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IGF2BP3 (IMP3) expression is a marker of unfavourable prognosis in colorectal cancer

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Abstract

Background—Evidence suggests that insulin-like growth factor 2 messenger RNA binding protein 3 (IGF2BP3, also known as IMP3) represents a promising cancer biomarker. However, the clinical, pathological, molecular, and prognostic features of IGF2BP3-positive colorectal cancers remain uncertain.

Materials and methods—We evaluated IGF2BP3 expression by immunohistochemistry in 671 rectal and colon cancer cases that form part of a molecular pathological epidemiology database. Cox proportional hazards regression models were used to compute mortality hazard ratio (HR), adjusting for clinical, pathological, and molecular features, including microsatellite instability, the CpG island methylator phenotype, LINE-1 methylation, and *KRAS*, *BRAF,* and *PIK3CA* mutations.

Results—Among 671 colorectal cancers, 234 (35%) tumours were positive for IGF2BP3. In contrast, normal colorectal epithelium was negative for IGF2BP3 in all 403 specimens of normal mucosa adjacent to carcinoma. IGF2BP3 positivity was associated with poor differentiation (p=0.0003), stage III–IV disease (p=0.0081), *BRAF* mutation (p=0.031), and LINE-1 hypomethylation (p=0.020). IGF2BP3 positivity was significantly associated with shorter colorectal cancer-specific [log-rank p<0.0001; multivariate HR, 1.37; 95% confidence interval (CI), 1.02–1.84] and overall survival (log-rank p=0.0004; multivariate HR, 1.32; 95% CI, 1.05–1.66).

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Conclusions—IGF2BP3 expression in colorectal cancer is associated with adverse clinical outcome. Our findings support a role for IGF2BP3 as a diagnostic and/or prognostic biomarker in colorectal cancer.

Keywords

adenocarcinoma; rectal cancer; cancer testis antigen; MAGE; carcinogenesis; diagnostic marker; pathology; therapeutic target; personalized medicine

Introduction

During embryogenesis, insulin-like growth factor 2 messenger RNA binding protein 3 (IGF2BP3, also known as IMP3) plays an important role in RNA trafficking and stabilization, cell growth, and cell migration.[1,2] IGF2BP3 is expressed by a variety of malignant tumours including pancreatic, lung, oesophageal, thyroid, and gynaecological cancers, and melanomas.[3] In addition, it has been shown that expression of IGF2BP3, determined by immunohistochemistry, has prognostic significance in a number of cancers,[4–10] including colorectal cancer.[11,12] Consequently, it has been proposed that IGF2BP3 represents a promising cancer biomarker.[3] Nonetheless, no previous study has comprehensively examined the clinical, pathological, and molecular features that may influence IGF2BP3 expression in colorectal cancer. Furthermore, previous studies[11,12] on IGF2BP3 expression and colorectal cancer survival have been limited by relatively small sample sizes (N=186 and 203), and by the inability to control for tumour molecular features, such as microsatellite instability (MSI), and *KRAS* and *BRAF* mutations, which may represent molecular confounders. Thus, the prognostic role of IGF2BP3 expression in colorectal cancer remains uncertain, and the molecular associations of IGF2BP3 expression, which are important in elucidating the role of IGF2BP3 in colorectal carcinogenesis, remain obscure.

We therefore examined the prognostic role, and clinical, pathological, and molecular associations of IGF2BP3 expression utilizing a molecular pathological epidemiology database[13,14] containing 671 colorectal cancers from two prospective cohort studies. Because our database contains information on tumour variables, including *KRAS*, *BRAF* and *PIK3CA* mutations, MSI, the CpG island methylator phenotype (CIMP), and LINE-1 methylation, we could robustly evaluate associations between IGF2BP3 and these variables, as well as examine the prognostic association of IGF2BP3 independent of potential confounders.

Materials and Methods

Study population

We utilized a database of two U.S. nationwide prospective cohort studies, the Nurses' Health Study (N=121,701 women followed since 1976), and the Health Professionals Follow-up Study (N=51,529 men followed since 1986).[15] Colorectal cancer cases were ascertained by means of biennial questionnaire and the National Death Index, which was also used for ascertainment of death. The cause of death was determined through medical record review by study physicians. We collected formalin-fixed paraffin-embedded tissue blocks from hospitals where surgical resections had been performed.[15] We collected diagnostic biopsy specimens from rectal cancer patients who had received preoperative treatment, to minimize treatment-induced bias or artefact. Based on tissue availability and IGF2BP3 expression data, we included 671 stage I–IV colorectal cancer cases diagnosed up to 2004 (Table 1). Patients were observed until death or January 1, 2011, whichever came first. Informed consent was obtained from all study subjects. This study was approved by the

Human Subjects Committees of Harvard School of Public Health and Brigham and Women's Hospital.

Histopathological evaluation

Haematoxylin and eosin-stained sections of all cases were examined by a pathologist (S.O.) unaware of other data. Tumour differentiation was categorized as well-moderate or poor (>50% vs. ≤50% gland formation). Tumour growth pattern at the tumour margin was categorized as expansile, intermediate, or infiltrative, as previously described.[16] The presence and extent of mucinous and/or signet ring cell component was recorded.[17] A subset of cases (N>100) was reviewed by a second pathologist (T.M.), and concordance was as follows (all $p<0.0001$): $\kappa=0.72$ for tumour differentiation; $\kappa=0.73$ for tumour growth pattern; Spearman r=0.87 for mucin (%); Spearman r=0.65 for signet ring cells (%).[16]

DNA extraction, Pyrosequencing of *KRAS*, *BRAF* and *PIK3CA*, and microsatellite instability (MSI) analyses

DNA was extracted from paraffin-embedded tissue. Mutation status for *KRAS* (codons 12 and 13),[18] *BRAF* (codon 600),[19] and *PIK3CA* (exons 9 and 20)[20] was determined by Pyrosequencing. PCR-based MSI analysis was performed using 10 microsatellite markers (D2S123, D5S346, D17S250, BAT25, BAT26, BAT40, D18S55, D18S56, D18S67 and D18S487).[21] MSI-high was defined as instability in ≥30% of the markers, and MSI-low/microsatellite stability (MSS) as instability in <30% of the markers.[21]

Methylation analyses for CpG islands and LINE-1

Using real-time PCR (MethyLight) on bisulfite-converted DNA, we quantified DNA methylation in eight CIMP-specific promoters (*CACNA1G, CDKN2A, CRABP1, IGF2, MLH1, NEUROG1, RUNX3* and *SOCS1*).[22] CIMP-high was defined as the presence of ≥6/8 methylated promoters, and CIMP-low/0 as 0–5/8 methylated promoters, in accordance with previously established criteria.[22] In order to accurately quantify methylation changes within LINE-1, we used Pyrosequencing as previously described.[23]

Immunohistochemical analysis

Tissue microarrays (TMA) were constructed as previously described.[24,25] Each tumour was represented by a minimum of two 0.6 mm tissue cores, which has previously been shown to be sufficient for assessment of IGF2BP3 expression.[26] Immunohistochemistry was performed as previously described for TP53,[27] CTNNB1 (β-catenin),[15] PTGS2 (cyclooxygenase-2)[24] and FASN.[17] For IGF2BP3, antigen retrieval from deparaffinised tissue sections was achieved by microwaving in a pressure cooker for 10 minutes in Citra Antigen Retrieval Solution, pH 6 (BioGenex, San Ramon, CA). Tissue sections were incubated for 15 minutes with Peroxidase Blocking Reagent (Dako North America, Carpinteria, CA). A mouse monoclonal antibody against IGF2BP3 (IMP3; clone 69.1; Dako; 1:100 dilution) was applied, and the sections were incubated for 16 hours at 4°C. Visualization was achieved using EnVision™+/HRP (Dako), diaminobenzidine (Dako), and hematoxylin counterstain. Human placenta was used as a positive control tissue. Omission of primary antibody served as a negative control. Cytoplasmic staining for IGF2BP3 was scored as absent, weak, moderate, or intense. For subsequent analyses, absent or weak staining was categorized as negative, while moderate or intense staining was categorized as positive.

Each immunohistochemical maker was interpreted by one of the investigators (IGF2BP3 and CTNNB1 by T.M.; TP53, PTGS2 and FASN by S.O.) unaware of other data. For agreement studies, over 100 randomly-selected cases were examined for each marker by a

second observer (IGF2BP3 by P.L.; CTNNB1 by S.O.; TP53, PTGS2 and FASN by T.M.) unaware of other data. The concordance between observers (all $p<0.0001$) was $\kappa=0.70$ for IGF2BP3, $\kappa=0.78$ for TP53, $\kappa=0.80$ for CTNNB1, $\kappa=0.69$ for PTGS2, $\kappa=0.80$ for FASN, indicating substantial agreement.

Statistical analysis

All statistical analyses were performed using SAS (Version 9.2, SAS Institute, Cary, NC). All p values were two-sided. Our primary hypothesis was that IGF2BP3 expression was associated with worse clinical outcome. When multiple hypothesis testing was performed for exploratory analyses of clinicopathological and molecular associations, or interactions, the p value for significance was adjusted by Bonferroni correction to $p=0.0024$ (=0.05/21). For categorical data, the chi-square test was performed. Mean age was compared using a t-test.

Kaplan-Meier method and log-rank test were used for survival analyses. For analyses of colorectal cancer-specific mortality, deaths as a result of other causes were censored. To control for confounding, multivariate Cox proportional hazards regression models were used. The multivariate model initially included sex, age at diagnosis (continuous), year of diagnosis (continuous), BMI (<30 vs. ≥30 kg/m^2), family history of colorectal cancer in any first-degree relative (present vs. absent), tumour location (proximal vs. distal), tumour differentiation (well-moderate vs. poor), tumour growth pattern (expansile-intermediate vs. infiltrative), mucinous component (continuous), signet ring cell component (continuous), MSI (high vs. low/MSS), CIMP (high vs. low/0), *KRAS*, *BRAF*, and *PIK3CA* mutation, LINE-1 methylation (continuous), and expression of TP53, nuclear CTNNB1, PTGS2 and FASN. Because we observed non-proportionality in the hazards for disease stage over time, and to avoid overfitting, stage (I, II, III, IV or unknown) was used as a stratifying variable using the “strata” option in the SAS “proc phreg” command. A backward elimination was performed with a threshold of $p=0.20$, to avoid overfitting. Cases with missing information in any of the covariates were assigned to the majority category of the given covariate to avoid overfitting. We confirmed that excluding cases with missing covariate information did not substantially alter results (data not shown). The proportionality of hazards assumption was satisfied for all variables in the multivariate models by evaluating time-dependent variables, which were the cross-product of a given variable and survival time. Interaction was assessed by the Wald test on the cross product of IGF2BP3 and another variable of interest (without data-missing cases) in a multivariate Cox model.

Results

IGF2BP3 status in colorectal cancer and normal colorectal mucosa

Among the database of 671 stage I–IV colorectal cancers in the two prospective cohort studies, 234 (35%) showed IGF2BP3 positivity (Figure 1). In contract, IGF2BP3 was negative in normal colorectal epithelial cells in all the 403 adjacent normal mucosal specimens that were evaluable. In normal colorectal tissue, only lymphocytes in the germinal centres of mucosa-associated lymphoid tissue stained positively for IGF2BP3 (Figure 1). As demonstrated in Table 1, IGF2BP3 positivity was significantly associated with poor differentiation ($p=0.0003$). Because multiple hypothesis testing required a Bonferroni corrected significance level of $p=0.0024$, the relationships between IGF2BP3 positivity and stage III–IV disease ($p=0.0081$), *BRAF* mutation ($p=0.031$), and LINE-1 hypomethylation ($p=0.020$) were considered of borderline significance; these findings require validation in an independent dataset.

IGF2BP3 positivity and survival of colorectal cancer patients

Among the 671 patients, there were 328 deaths, including 194 colorectal cancer-specific deaths, during a median follow-up of 160 months (interquartile range, 123–207 months) for censored cases. In Kaplan-Meier analysis, IGF2BP3 positivity was significantly associated with shorter colorectal cancer-specific (log-rank $p<0.0001$) and overall survival (log-rank $p=0.0004$) (Figure 2).

We used Cox multivariate regression models to assess the prognostic influence of IGF2BP3 positivity independent of clinical, pathological, and other tumour molecular features (Table 2). Univariate hazard ratio (HR) for IGF2BP3 positivity was 1.76 [95% confidence interval (CI), 1.32–2.33] for colorectal cancer-specific survival, and 1.48 (95% CI, 1.19–1.85) for overall survival. The multivariate HR for IGF2BP3 positivity was 1.37 (95% CI, 1.02–1.84) for colorectal cancer-specific survival, and 1.32 (95% CI, 1.05–1.66) for overall survival. The attenuation of the effect of IGF2BP3 positivity in the multivariate analysis occurred largely as a result of adjusting for disease stage. When adjusted for disease stage alone, the HR for IGF2BP3 positivity was 1.39 (95% CI, 1.04–1.85) for colorectal cancer-specific survival, and 1.30 (95% CI, 1.04–1.63) for overall survival (Table 2). No other major confounder was present.

Given that stage appeared to be a mediator of the association between IGF2BP3 expression and survival, we performed Kaplan-Meier analyses stratified by disease stage (Supplementary figures 1 and 2). A significant difference in survival time distribution according to IGF2BP3 expression was observed only for colorectal cancer-specific survival in stage II disease (Supplementary figure 1, B; log-rank $p=0.0005$), and with borderline significance for stage II overall survival (Supplementary figure 2, B; log-rank $p=0.071$). Similarly, in stage-specific Cox regression, IGF2BP3 was significantly associated with colorectal cancer-specific survival only in stage II disease (multivariate HR=2.50; 95% CI, 1.14–5.47). We did not, however, observe a significant interaction between disease stage and IGF2BP3 positivity, indicating that the prognostic association of IGF2BP3 did not differ significantly by stage.

Exploratory analyses of interactions between IGF2BP3 and other covariates

We further examined whether any of the clinical, pathological, or molecular variables modified the influence of IGF2BP3 positivity on cancer-specific mortality. We found no significant interaction between IGF2BP3 and any of the covariates examined (all $P_{interaction}>0.10$). Notably, the effect of IGF2BP3 positivity did not significantly differ between the two cohort studies ($P_{interaction}=0.33$).

Discussion

Here, we report our findings on IGF2BP3 expression in colorectal cancer in relation to patient survival as well as clinical, pathological, and molecular features, in two large prospective cohort studies. We found significant associations between IGF2BP3 positivity, poor differentiation, and increased mortality, independent of patient characteristics or tumour molecular variables. Our results suggest that IGF2BP3 positivity may define a colorectal cancer phenotype with more aggressive biological behaviour.

It has been reported that IGF2BP3 represents a novel biomarker that can differentiate normal from cancerous tissue in a variety of organ systems.[3] In the present study, IGF2BP3 positivity was observed in approximately 35% of colorectal cancer cases, while normal colorectal epithelial cells were always negative for IGF2BP3. Thus, IGF2BP3 positivity in small biopsy specimens may provide a pathologist with greater confidence in the diagnosis of colorectal cancer, especially when substantial crush artefact is present. The frequency of

IGF2BP3 positivity in colorectal adenomata and specimens with inflammation or reactive atypia must, however, be determined in future studies.

We found that germinal centre lymphocytes in gut-associated lymphoid tissue also stained positively for IGF2BP3. Consistent with this observation, IGF2BP3 positivity in germinal centre B cells has been reported in normal lymphoid tissues including lymph node, tonsil, spleen, and thymus.[28] To the best of our knowledge, IGF2BP3 positivity in gut-associated lymphoid tissue has not previously been reported. This pattern of IGF2BP3 positivity may generate a potential diagnostic pitfall, and pathologists should be aware of it, especially where a nest of IGF2BP3-positive cells lacking glandular structure occurs in a biopsy specimen. Careful histological evaluation together with immunohistochemistry for epithelial and lymphocytic markers would help confirm the correct diagnosis in such cases.

It has recently been reported that IGF2BP3 protein expression is associated with a poorer prognosis in colorectal cancer patients.[11,12] However, both of the two previous studies[11,12] on IGF2BP3 expression and colorectal cancer survival were limited by relatively small sample sizes (N=186 and 203). Furthermore, neither of these studies[11,12] took into account the potential confounding effects of established colorectal cancer molecular biomarkers. In the present study, IGF2BP3 positivity appeared to be associated with *BRAF* mutation and LINE-1 hypomethylation, both of which are associated with poor survival.[29–33] We have shown that IGF2BP3 positivity is associated with poorer survival in patients with colorectal cancer, independent of these molecular features.

Experimental data generally support an oncogenic role for IGF2BP3.[10,34–37] Overexpression of IGF2BP3 in lung and breast cancer cell lines enhances tumour cell invasiveness.[35] Similarly, in a mouse melanoma model, Igf2bp3 overexpression in melanoma cells enhances tumour growth, angiogenesis, and metastasis, resulting in poorer survival.[37] In leukaemia,[34] hepatocellular carcinoma,[35] melanoma,[36] glioma,[37] and cervical cancer[10] cell lines *in vitro,* small interfering RNA-mediated knockdown of IGF2BP3 attenuates malignant characteristics, including cell proliferation, migration, anchorage-independent growth, and invasion [10,34–37]. Our observational data certainly support a role of IGF2BP3 in colorectal cancer progression.

Since the prognostic association of IGF2BP3 overexpression might be related to biological effects on invasion and metastasis,[35,36] it might have been illuminating to evaluate the influence of IGF2BP3 expression on disease-free survival or time to metastasis; unfortunately, these data were not available for our cohorts. Further studies are therefore needed to determine exactly how IGF2BP3 influences the biological behaviour of colorectal cancer cells.

IGF2BP3 is not only a diagnostic/prognostic marker, but also a potential therapeutic target.[38] Cytotoxic T-cell clones, generated by *in vitro* exposure to an HLA-A24-restricted peptide epitope derived from IGF2BP3, exert a considerable cytotoxic response against lung and oesophageal cancer cells that express endogenous IGF2BP3.[39] Moreover, in a phase I study, vaccination therapy using an IGF2BP3-derived peptide, together with two other peptides, induced clinical response in five out of 10 oesophageal cancer patients who were resistant to chemotherapy and/or radiotherapy.[40] Tissue expression of IGF2BP3 in colorectal cancer might serve as a predictive biomarker, and could be used to identify patients who may benefit from vaccination-based therapies that target IGF2BP3.

In summary, IGF2BP3 is a promising diagnostic biomarker candidate for colorectal cancer and its expression is independently associated with poorer prognosis. Our findings may have broader clinical implications by facilitating the evolution of IGF2BP3 as a therapeutic target. Further studies are required to validate our findings and elucidate the molecular mechanisms

and pathways through which IGF2BP3 affects the biological phenotype of colorectal cancer cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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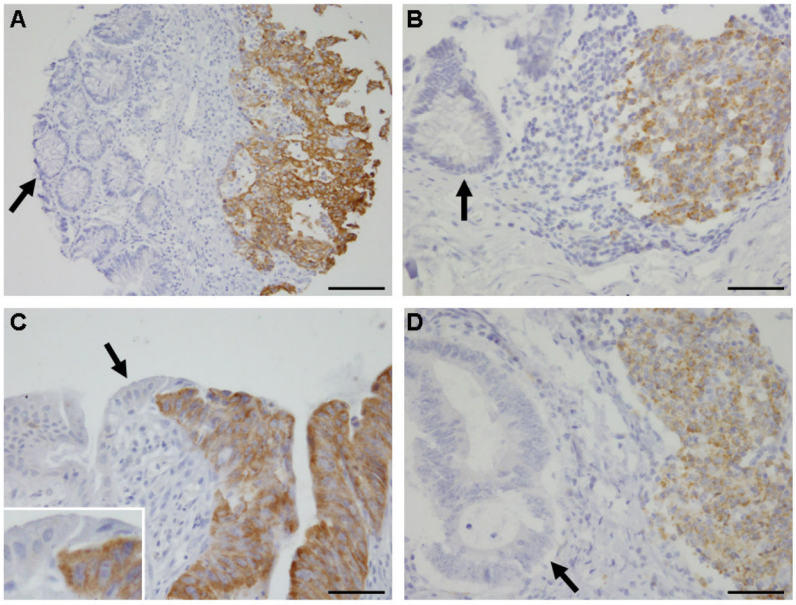
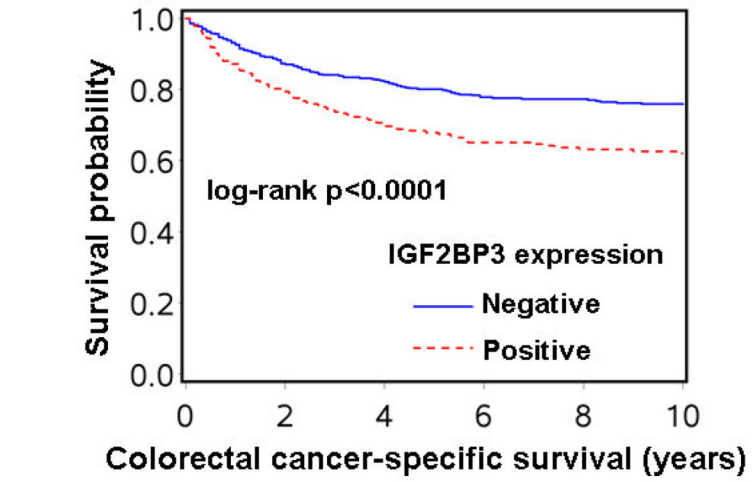
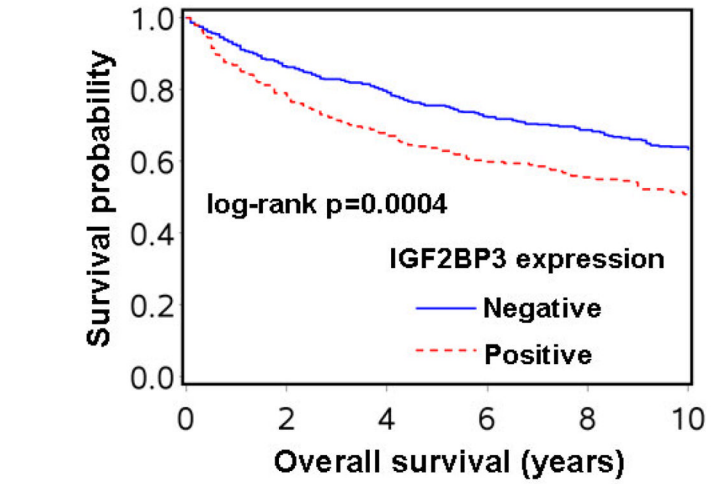


Figure 1.
(A) IGF2BP3-positive colon cancer (right). Adjacent normal glands are negative for IGF2BP3 (left, arrrow). Bar, 100 μm. (B) Germinal center lymphocytes in normal colonic mucosa are positively stained for IGF2BP3 (right). Normal glands are negative for IGF2BP3 (left, arrow). Bar, 50 μm. (C) IGF2BP3-positive colon cancer (right). Adjacent normal colonic epithelial cells are negative for IGF2BP3 (left, arrow). Inset, high-power view of the boundary between normal epithelial cells and cancer cells. Bar, 50 μm. (D) IGF2BP3-negative colon cancer (left, arrow). Germinal center lymphocytes are positive for IGF2BP3 (right). Bar, 50 μm.





Number at risk

Year	0	2	4	6	8	10
Negative	437	377	346	314	279	219
Positive	234	182	157	140	124	101

Figure 2.

Kaplan-Meier curves for colorectal cancer-specific survival (upper) and overall survival (lower) according to IGF2BP3 status in colorectal cancer. The table (bottom) shows the number of patients who remained alive, and at risk, at each time point after the diagnosis of colorectal cancer. The number at risk is applicable to both colorectal cancer-specific and overall survival.

Table 1

Clinical, pathological or molecular characteristics according to IGF2BP3 status in colorectal cancer

		IGF2BP3		
Clinical, pathological or molecular feature	**Total *N***	**negative**	**positive**	**p value**
All cases	671	437	234	
Sex				0.61
Male	241 (36%)	160 (37%)	81 (35%)	
Female	430 (64%)	277 (63%)	153 (65%)	
Mean age ± SD	67.4 ± 8.4	67.5 ± 8.3	67.1 ± 8.5	0.58
Body mass index (kg/m^2)				0.38
<30	536 (80%)	345 (79%)	191 (82%)	
≥30	133 (20%)	91 (21%)	42 (18%)	
Family history of colorectal cancer in any first degree relative				0.70
(−)	536 (80%)	351 (80%)	185 (79%)	
(+)	135 (20%)	86 (20%)	49 (21%)	
Year of diagnosis				0.32
Prior to 1997	336 (50%)	225 (51%)	111 (47%)	
1997–2004	335 (50%)	212 (49%)	123 (53%)	
Tumour location				0.58
Proximal colon (cecum to transverse)	323 (48%)	204 (47%)	119 (51%)	
Distal colon (splenic flexure to sigmoid)	206 (31%)	139 (32%)	67 (29%)	
Rectum	138 (21%)	91 (21%)	47 (20%)	
Disease stage				0.0081
I	146 (22%)	104 (24%)	42 (18%)	
II	212 (32%)	142 (32%)	70 (30%)	
III	183 (27%)	111 (25%)	72 (31%)	
IV	93 (14%)	50 (11%)	43 (18%)	
Unknown	37 (5.5%)	30 (6.9%)	7 (3.0%)	
Tumour differentiation				0.0003
Well to moderate	606 (91%)	408 (94%)	198 (85%)	
Poor	63 (9.4%)	28 (6.4%)	35 (15%)	
Tumour growth pattern				0.56
Expansile	201 (34%)	134 (35%)	67 (32%)	
Intermediate	313 (53%)	205 (53%)	108 (52%)	
Infiltrative	80 (13%)	48 (12%)	32 (15%)	
Mucinous component				0.84
0%	420 (63%)	270 (62%)	150 (64%)	
1–50%	174 (26%)	116 (27%)	58 (25%)	
>50%	77 (11%)	51 (12%)	26 (11%)	
Signet ring cell component				0.49
0%	612 (91%)	400 (91%)	212 (91%)	
1–50%	53 (7.9%)	32 (7.3%)	21 (9.0%)	

Clinical, pathological or molecular feature	Total *N*	IGF2BP3		p value
		negative	positive	
>50%	6 (0.9%)	5 (1.1%)	1 (0.4%)	
MSI status				0.73
MSI-low/MSS	540 (82%)	352 (83%)	188 (82%)	
MSI-high	115 (18%)	73 (17%)	42 (18%)	
CIMP status				0.091
CIMP-low/0	548 (83%)	365 (85%)	183 (80%)	
CIMP-high	110 (17%)	64 (15%)	46 (20%)	
KRAS mutation				0.13
(−)	424 (64%)	268 (62%)	156 (68%)	
(+)	239 (36%)	165 (38%)	74 (32%)	
BRAF mutation				0.031
(−)	557 (84%)	372 (87%)	185 (80%)	
(+)	104 (16%)	58 (13%)	46 (20%)	
PIK3CA mutation				0.84
(−)	506 (84%)	332 (84%)	174 (85%)	
(+)	93 (16%)	62 (16%)	31 (15%)	
LINE-1 methylation level (Mean ± SD)	61.3 ± 9.5	62.0 ± 8.9	60.1 ± 10.4	0.020
TP53 expression				0.33
(−)	388 (58%)	259 (60%)	129 (56%)	
(+)	279 (42%)	176 (40%)	103 (44%)	
Nuclear CTNNB1 expression				0.62
(−)	339 (53%)	224 (54%)	115 (52%)	
(+)	299 (47%)	192 (46%)	107 (48%)	
PTGS2 expression				0.33
(−)	249 (37%)	168 (39%)	81 (35%)	
(+)	419 (63%)	267 (61%)	152 (65%)	
FASN expression				0.17
(−)	249 (38%)	170 (39%)	79 (34%)	
(+)	414 (62%)	261 (61%)	153 (66%)	

(%) indicates the proportion of cases with a specific clinical, pathological or molecular feature among each expression group. A p value for significance was adjusted for multiple hypothesis testing to p=0.05/21=0.0024. Thus, a p value between 0.05 and 0.0024 should be regarded as of borderline significance.

CIMP, CpG island methylator phenotype; MSI, microsatellite instability; MSS, microsatellite stable; SD, standard deviation.

Table 2

IGF2BP3 positivity in colorectal cancer and patient mortality

	Total *N* (%)	Colorectal cancer-specific mortality				Overall mortality			
		Number of events	Univariate HR (95% CI)	Stage-stratified HR (95% CI)	Multivariate stage-stratified HR (95% CI)	Number of events	Univariate HR (95% CI)	Stage-stratified HR (95% CI)	Multivariate stage-stratified HR (95% CI)
IGF2BP3 (−)	437 (65%)	105	1 (referent)	1 (referent)	1 (referent)	194	1 (referent)	1 (referent)	1 (referent)
IGF2BP3 (+)	234 (35%)	89	1.76 (1.32–2.33)	1.39 (1.04–1.85)	1.37 (1.02–1.84)	134	1.48 (1.19–1.85)	1.30 (1.04–1.63)	1.32 (1.05–1.66)
p value			<0.0001	0.028	0.039		0.0005	0.022	0.016

We tested the specific study hypothesis on the prognostic role of IGP2BP3 positivity. Thus, a p value for significance was set at p=0.05. The multivariate, stage-stratified Cox regression model initially included the IGF2BP3 variable (negative or positive), age, sex, year of diagnosis, body mass index, family history of colorectal cancer in any first degree relative, tumour location, tumour differentiation, tumour growth pattern, mucinous component, signet ring cell component, microsatellite instability, CpG island methylator phenotype, *KRAS*, *BRAF*, *PIK3CA*, LINE-1 methylation, TP53, nuclear CTNNB1, PTGS2 and FASN. A backward elimination with threshold of p=0.20 was used to select variables in the final models.

CI, confidence interval; HR, hazard ratio.