Profiling Markers of Prognosis in Colorectal Cancer

Matthew S. Lyall, Sinclair R. Dundas, Stephanie Curran, and Graeme I. Murray

Abstract

Purpose: Colorectal cancer is one of the most common forms of cancer in developed nations and the incidence of this disease is increasing. There is a need to further stratify prognostically distinct groups of colorectal cancer, and the purpose of this study was to identify prognostically significant immunohistochemical marker profiles in colorectal cancer.

Experimental Design: In this study, a range (n = 23) of markers [pRb, p16, p21, p27, p53, proliferating cell nuclear antigen, cyclin D1, bcl-2, epidermal growth factor receptor, C-erb-B2, topoisomerase-I, liver fatty acid – binding protein, matrix metalloproteinases (MMP) 1-3, 7, 9, and 13, MT1-MMP, MT2-MMP, and tissue inhibitors of MMP 1-3] of putative prognostic significance have been investigated by immunohistochemistry on formalin-fixed, wax-embedded sections in a series (n = 90) of stage III (Dukes C) colorectal cancers. An immunohistochemical score based on the intensity of immunoreactivity and, where relevant, the proportion of immunoreactive cells was established for each marker.

Results: Unsupervised two-dimensional hierarchical cluster analysis identified three distinct cluster groups (designated groups 1-3) with different marker profiles. There were significant survival differences between groups 1 and 2 (log rank = 11.48; P = 0.0007) and between groups 1 and 3 (log rank = 8.32; P = 0.0039). Multivariate analysis showed that the complete marker profile was independently the most significant prognostic factor (hazard ratio, 2.27; 95% confidence interval, 1.15-4.48; P = 0.004).

Conclusions: This study has identified an immunohistochemical marker profile of colorectal cancer and showed that it is an independent indicator of prognosis in this type of cancer.

Colorectal cancer is one of the most common forms of cancer in developed nations, and the incidence of this disease is continuing to increase (1). It is the third most commonly diagnosed cancer in both men and women, and its 5-year survival rate, although slowly improving, is still poor at 40%. As well as genetic susceptibility, environmental influences make important contributions to the development of colorectal cancer, and epidemiologic data suggest that dietary factors are important in the pathogenesis of this tumor type (2, 3). Most large bowel cancers arise from adenomas and ~5% of adenomatous polyps progress to malignant tumors within 5 to 10 years. The majority arise within the rectum, the next most common site being the sigmoid colon, and the remainder are fairly equally distributed between the descending, transverse, and ascending colon. Histologically, the tumors are virtually all adenocarcinomas (1).

Currently, the most useful and widespread method of obtaining a guide to the prognosis in each individual case is through histopathologic confirmation of the adequacy of excision and the tumor stage as assessed by invasion of the tumor through the intestinal wall and determination of lymph node metastasis. This process is the basis for all of the major staging methods used for this particular type of cancer and has been proven to provide the best indication of prognosis at the time of presentation. However, it is widely recognized that tumors of the same pathologic stage can produce considerably different clinical outcomes, thereby highlighting the necessity for further identification of other factors that influence prognosis independent of tumor stage (i.e., independent prognostic markers; refs. 1, 4).

The association between various tumor biomarkers and colorectal cancer has been extensively studied and investigations have included a wide range of markers representing proteins involved in many aspects of tumor development and progression, including cell cycle regulatory proteins, growth factors and their receptors, cell death – associated proteins, and proteins related to tumor invasion and metastasis (5 – 35).

In this study, we have investigated, by immunohistochemistry, the expression of a wide array of proteins that have previously been individually suggested to be of prognostic significance in colorectal carcinomas. These include matrix metalloproteinases (MMP), tissue inhibitors of MMPs (TIMP), cell cycle control proteins, apoptosis-related proteins, and growth factor receptors. Analysis based on the clustered expression of these markers defines groups of colorectal cancers with specific phenotypes and distinct clinical outcomes.

Authors' Affiliation: Department of Pathology, University of Aberdeen, Aberdeen, United Kingdom

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Requests for reprints: Graeme I. Murray, Department of Pathology, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, United Kingdom. Phone: 44-1224-553794; Fax: 44-1224-663002; E-mail: g.i.murray@abdn.ac.uk.

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Materials and Methods

Tumor samples

Tumor samples were collected from patients undergoing curative resection for colorectal cancer between 1994 and 1998 at Aberdeen Royal Infirmary (Aberdeen, United Kingdom). All the tumors were fixed in formalin and representative blocks were embedded in wax. Two expert gastrointestinal pathologists (S.C. and G.I.M.) reviewed the histopathology of each case by light microscopic examination of H&E-stained sections. All of the tumors in the study were Dukes stage C adenocarcinomas and International Union Against Cancer stage III (i.e., tumors with lymph node metastases), as these constitute a well-defined group of tumors (n = 90; Dukes C1 = 69 and Dukes C2 = 21; pN1 = 64 and pN2 = 26). The majority of tumors 75 (83%) were moderately differentiated, whereas the remaining 15 (17%) were poorly differentiated. The age range of the patients was 33 to 89 years (mean, 65 years); 42 (47%) of the patients were female, whereas 48 (53%) were male. There were 29 (32%) proximal colon cancers, 36 (40%) distal colonic cancers, and 25 (28%) rectal cancers. Follow-up ranged between 60 and 100 months, at which time 55 (61%) of the patients had died with a median survival of 42 months [95% confidence interval (95% CI), 27-57] and a mean survival of 53 months (95% CI, 45-61). This study had the approval of the local research ethics committee.

Immunohistochemistry

Formalin-fixed, wax-embedded whole tumor sections (4 μm thick), which included the deep invasive margin, were used. The sections were dewaxed in xylene and rehydrated and an antigen retrieval step was done. Antigen retrieval was required for all the antibodies, except MMP7 and MMP13, and was achieved by microwaving the sections in 0.01 mol/L citrate buffer at pH 6.0 for 20 minutes in an 800 W microwave oven. For cyclin D1 staining, sections were microwaved in 1 mmol/L EDTA (pH 8.0) for 20 minutes. The sections were then allowed to cool to room temperature and were then immunostained using a Dako TechMate 500 autostainer (Dako, Ely, United Kingdom). Details of antibodies, antibody dilutions, and positive controls are noted in Table 1. Primary antibody appropriately diluted was applied for 50 minutes at room temperature followed by washing with buffer (Dako) and peroxidase blocking. Biotinylated goat anti-mouse/rabbit secondary antibody (1/1,000; Dako) was applied for 25 minutes at room temperature followed by further washing with buffer to remove unbound antibody. A complex of avidin with horseradish peroxidase (Dako) and diaminobenzidine were used as the detection staining system (18, 36). The sections were then lightly counterstained with hematoxylin, dehydrated, and mounted. Omitting the primary antibody from the immunohistochemical procedure and replacing it with antibody diluent or nonimmune rabbit serum acted as negative controls for monoclonal and polyclonal antibodies, respectively. Immunoreactivity for each antigen was evaluated by examination of the sections by bright-field light microscopy independently by two experienced observers. For each marker, there was high degree of concordance between the two observers and any discrepancies were resolved by simultaneous reevaluation of the sections by both observers using a multihead microscope. All the markers were evaluated and sections were scored before obtaining outcome (survival) data for each case.

Immunohistochemical scoring

MMPs/TIMPs. A scoring system was used to describe both intensity (negative, weak, moderate, and strong) and proportion (0%, 1-5%, 6-75%, and 76-100%) of tumor cells staining in each case as described previously (18, 37). To enable analysis of the individual immunostaining results, integer values were assigned to the intensity scores (0-3) and the proportion of cells stained (0-3). These values were multiplied together to provide a single integrated score for each MMP or TIMP and the data were reduced to an ordinal scale of 0 to 6 as described previously (18, 37).

Table 1. Details of the antibodies used in this study

Antibody	Type	Source and ID (ref.)	Positive control	Antigen retrieval	Antibody dilution
LFABP	Monoclonal	Novocastra, NCL-LFABP	Liver	Yes	1:200
Topoisomerase-I	Monoclonal	Novocastra, NCL-TOPO1	Tonsil	Yes	1:25
Cyclin D1	Monoclonal	Novocastra, NCL-P2D11F11	Breast cancer	Yes (1 mmol/L EDTA, pH 8)	1:10
p16	Monoclonal	Pierce and Warriner, G175-405	Esophagus	Yes	1:25
p21	Monoclonal	Oncogene Research Products, EA10	Breast cancer	Yes	1:40
p27	Monoclonal	Novocastra, NCL-1B4	Tonsil	Yes	1:40
p53	Monoclonal	Dako, DO-7	Colon cancer	Yes	1:200
PCNA	Monoclonal	Dako, PC10	Colon cancer	No	1:50
pRb	Monoclonal	Dako, pRB1	Tonsil	Yes	1:50
bcl-2	Monoclonal	Dako, clone 124	Tonsil	Yes	1:100
C-erb-B2	Monoclonal	Novocastra, NCL-CB11	Breast cancer	Yes	1:100
EGFR	Monoclonal	Novocastra, NCL-EGFR	Placenta	Yes	1:20
MMP1	Monoclonal	Own laboratory, 3B6 (16, 18)	Colon cancer	Yes	1:2
MMP2	Monoclonal	Own laboratory, 4D3 (18)	Colon cancer	Yes	1:20
MMP3	Monoclonal	Own laboratory, 1B4 (18)	Colon cancer	Yes	Neat
MMP7	Monoclonal	Chemicon, MAB3315	Colon cancer	No	1:3,200
MMP9	Monoclonal	Own laboratory, 2C3 (18)	Colon cancer	Yes	1:2
MMP13	Monoclonal	Chemicon, MAB181-15A12	Breast cancer	No	1:200
MT1-MMP	Polyclonal	Chemicon, AB815	Placenta	Yes	1:200
MT2-MMP	Monoclonal	Chemicon, MAB3320	Colon cancer	Yes	1:400
TIMP1	Monoclonal	Own laboratory, 2A5 (18)	Colon cancer	Yes	1:2
TIMP2	Monoclonal	Own laboratory, 3A4 (18)	Colon cancer	Yes	1:64
TIMP3	Monoclonal	Chemicon, MAB3318	Colon cancer	Yes	1:2,000

Cell cycle regulatory proteins. Sections immunostained for cyclin D1, pRb, p16, p21, p27, and p53 were scored semiquantitatively by estimating the percentage of tumor cell nuclei staining (0-5%, 6-25%, 26-50%, 51-75%, and 76-100%; ref. 36). All immunoreactive nuclei were regarded as positive irrespective of the intensity of staining. The proliferating cell nuclear antigen (PCNA) labeling index was calculated as the percentage of positive nuclei divided by the total number of nuclei counted (>500 nuclei in ≥3 high-power fields; ref. 36).

C-erb-B2. Sections were scored semiquantitatively according to the following Food and Drug Administration – approved scoring system

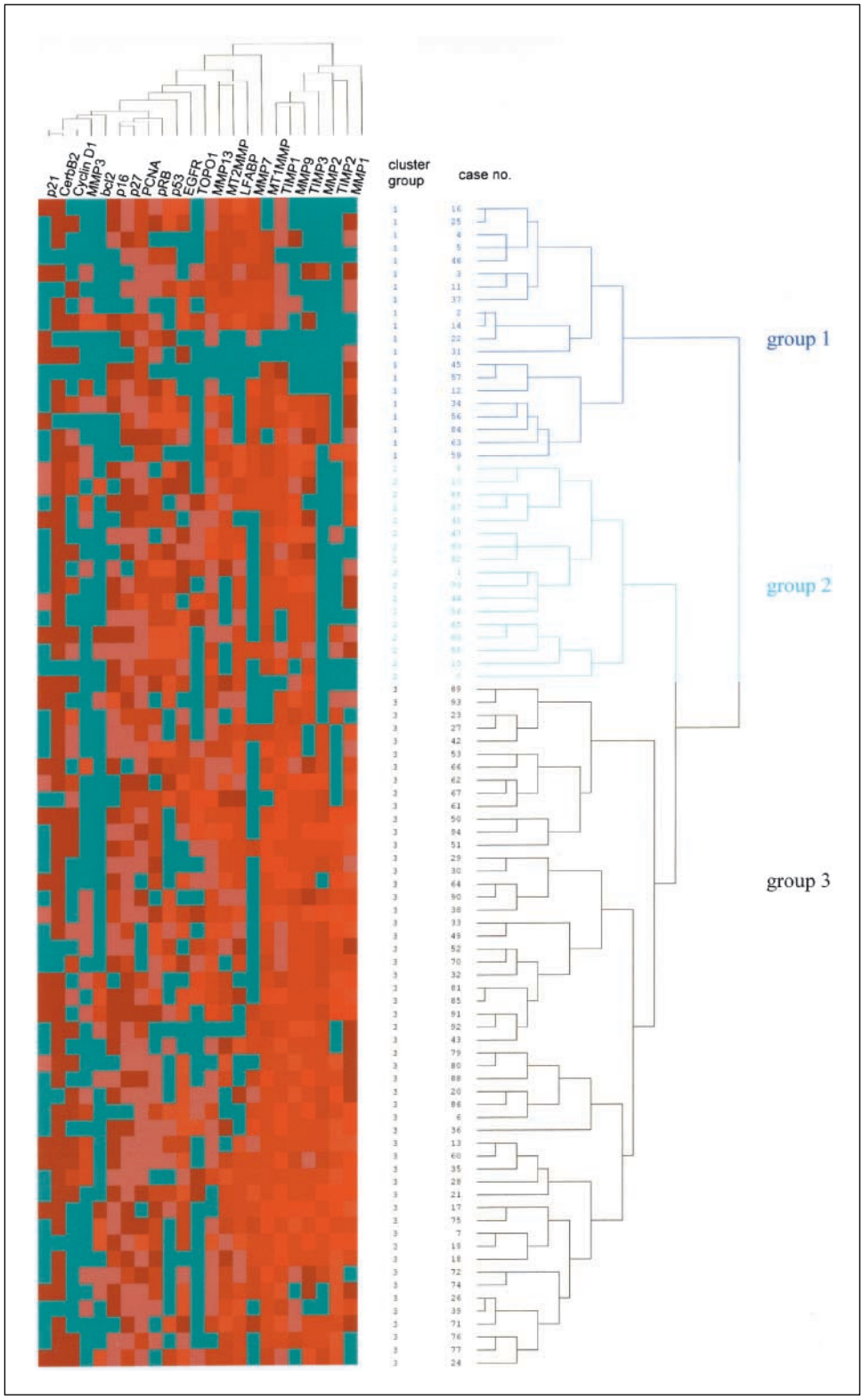

Fig. 1. Two-dimensional hierarchical cluster analysis of marker profile in colorectal cancer. Left, a graphical representation of the marker immunohistochemistry score; right, dendrogram produced by the hierarchical cluster analysis. Columns, specific markers; rows, individual cases. In the graphical representation, blue denotes zero and red denotes positive values. Brighter shades of red represent a higher immunohistochemistry score. Three distinct clusters are identified (*blue,* group 1; *turquoise,* group 2; *black,* group 3). All but one of the patients in group 1 were dead at the time of censoring the patient survival data.

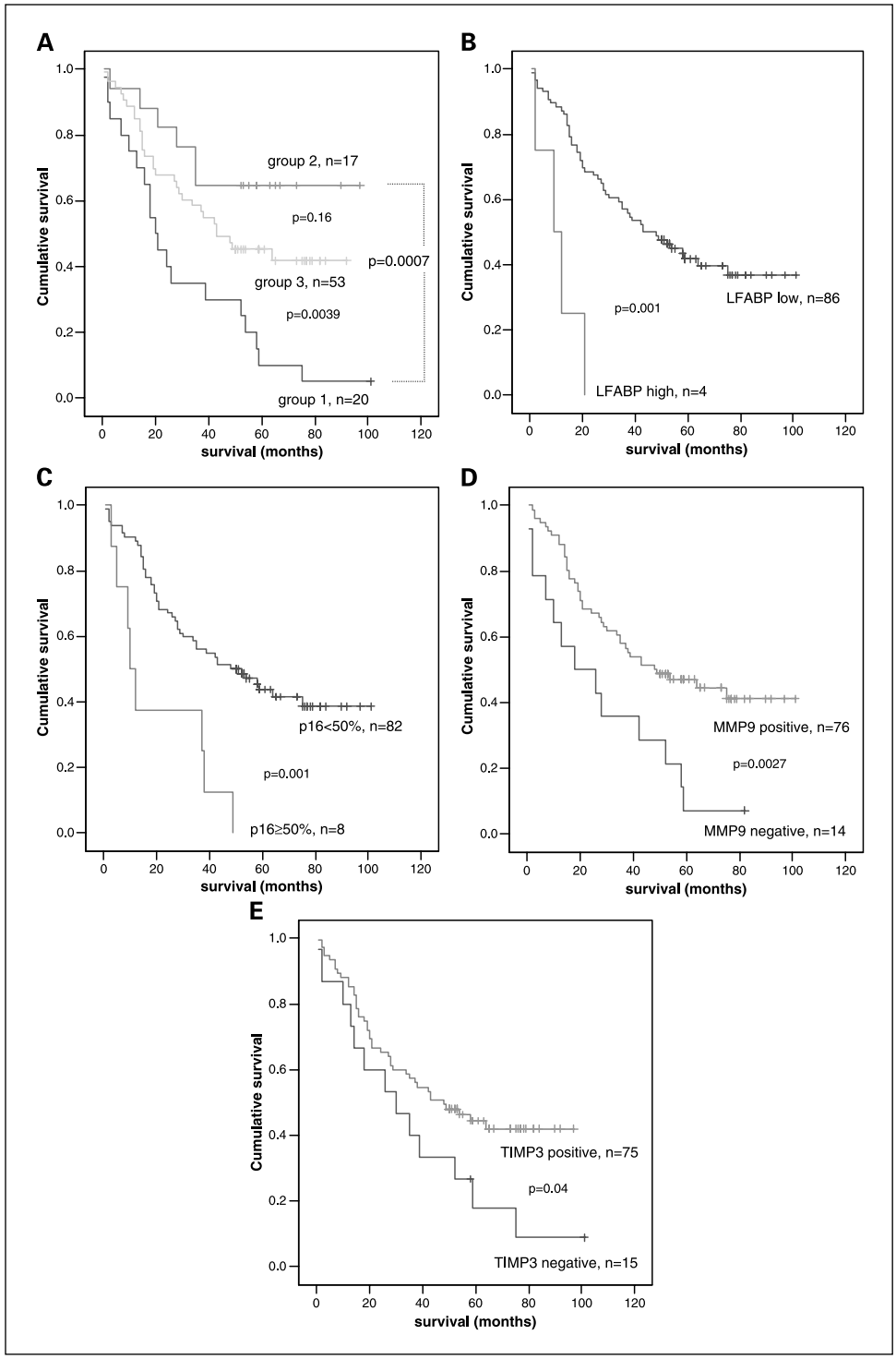

Fig. 2. *A,* Kaplan-Meier survival plot of the three groups identified by hierarchical cluster analysis (groups 1-3). There is a highly significant difference in survival between patients in groups 1 and 2 (log rank = 11.48; $P = 0.0007$) and between groups 1 and 3 (log rank = 8.32; $P = 0.0039$). The difference in survival between groups 2 and 3 does not reach statistical significance (log rank = 1.99; $P = 0.16$). Kaplan-Meier survival plots of individual markers identified to show significant prognostic differences. *B,* LFABP. *C,* p16. *D,* MMP9. *E,* TIMP3.

(38, 39): 0, no immunostaining; 1, complete membranous immunostaining of <10% of tumor cells; 2, weak complete membranous staining of >10% of tumor cells; 3, strong complete membranous staining of >10% of tumor cells. Scores of 0 or 1 indicate a negative tumor, whereas scores of 2 and 3 were regarded as positive expression of C-erb-B2.

Epidermal growth factor receptor. Epidermal growth factor receptor (EGFR) immunohistochemistry was assessed according to the distribution (≤5%, 6-25%, 26-50%, 51-75%, and 76-100%) and localization of the immunoreactivity (membranous or cytoplasmic; ref. 40).

Liver fatty acid–binding protein. The cellular localization of liver fatty acid–binding protein (LFABP) immunoreactivity was determined and scored for both intensity (0, negative; 1, weak; 2, moderate; 3, strong) and proportion of stained cells (0, 0%; 1, 1-5%; 2, 6-25%; 3, 26-50%; 4, 51-75%; 5, 76-100%). The values for intensity and proportion of tumor cells stained were combined together to provide a single LFABP score (41).

Topoisomerase-I. Immunostaining for topoisomerase-I was scored as the percentage of positive nuclei (≤5%, 6-25%, 26-50%, 51-75%, 76-100%; ref. 42).

Statistical analysis

Statistical analysis, including hierarchical cluster analysis, χ^2 test, Kaplan-Meier survival analysis, and Cox multivariate regression analysis, was done using SPSS version 12.0.1 for Windows XP (SPSS UK Ltd., Woking, Surrey, United Kingdom). Unsupervised two-dimensional hierarchical cluster analysis of the immunohistochemical score data was done using the furthest neighbor linkage method with the χ^2 measure to identify individual groups of tumors with specific marker profiles. The log-rank test was used to determine survival differences between individual groups. For survival analysis of individual markers, dichotomization of the immunohistochemical scores was based on visual inspection of Kaplan-Meier survival curves generated using multiple cutoff points of the raw scores in combination with the evaluation of the resultant log rank and *P* values. The cutoff points of each marker selected for further analysis were those which gave the most significant discrimination (in terms of survival) between the two groups. $P < 0.05$ was regarded as significant.

Results

Hierarchical cluster analysis. Unsupervised two-dimensional hierarchical cluster analysis identified three distinct cluster groups (designated groups 1-3; Fig. 1). Group 1 (poor prognosis) consisted of 20 patients, only 1 of whom was still alive at the census point, with a median survival of 20 months (95% CI, 13-27; mean, 31 months; 95% CI, 19-42). Group 2 (good prognosis) contained 17 patients, of whom 11 patients were alive at the census point, with a median survival of >100 months (mean, 71 months; 95% CI, 54-88). There were 53 patients in group 3 (intermediate prognosis), of whom 23 were alive at the census point, with a median survival of 43 months (95% CI, 18-68; mean, 53 months; 95% CI, 43-63). There were significant survival differences between groups 1 and 2 (log rank = 11.48; $P = 0.0007$; Fig. 2A) and between groups 1 and 3 (log rank = 8.32; $P = 0.0039$). There was no significant survival difference between groups 2 and 3 (log rank = 1.99; $P = 0.16$).

Analysis of the individual groups identified the following markers as having the most significantly different expression between the individual groups: LFABP (low in group 1, high in group 2), p16 (low in group 1, high in group 2), p53 (low in group 1, high in group 2), MMP2 (low in group 1, high in group 2), MMP7 (high in group 1, low in group 2), MT1-MMP (low in group 1, high in group 2), TIMP1 (low in group 1, high in group 2), TIMP2 (low in group 1, high in group 2), and TIMP3 (low in group 1, high in group 2; Table 2). There were no significant differences between the three groups in terms of the distribution of tumor site, degree of tumor differentiation, nodal status (i.e., Dukes C1 versus Dukes C2 or pN1 versus pN2), or patient gender.

Univariate survival analysis showed that, apart from cluster group, the only other significant survival factors were apical lymph node positivity (i.e., Dukes C2; log rank = 4.56; $P = 0.03$) and gender (log rank = 4.12; $P = 0.04$, poorer outcome in males). Other factors, such as tumor site (i.e., proximal versus distal versus rectum), patient age (i.e., ≤65 versus >65 years), number of involved lymph nodes (i.e., pN1 versus pN2), and tumor differentiation (moderate versus poor), showed no significant survival differences (Table 3).

Multivariate analysis, including all the factors studied by univariate analysis in the Cox regression model, showed that cluster group (hazard ratio, 2.27; 95% CI, 1.15-4.48; $P = 0.004$) was the most significant prognostic factor, whereas the only other independently significant factor was gender (hazard ratio, 0.56; 95% CI, 0.32-0.98; $P = 0.04$). Apical lymph node positivity was not an independent prognostic factor.

Analysis of individual markers. The results of survival analysis for each of the markers individually are shown in Table 4 and Fig. 2B-E. Only LFABP (log rank = 14.4; $P = 0.001$), p16 (log rank = 23.9; $P = 0.001$), MMP9 (log rank = 8.98; $P = 0.0027$), and TIMP3 (log rank = 4.05; $P = 0.04$) displayed prognostic significance in the whole cohort of patients. The median survival in the LFABP high group ($n = 4$) was 9 months (95% CI, 0-19; mean, 11 months; 95% CI, 3-19), whereas in the LFABP low group ($n = 86$) the median survival was 43 months (95% CI, 26-60; mean, 55 months; 95% CI, 47-63). In the p16 ≥50% group ($n = 8$), the median survival was 10 months (95% CI, 6-14; mean, 20 months; 95% CI, 8-33), whereas in the p16 <50% group ($n = 82$) the median survival was 48 months (95% CI, 27-69; mean, 56 months; 95% CI, 48-65). The median survival in the MMP9-negative group ($n = 14$) was 18 months (95% CI, 0-42; mean, 29 months; 95% CI, 15-42), whereas in the MMP9-positive group ($n = 76$) the median survival was 48 months (95% CI, 21-75; mean, 58 months; 95% CI, 49-67). In the TIMP3-negative group ($n = 15$) the median survival was 30 months (95% CI, 9-51; mean, 37 months; 95% CI, 22-52), whereas in the TIMP3-positive group ($n = 75$) the median survival was 48 months (95% CI, 27-69; mean, 55 months; 95% CI, 46-64).

Discussion

Many markers of prognosis have been described in colorectal cancer. Most studies usually focus on either a single marker or very small group of markers; often, individual studies produce conflicting results (4–35). In this study, we have investigated, by immunohistochemistry on formalin-fixed, wax-embedded whole tumor sections, the presence of a range of markers simultaneously in a large series of well-characterized colorectal cancers of a single pathologic stage (stage III, Dukes C), all with long-term follow-up.

The proteins investigated in this study included markers of tumor invasion, cell cycle regulatory proteins, and growth factor receptors. All of these proteins have important roles in tumor progression and previous studies have suggested that they could be markers of prognosis in colorectal cancer. The present analysis has permitted the evaluation of specific groups of markers in a broader biological context and has determined the interplay of these molecules and their prognostic relevance. MMPs are able to degrade elements within basement membrane and extracellular matrix, whereas the TIMPs are understood to regulate the activation and function of the MMPs. The relationship between MMPs and TIMPs plays a fundamental role in regulating the degradation of extracellular matrix, and this interaction is important with regard to the promotion of tumor invasion and metastasis (17, 18). In addition, the MMPs also have many other specific biological actions distinct from their role in degrading matrix proteins, including promotion of cell proliferation and inhibition of apoptosis. Some studies have shown the prognostic relevance of these enzymes (MMP1, MMP7, MMP9, TIMP1, and TIMP2; refs. 16, 17) in colorectal cancer. We have

Table 2. Relationship of individual markers in the different cluster groups

Marker	χ^2	*P*	Group 1 phenotype
LFABP	26.2	0.01	Low
Topoisomerase-I	14.1	0.29	—
Cyclin D1	9.4	0.31	—
p16	23.4	0.003	Low
p21	0.9	0.98	—
p27	11.5	0.17	—
p53	17.1	0.02	Low
PCNA	17.5	0.03	—
pRb	9.9	0.27	—
bcl-2	6.4	0.60	—
C-erb-B2	1.1	0.59	—
EGFR	8.1	0.42	—
MMP1	14.8	0.14	—
MMP2	44.1	<0.0001	Low
MMP3	12.1	0.15	
MMP7	32.1	0.001	High
MMP9	34.5	0.001	Low
MMP13	16.1	0.19	
MT1-MMP	37.8	<0.0001	Low
MT2-MMP	17.5	0.13	
TIMP1	27.3	0.007	Low
TIMP2	60.4	<0.0001	Low
TIMP3	36.0	<0.0001	Low

examined previously the MMP/TIMP phenotype in the tumors of this cohort of patients and showed that a specific MMP/TIMP expression profile was associated with poor prognosis in colorectal cancer (18).

Other proteins believed to be of importance in neoplasia and in the development of colorectal cancer are those involved in cell cycle control. Precise control of the cell cycle is fundamental to the prevention of disordered cellular proliferation and carcinogenesis. Abnormal expression of one or more cell cycle regulators occurs commonly in colorectal cancer, although the prognostic value of these proteins in this type of tumor is disputed (4, 13, 20–23, 26–28, 30).

Analysis of biomarker data carried out using unsupervised hierarchical clustering does not depend on an *a priori* hypothesis and this approach creates clusters or groups of data solely based on their shared characteristics. In this particular study, an immunohistochemical score was devised for each individual marker to indicate their expression within each tumor and it was through this scoring scheme that the data analysis was done. Hierarchical cluster analysis identified three different patterns of biomarker expression leading to the hypothesis that individual groups of colorectal cancer with differing phenotypes had distinct survival outcomes.

Analysis of the three groups generated by cluster analysis with respect to patient survival showed that there were statistically significant survival differences between them (group 1 versus group 2, $P = 0.0007$; group 1 versus group 3, $P = 0.0039$). Survival analysis was also done with respect to established clinicopathologic prognostic indicators, specifically nodal status (Dukes C1 versus Dukes C2; pN1 versus pN2), gender, tumor site, age, and tumor differentiation. Of these factors, only apical node positivity (Dukes C1 versus C2) and gender were marginally significant ($P = 0.03$ and 0.04, respectively), emphasizing the value of the marker profile as an independent indicator of prognosis. With regard to cluster group membership group 1 (the very poor survival cluster), it contained all but one of the patients that were present in the poor survival group previously identified by MMP/TIMP profiling (18) as well as a further three patients.

We consider it important that all of the tumors analyzed in this study were of the same stage (i.e., Dukes stage C tumors). Many published studies of biomarkers in colorectal cancer have used cohorts of patients with different disease stages (5–35) and this introduces a further and highly significant confounding factor into the analysis of the markers. However, we focused this study on patients with a single disease stage (i.e., patients with nodal disease at diagnosis), as this provides an important and well-defined group of patients for investigation. In addition, few previous studies have focused on marker analysis in Dukes C cancer, although Garrity et al. (7) studied a few markers (p53, Ki-67, and bcl-2) in a cohort of predominantly Dukes C colorectal cancer. They found that a Ki-67 score of <27% was associated with poorer outcome.

Analysis of biomarker expression within and between each of our identified clusters revealed that the expression of LFABP, p16, p53, PCNA, MMP2, MMP7, MMP9, MT1-MMP, TIMP1, TIMP2, and TIMP3 were significantly different between the cluster groups. With the exception of MMP7, low expression of these specific markers characterized the very poor prognosis group (group 1). The overall profile was more significant than the expression of individual markers and it is interesting to note that the most statistically significant differences between cluster groups were seen for several of the MMPs and TIMPs (i.e., MMP2, MT1-MMP, TIMP2, and TIMP3). Even when prognostic significance is evaluated in a broader context with cell cycle regulatory proteins and growth factor receptor proteins included in the analysis, TIMP2 remains one of the key prognostically significant molecules in individual groups (18).

Of the cell cycle regulatory proteins, only p16, p53, and PCNA showed significant differences in expression between the individual prognostic groups. This contrasts with some studies that have shown that cell cycle control proteins may be prognostic factors in colorectal cancer (4, 36, 43–45).

Table 3. Results of univariate survival analysis

Factor	Log rank	*P*
Cluster group (overall)	14.00	0.0009
Group 1 vs group 2	11.48	0.0007
Group 1 vs group 3	8.32	0.0039
Group 2 vs group 3	1.99	0.16
Status of apical node (Dukes C1 vs Dukes C2)	4.56	0.03
Gender (male vs female)	4.12	0.04
Tumor site (proximal vs distal vs rectum)	3.08	0.21
Age (≤65 vs >65)	2.05	0.15
Nodal status (pN1 vs pN2)	0.33	0.57
Tumor differentiation (moderate vs poor)	0.07	0.79

Table 4. Survival analysis of individual markers

Marker (cutoff point)	Nos. in each group	Log rank	*P*
LFABP (0-5 vs 6)	86 vs 4	14.4	0.001
Topoisomerase-I (≤5% vs >5%)	52 vs 38	3.7	0.71
Cyclin D1 (≤5% vs >5%)	44 vs 56	2.99	0.56
p16 (≤50% vs >50%)	82 vs 8	23.9	0.001
p21 (≤5% vs >5%)	54 vs 46	5.99	0.11
p27 (≤5% vs >5%)	7 vs 83	2.38	0.66
p53 (≤5% vs >5%)	35 vs 45	2.13	0.71
PCNA (≤5% vs >5%)	5 vs 85	4.06	0.39
pRb (≤5% vs >5%)	15 vs 75	2.75	0.59
bcl-2 (≤5% vs >5%)	68 vs 22	3.54	0.47
C-erb-B2 (0 or 1 vs 2 or 3)	18 vs 72	0.1	0.99
EGFR (≤5% vs >5%)	21 vs 69	5.90	0.20
MMP1 (negative vs positive)	17 vs 73	0.68	0.4
MMP2 (negative vs positive)	33 vs 57	2.72	0.09
MMP3 (negative vs positive)	64 vs 26	0.1	0.99
MMP7 (negative vs positive)	33 vs 57	0.06	0.8
MMP9 (negative vs positive)	14 vs 86	8.98	0.0027
MMP13 (negative vs positive)	8 vs 82	0.37	0.54
MT1-MMP (negative vs positive)	9 vs 81	0.30	0.58
MT2-MMP (negative vs positive)	18 vs 72	3.61	0.06
TIMP1 (negative vs positive)	6 vs 84	0.03	0.86
TIMP2 (negative vs positive)	37 vs 53	0.63	0.42
TIMP3 (negative vs positive)	15 vs 75	4.05	0.04

Although several of the markers studied individually showed prognostic significance in the whole group of patients, this was achieved, as in other studies, with the use of arbitrary cutoff points for the dichotomization of the immunohistochemical scores and often with either relatively or absolutely small numbers of cases in a specific group. For example, the LFABP poor survival group contains only four patients, whereas the p16 poor survival patient group only contains eight patients. Hierarchical cluster analysis of the data using the "raw" immunohistochemical scores does not suffer from these disadvantages. The integrated analysis of markers by clustering the data produced more meaningfully sized groups, was more discriminatory in identifying prognostic groups, and enabled more appropriate conclusions regarding phenotype to be drawn.

This study places the expression of a range of candidate prognostic markers into a broader biological context, especially the relationship of MMPs and TIMPs to cell cycle regulatory and apoptosis-related proteins, and highlights the role of specific MMPs and TIMPs in influencing the prognosis of locally advanced colorectal cancer. Many of the proteins investigated in this study have not only been suggested to be prognostic markers but also been proposed to be therapeutic targets in colorectal cancer. Defining the expression of these proteins and determining their relationship between each other and to clinical outcome provides the rationale for therapeutic evaluation. These results reinforce the importance of specific MMPs and TIMPs as significant prognostic factors even when evaluated in a wider biological perspective. This fact provides the rationale for reexamining the use of anti-MMP therapy in colorectal cancer because the relationship of MMP expression with other biomarkers has not necessarily been adequately considered with early generations of anti-MMP therapeutics. This may have significantly contributed to their lack of clinical success (46, 47). Furthermore, although EGFR and c-erb-B2 molecules are currently being targeted for therapy in colorectal cancer, we found that expression of these two growth factor receptors did not significantly differ between prognostic groups.

In conclusion, we have defined the expression profile of a range of immunohistochemical markers in colorectal cancer and identified distinct profiles that correlate with patient survival. Whereas most cell cycle regulatory proteins and growth factor receptor proteins do not show significant correlation, individual MMPs and TIMPs seem to contribute most to the aggressive cancer phenotype.

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