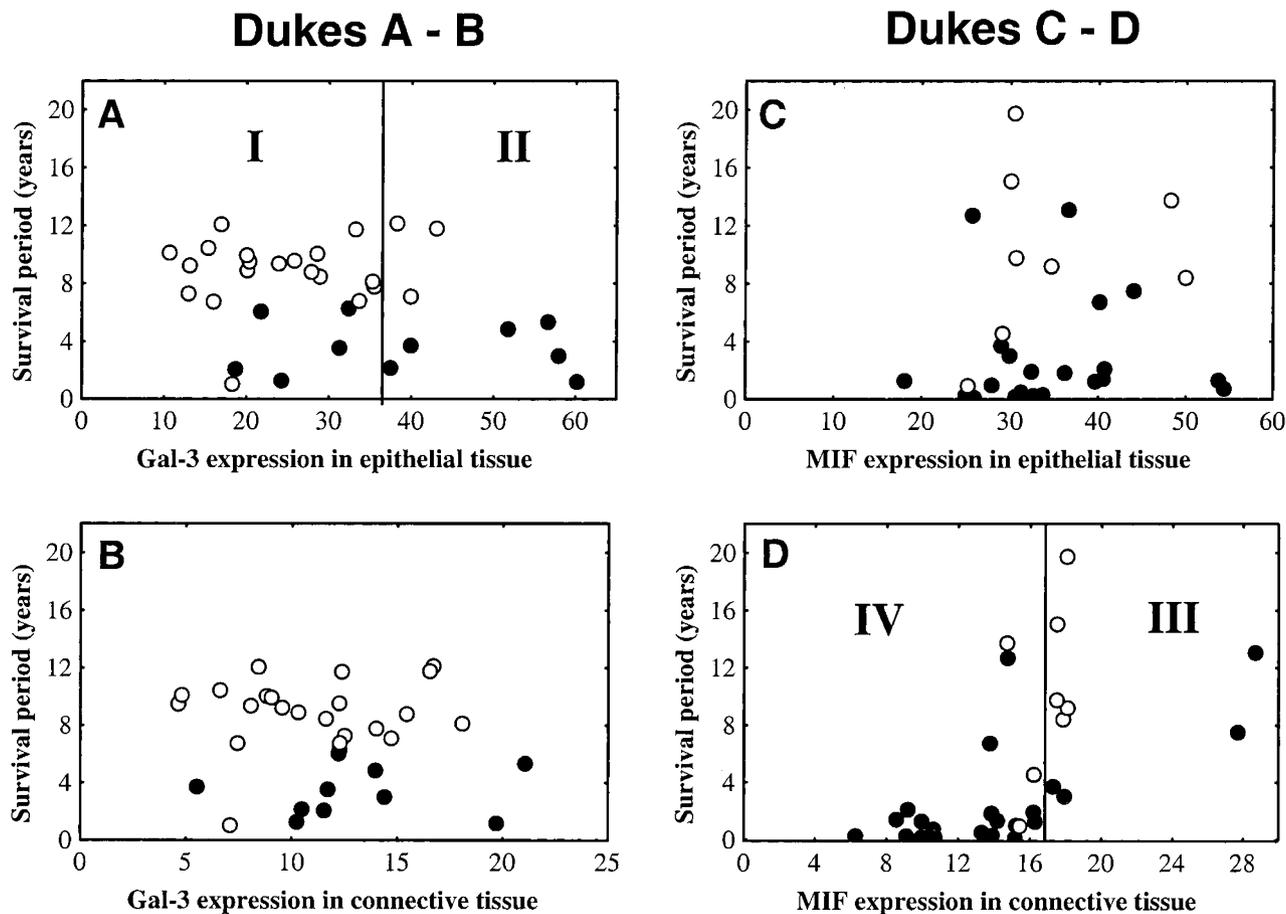


FIGURE 3. Distribution of the patients' survival periods as function either of the galectin-3 (frames A-B) or MIF (frames C-D) expression levels in the epithelial (A-C) and the connective (B-D) compartments. While frames A and B deal with patients suffering from Dukes A or B tumors, frames C and D show patients suffering from Dukes C or D tumors. The living patients are represented by open dots and the dead ones by black dots. In frames A and D the vertical lines (each of which defines a threshold value on the X-axis) define four new groups of patients as indicated: groups I and II in frame A (*i.e.*, from among the patients with tumors classified as Dukes A or B) and groups III and IV in frame D (*i.e.*, from among the patients with tumors classified as Dukes C or D).

D. While groups I and III included a majority of patients with long survival periods, a majority of patients with short survival periods belonged to groups II and IV. The prognostic value associated with each of these new four groups was demonstrated by means of standard survival time analyses. The results of these analyses are reported in Fig. 4. While Fig. 4A gives the survival curves associated with Dukes A and Dukes B tumors, Fig. 4B shows similar curves obtained when the two new groups I and II (defined as detailed in Fig. 3E) were considered. As is illustrated in Fig. 4B, this new classification enabled a majority of the patients suffering from Dukes B tumors and associated with long survival periods to be allocated to the new group I characterized by very good prognoses (nearly 80% of survivors after 10 years). As a consequence the patients remaining in the new group II were associated with bad prognoses (below 50% of survivors after 5 years). The levels of MIF expression (measured in epithelial and connective tumor tissue) exhibited no significant variations between

groups I and II (data not shown). Similarly, while Fig. 4C shows the survival curves associated with Dukes C and stage D tumors, Fig. 4D displays similar curves obtained when the two new groups III and IV (defined as detailed in Fig. 3D) are considered. This time, this new classification allocates to new group IV the patients suffering of Dukes C tumors and associated with short survival periods. This group IV is characterized by a generally very bad prognosis (below 20% of survivors after 5 years, see Fig. 4D). The levels of galectin-3 expression (measured in epithelial and connective tumor tissue) showed no significant variations between groups III and IV (data not shown).

Finally, Fig. 5 summarizes the complete set of prognostic data resulting from the new classification into groups I, II, III and IV (Fig. 5B) in comparison with the data associated with the standard Dukes classification (with an additional stage D, see Fig. 5A). Fig. 5, C-D details the case distribution when crossing the new prognostic groups (I, II, III and IV) and the clinical groups resulting from the

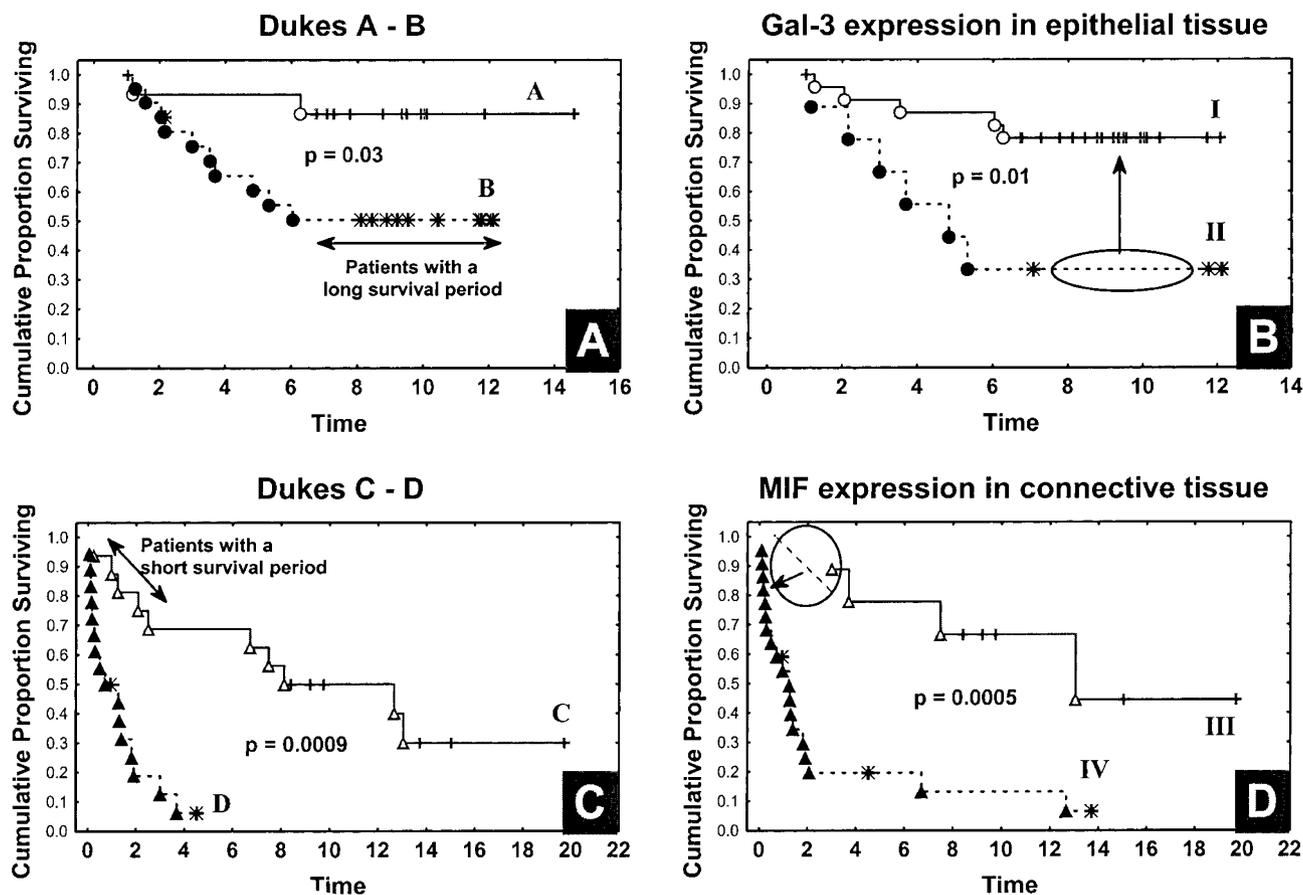


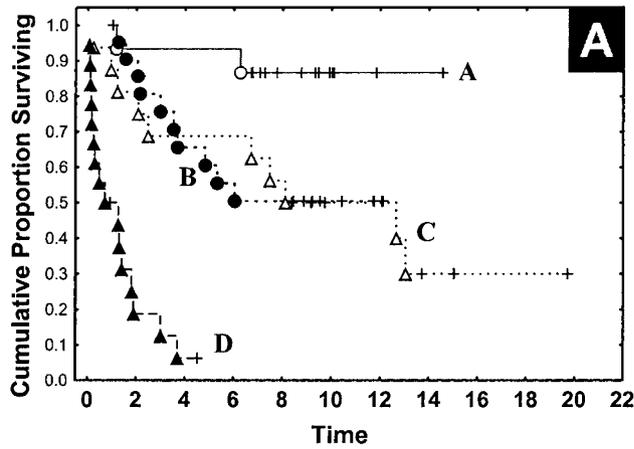
FIGURE 4. Illustration of the survival curves (expressed in years) associated with the Dukes classification (frames A and C) and the new prognostic groups (frames B and D) defined as indicated in Figs. 3A-D. The dead patients are indicated by means of symbols and the living ones by crosses (+) or stars (*). The p-values reported are computed by means of a standard log-rank test. Frames A-B show that the new distribution of Dukes A and B cases in terms of the new groups I and II enabled the Dukes B cases associated with long survival periods to be allocated to the group I associated with favorable prognoses. Frames C-D show that the new distribution of Dukes C and D cases in terms of the new groups III and IV enabled to allocate the Dukes C cases associated with short survival periods to the group IV associated with very bad prognoses.

Dukes' classification combined with the survival data (*i.e.*, survival periods shorter than 5 years and longer than 6 years). It will be noted that as in Fig. 2, Fig. 5, C-D does not take account of the cases corresponding to living patients with survival periods of fewer than 6 years. This shows that the Dukes B curve (which concerns patients who did not receive chemotherapy) is similar to the Dukes C curve (which concerns patients who did). In contrast, the new group II, which encompasses patients suffering from Dukes A or B tumors (see Fig. 5C) who did not receive chemotherapy, is characterized by a survival curve that is worse than the curve for the new group III, which includes patients suffering from Dukes C or D tumors (see Fig. 5D) who did receive chemotherapy.

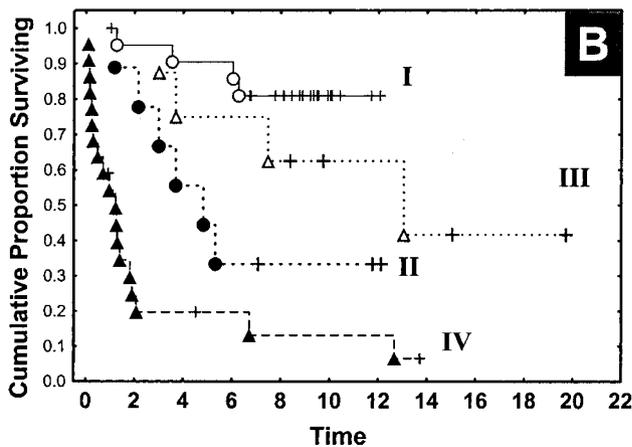
Combination of the Prognostic Values Provided by Galectin and MIF Expression in Addition to Standard Clinical Features

Cox regression analyses were carried out to determine the relationship between the survival times and the different variables identified above as po-

tentially informative, *i.e.*, the galectin and MIF mean optical densities (MOD) measured in epithelial and connective tissue, as well as the patients' ages, the Dukes tumor stages and the tumor location, giving a total of 7 variables (see Table 1). Table 1 summarizes the best models generated by considering either all the tumor cases together (Models A-B), Dukes A-B tumors (Model C) or Dukes C-D tumors (Models D-E). Each model in Table 1 is characterized by its level of significance, the list of the variables selected, their respective coefficients in the models (in logarithmic and exponential form, respectively) and the corresponding p-values evaluating the significance of the predictive contribution of the variables to the overall model. Model A (taking all the case into account) show that of the 7 variables considered, the most contributive variables to the prediction of the survival times concerned galectin-3 expression (in epithelium and, in a lesser degree, connective tissue). Of the clinical variables, only the patients' ages significantly contributed to the model. Model B presents the best model that we evidenced on the complete series of



C	Dukes A		Dukes B	
	S	L	S	L
I	0	10	2	9
II	1	1	4	2



D	Dukes C		Dukes D
	S	L	(S)
III	0	6	2
IV	4	3	14

FIGURE 5. Illustration of the survival curves (expressed in years) associated with the Dukes classification (frame A) and the new prognostic groups I, II, III, and IV (frame B) defined as indicated in Figs. 3A-D. Frames C-D detail the composition of these prognostic groups in terms of the clinical groups defined on the basis of the Dukes classification and the patients' survival periods. S = short period (less than 5 years) and L = long period (more than 6 years). In contrast to the survival curves, these latter two tables do not take into account the living patients with survival periods of fewer than 6 years.

tumor cases. This confirms that galectin-3 expression provided highly significant contributions to the predictive model.

Model C also confirms that the most contributive variable concerned galectin-3 expression in epithelial tissue in the specific case of tumors classified as Dukes A or B. In contrast, when Dukes C or D cases were specifically considered Model D indicated that the Dukes stages (*i.e.*, the difference between Dukes C and D stages) was the most contributive variable. This was not surprising as shown in Fig. 4C. However, the best model that we generated to predict the survival times of patients suffering from Dukes C or D tumors (detailed in Model E) revealed that MIF expression in connective tissue was able to add significant prognostic information to the difference between the tumor stages.

DISCUSSION

Galectin-3 is expressed in almost all types of epithelia, chondrocytes and in various inflammatory

cells (20–24), and the present study shows that the prognostic value that we delineated for galectin-3 concerns the epithelial component of colorectal tumors rather than the connective tissue. In contrast, galectin-1 is primarily expressed in the stromal cells of human colorectal tissue, with the frequency of strong stromal galectin-1 expression increasing in the normal-adenoma-carcinoma progression (31, 48). In the present study we observed a slight increase in the level of galectin-3 expression (in terms of immunostaining intensity) in the connective tissues of colorectal tumors, whereas the metastases showed a decrease in galectin-3 expression when compared with the normal samples (Fig. 2B).

The prognostic value associated in the present study to galectin-3 expression in epithelial tissue of human colorectal cancers concerns specimens associated with low clinical stages (Dukes A and B; see Fig. 3A). In these specimens we observed that higher levels of galectin-3 expression were associated with shorter periods of survival (Fig. 4B). In other words, these data mean that increased levels

of galectin-3 are associated with bad prognoses in human colorectal cancers with low clinical stages (Dukes A and B), but no longer in those with higher clinical stages (Dukes C and D). These data therefore suggest that modifications to galectin-3 expression concern the early steps of colorectal cancer progression and no longer the later steps, in which MIF seems to play a significant role, as explained below. These different results were confirmed by means of multivariate analyses taking into account standard clinical variables (Table 1). These analyses also enhanced a strong prognostic contribution for galectin-3 expression when all cases were considered (Table 1).

Our data on galectin-3 partly corroborate those of Sanjuan *et al.* (48), who observed that patients with stage II and III colorectal cancers whose tumors exhibited strong galectin-3 expression had worse overall and short relapse-free survival periods than those with low galectin-3 expression. Sanjuan *et al.* (48) also observed that galectin-3 expression is down-regulated in the initial stages of neoplastic progression. We did not observe any such feature but, as discussed below, the term “galectin-3 expression” is very ambiguous: does it refer to the

number of cells with galectin-3 expression (corresponding to the LI variable), to the galectin-3 staining intensity per immunopositive cell (corresponding to the MOD variable) or to both? The fact, already emphasized by Itzkowitz (28), remains valid that the data published in the literature on the diagnostic and/or prognostic value of galectin-3 expression in human colorectal carcinomas are very inconsistent. Indeed, as already mentioned in the Introduction, Lotz *et al.* (29) and Castronovo *et al.* (30) report decreasing levels of galectin-3 expression in colon carcinomas as compared with normal colon tissue while Irimura *et al.* (31), Schoeppner *et al.* (32) and Nakamura *et al.* (33) report the opposite. The data from our study can be reconciled with those of the second group of authors because we also observed increasing levels of galectin-3 (staining intensity) in colorectal tumors as compared with normal colon tissue. In the mentioned editorial, Itzkowitz (28) reports that the most comprehensive of the above-mentioned studies was the one published by Schoeppner *et al.* (32), who showed weak or no galectin-3 expression in normal colonic mucosa remote from colon cancers, with the expression progressively increasing in colonic adenomas, colon cancers at more advanced stages, and colon cancer metastases as compared with their primary tumors. While increased galectin-3 expression in colorectal cancers was associated with lower survival rates, this effect was not found to be independent of, or dependent on, tumor stage (32). Our study clearly indicates that this effect is dependent on the clinical stages.

It has been shown recently that circulating galectin-3 might indicate tumor progression (and not only of colorectal origin) because the galectin-3 concentration in sera from patients with metastatic pathologies is higher than in sera from patients with localized tumors (49). However, because only 25 patients with gastrointestinal cancer were included and the source of the serum galectin-3 is unclear, further studies are warranted to delineate a correlation between the metastasizing phenotype and this serum parameter. Itzkowitz (28) asserts that possible explanations for the varying conclusions in the mentioned studies published in the literature on the diagnostic and/or prognostic values of galectin-3 expression include differences in sample size and selection, different methodological approaches, the use of different antibodies and differences in the interpretation of staining. The present study clearly indicates that the significant variations observed in terms of MIF and galectin-3 expression at protein level across the different groups analyzed in the Results section were connected with immunostaining intensity quantified by means of the Mean Optical Density variable (MOD, see Materials and Methods). In contrast, the

TABLE 1. Cox Regression Models

Model/ <i>P</i> -Value	Variable	β	exp(β)	<i>P</i> -value
In all cases				
A) <i>P</i> = 0.009	Dukes (A-D)	0.111	1.118	0.5
	Tumor Location	-0.401	0.670	0.4
	Patient's age	0.053	1.055	0.02
	Gal3-Epith	0.065	1.068	0.001
	Gal3-Conn	-0.154	0.857	0.01
	MIF-Epith	0.008	1.008	0.7
	MIF-Conn	-0.005	0.995	0.9
B) <i>P</i> = 0.0006	Patient age	0.052	1.053	0.01
	Gal3-Epith	0.065	1.067	0.0004
	Gal3-Conn	-0.162	0.851	0.002
In Dukes A and B tumors				
C) <i>P</i> = 0.002	Dukes (A-B)	1.026	2.790	0.07
	Patient's age	0.084	1.088	0.2
	Gal3-Epith	0.137	1.147	0.001
	Gal3-Conn	-0.123	0.884	0.4
	MIF-Epith	0.181	1.198	0.04
MIF-Conn	-0.094	0.910	0.2	
In Dukes C and D tumors				
D) <i>P</i> = 0.001	Dukes (C-D)	0.836	2.307	0.008
	Patient's age	0.051	1.052	0.1
	Gal3-Epith	0.031	1.092	0.4
	Gal3-Conn	0.014	1.014	0.9
	MIF-Epith	-0.020	0.980	0.4
	MIF-Conn	-0.132	0.876	0.1
E) <i>P</i> = 0.00007	Dukes (C-D)	0.869	2.385	0.003
	Patient's age	0.033	1.033	0.1
	MIF-Conn	-0.145	0.865	0.03

The “Model/*P* value” indicates the overall level of significance of the model. The variables related to MIF and galectin-3 expression are the mean optical densities measured either in tumor epithelium (“Epith”) or in peritumoral connective tissue (“Conn”). The equation at the basis of the Cox Regression model is an exponential function of a linear combination of the variables considered, where β indicates the coefficient of each variable in the linear combination, and exp(β) its exponential value. The *P*-value indicates the level of significance of the contribution of each variable to the model (so enabling to be concluded that β is significantly different from zero).

Labeling Index (the LI variable that refers to the percentage of immunopositive cells) for the MIF and galectin-3 expression did not significantly vary across the different groups. Thus, in our study the MIF and galectin-3 staining intensities related to significant prognostic values, while the percentages of cells immunopositive for these proteins did not.

The present study strengthens the case that galectin-3 expression is associated with colorectal cancer development. In principle, galectin-3 can interfere with tumor progression at different levels. Galectin-3 can for example maintain tumor growth by decreasing cell loss via apoptosis, although it is emerging that the profile of galectin-3 activity critically depends on the cell type (50, 51). Galectin-3 may also be a critical determinant for the anchorage-independent survival of disseminating cancer cells in the circulation during metastases (51). Galectin-3 can also significantly modify the metastatic capacities of colorectal cancer cells: using lectin blocking by sugar or antibody or transfected cell lines a marked decrease in liver colonization and the spontaneous metastasis of the sense-transfected colon cancer cells were observed (52, 53). Another level where galectin-3 can be functional is in interfering with activities of other galectins, as recently shown for galectin-1 as negative growth regulator (54). Because we have previously documented that it is essential to monitor presence of more than galectin-1 and -3 in colorectal cancer by delineating galectin-8 as suppressor activity (43), this aspect will deserve special attention in further studies.

It should be emphasized that data obtained from *in vitro* with cell lines, whose biological behavior dramatically differs from human clinical samples may not be readily extrapolated to the clinical situation.

MIF was one of the first cytokine activities to be discovered and was originally described as a T lymphocyte product that inhibited the random migration of macrophages (36, 37). Similar to other proinflammatory cytokines, it not only modulates immune functions but also has a bearing on angiogenesis and tumor growth, rendering tumor-growth-promoting activities likely besides an expected role in anti-tumor immunity (36, 37). MIF has already been proved to be over-expressed in colon cancers as compared with normal colon tissues (38), and to participate in colon cancer progression (39). Takahashi *et al.* (55) studying the murine colon cancer line colon 26 substantiated that in this model MIF is profoundly involved in tumor growth stimulation. Elicitors for increase of intracellular MIF presence were suggested to serve as potential targets for therapy, a conclusion extended to B cell lymphoma and melanoma in ensuing studies (56, 57). Corroborating these reports and underlining the asserted role of MIF in tumor

progression, Takahashi *et al.* suggested the possibility that MIF promotes tumor cell growth in conjunction with other growth factors. In addition, Ogawa *et al.* (58) found that MIF activity may be involved in tumorigenesis via the promotion of angiogenesis. Thus, MIF—like other proinflammatory cytokines such as interleukin-6 (for reviews, see 59–61; for recent report on this cytokine as growth factor in the case of prostate cancer, see 62)—is not only involved in immunoregulation but also in tumor cell growth. Our report clearly substantiates that MIF is present in human colorectal cancer tissue *in situ* and encourages further histopathological investigations targeting MIF presence, an emerging topic with potential therapeutic implications. The prognostic value that we evidenced for MIF concerns the connective tissue of colorectal tumors associated with high clinical stages (Dukes C and D; see Figs. 3D and 4D). These data therefore suggest that MIF detected in the vicinity can influence the biological behavior of highly malignant colorectal epithelial cells. Data on the Kendall correlation tests shown in Fig. 2D and F even intimate a functional cooperation with galectin-3 present in this compartment for patients with short survival periods. Remarkably, sustained growth in cholesteatomas had been correlated by the same statistical procedure to the score of immunopositive cells for these two effectors (34). Emergence of the same correlation in these two tissue types warrants to broaden the data basis on this aspect, keeping in mind the suggestive functional overlap of MIF and galectin-3 in growth regulation and angiogenesis.

In conclusion, the data from the present study show that the survival curve associated with the Dukes B colorectal cancer patients (who did not receive chemotherapy) is similar to the survival curve of the Dukes C colorectal cancer patients (who did receive chemotherapy). In contrast, the new group II that we identified in the present study on the basis of galectin-3 expression in the epithelial tissue of colorectal cancers associated with Dukes A and B clinical stages, includes patients who did not receive chemotherapy. The survival curve associated with this group is worse than the curve characterizing the new group III, which includes patients suffering from a Dukes C or D colorectal tumor (see Fig. 5D) who received chemotherapy. These data therefore enabled us to identify from among the patients suffering from a Dukes A or B colorectal tumor the group II of patients for whom chemotherapy would be recommended in our view. Similarly, the new group IV identified in the present study could be considered as identifying cases requiring more “aggressive” adjuvant therapy than that currently provided. Lastly, our data suggest that the pro-inflammatory MIF cytokine could influence the late stages of colorectal

cancer development. Immunomodulatory compounds, in particular those affecting MIF presence, could therefore be assayed in addition to standard chemotherapy for patients whose colorectal cancers are associated with a high clinical stage. As suggested from model animal studies on colon carcinoma, lymphoma and melanoma, tumor progression could be reduced by targeting MIF expression. In this respect, our data provide first histopathological evidence in colorectal cancer supporting this emerging concept.

Acknowledgments: We gratefully acknowledge Dr. J.L. Wang for kindly providing the galectin-3 expression vector. This work has been carried out on the basis of grants awarded by the "Amis de l'Institut Bordet" (Brussels, Belgium), the "Fonds de la Recherche Scientifique Médicale" (FRSM, Brussels, Belgium), the "Fondation Yvonne Boël" (Brussels, Belgium) and the Wilhelm Sander-Stiftung (Munich, Germany).

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