The expression and prognostic significance of bcl2 associated transcription factor (BCLAF1) in rectal cancer following neoadjuvant therapy

Short title: bcl associated factor and rectal cancer

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Conflict of interest: BC and AA are employees of Vertebrate Antibodies. GIM is a scientific advisor to Vertebrate Antibodies.

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Abstract

Aims

Bcl2 associated transcription factor (BCLAF1) is a nuclear protein that binds to bcl related proteins and can induce apoptosis and autophagy. This study has investigated the expression of BCLAF1 in a series of rectal cancers following neoadjuvant therapy.

Methods and results

Immunohistochemistry was performed on a post-neoadjuvant therapy rectal cancer tissue microarray. It contained rectal cancers (n=248), lymph node metastasis (n=76) and non-neoplastic rectal mucosal samples (n=73). A monoclonal antibody which we have developed to BCLAF1 was used.

Non-neoplastic rectal epithelium showed nuclear localisation of BCLAF1 in both crypt and surface epithelial cells whereas rectal cancers showed both nuclear and cytoplasmic BCLAF1 expression. Most rectal cancers showed moderate or strong nuclear immunoreactivity but showed weak cytoplasmic immunoreactivity. Cytoplasmic BCLAF1 expression was increased in primary rectal cancers compared with non-neoplastic rectal mucosa (p=0.008). Negative and weak nuclear BCLAF1 expression was associated with poor prognosis (HR=0.502, 95%CI=0.269-0.939, χ^2=4.876, p=0.027). Nuclear BCLAF1 was independently prognostic in a multivariate model (HR=0.431, 95%CI=0.221-0.840, p=0.013).

Conclusions

This study has shown that both cytoplasmic and nuclear BCLAF1 are increased in post neoadjuvant therapy rectal cancer and that negative and weak nuclear BCLAF1 expression is independently associated with poor prognosis.

Key words: bcl2 associated transcription factor, biomarker, immunohistochemistry, monoclonal antibody, neoadjuvant therapy, prognosis, rectal cancer

Introduction

Colorectal cancer is one of the commonest types of cancer with an increasing incidence. While primary surgery remains the mainstay of colon cancer treatment, rectal cancer is increasingly being treated by neoadjuvant chemo-radiotherapy to down stage the tumour and increase the opportunity for curative surgery. There are also studies suggesting that in some circumstances, after pelvic chemo-radiotherapy and close clinical & imaging follow up, surgery can be used as a 'salvage' procedure in those patients that relapse. High resolution pelvic MRI is used to guide patient selection for neoadjuvant therapy using criteria including high stage tumours, possible involvement of circumferential resection margin (CRM) and the presence of extramural vascular invasion. In rectal cancer neoadjuvant therapy can achieve a 25% complete pathological response rate, with approximately 65% of tumours showing some response to chemo-radiotherapy. Approximately 10% of patients show no significant response to such therapy. [1-4] The outcome in patients with a complete pathological response appears to be good with a very low rate of local recurrence. The position regarding those patients whose tumours do not show a complete response is less certain, as is the value of adjuvant chemotherapy. There is still a requirement for prognostic as well as predictive markers biomarkers in those patients, to aid decisions around the use of adjuvant chemotherapy.

Bcl2 associated transcription factor (BCLAF1) is a nuclear protein whose homologue (btf) was originally identified in a screen of adenovirus proteins that binds to bcl related proteins. [5] Studies have shown that BCLAF1 can induce apoptosis, autophagy and repress transcription. [6] It has also been suggested to have cellular roles including the regulation of T cell activation and mRNA distribution which are distinct from its interactions with bcl related proteins. [7,8]

This study has investigated the expression of bcl2 associated transcription factor (BCLAF1) in a series of rectal cancers following neoadjuvant therapy using a well characterised rectal cancer tissue microarray.

Materials and methods

Development of BCLAF1 monoclonal antibody

A monoclonal antibody to BCLAF1 was produced in collaboration with Vertebrate Antibodies Ltd (Aberdeen, UK) using a synthetic peptide as the immunogen. Briefly, a 10 amino acid sequence (KYQGDGIVED) corresponding to amino acids 891-900 of the BCLAF1 sequence was identified which was antigenic, exposed on the surface and unique to the target protein. The amino acid sequence lies in a region of the protein which is present in all the splice variants of BCLAF1. The peptide was obtained commercially (Almac Sciences Ltd, East Lothian, UK) and conjugated to ovalbumin for the immunisation and bovine serum albumin for ELISA. [9,10] The immunisation of mice, production of hybridoma cells and ELISA screening were carried out essentially as described previously. [9,10] The hybridomas were cloned by limiting dilution until a single ELISA positive colony was grown in a 96 well plate. The hybridoma cell line designated M33-P5B11 was then grown at high cell density for the preparation of antibody stock which was used subsequently for its characterisation by immunoblotting and immunohistochemistry.

Immunoblotting

Whole cell lysate from cells (human embryonic kidney cells) overexpressing BCLAF1 was used as positive control for immunoblotting while lysate from cells containing vector only was used as a negative control. The lysates were bought from (Novus Biologicals, Cambridge, UK). Cell lysates (5µg protein/lane) were resolved by electrophoresis on NuPAGE 4-12% Bis-Tris gels (Fisher Scientific, Loughborough, UK,). The membranes were blocked for 1 hour at room temp in PBS-Tween-20 (PBST) containing 3% (w/v) skim milk powder. Blots were incubated overnight at 4 C with anti-BCLAF1 monoclonal antibody diluted in PBST (1/2 dilution). Membranes were washed (6 times) for 1 hour in 1% skim

milk. Blots were subsequently probed for 1 hour with a secondary antibody conjugated horseradish-peroxidase-conjugated anti-mouse IgG (Sigma-Aldrich, Dorset, UK) (1:2000). The membranes were washed (6 times) for 1 hour in 1% skim milk and protein bands visualized using the enhanced chemiluminescence detection system (Fisher Scientific).

Patient cohort

A tissue microarray was constructed to include samples from 321 patients recruited consecutively over a seven-year period (2005-2011). Each of these patients had a surgical resection of a primary rectal tumour which had been treated with neoadjuvant therapy prior to surgery (table 1). The neoadjuvant treatment was 5 weeks of pelvic radiotherapy, using intravenous contrast and CT planning with the diagnostic thin slice MRI scan fused with the planning CT scan to aid in identification of the tumour. Using these images the oncologist (LMS) contoured the relevant anatomy to be treated (primary tumour plus a margin, local mesorectal lymph nodes and lymph nodes following the internal iliac artery up to the S1/S2 vertebral area). The dose prescribed was 45Gy to the 100% isodose point, and this was delivered in 25 daily fractions of 1.8Gy, Monday to Friday for 5 weeks. Concurrent chemotherapy, using oral capecitabine (825mg/m2 bd) was also taken by the patients on the same days (Monday to Friday) as the radiotherapy for 5 weeks. Surgery was scheduled for about 8 to 10 weeks after completion of the neoadjuvant therapy. Repeat imaging of the pelvis with thin slice MRI was only carried out on a minority of the patients whose tumours on the diagnostic imaging suggested they were initially inoperable, as opposed to those who were at risk of having an involved circumferential resection margin but would be otherwise resectable.

Adjuvant therapy was considered for patients who had adverse pathological factors including poor differentiation, extramural vascular invasion, lymph node metastases,

involved CRM, partial or minimal response to neoadjuvant therapy in their resected rectal cancer specimen.

The resected rectal cancer specimens were opened anteriorly along the anti-mesenteric border of the sigmoid colon, washed in water and fixed in formalin for at least 48 hours. The rectum was left intact to facilitate assessment of potential serosal surface involvement and circumferential margin involvement. The fixed specimens were then further dissected and appropriate tissue blocks taken for histopathological assessment according to the guidelines of The Royal College Pathologists for reporting of colorectal cancer excision specimens. [11] Appropriate guidance from TNM5 was also followed and reported by an expert gastro-intestinal pathologist (GIM).

The response of rectal cancer to neoadjuvant therapy was assessed using the following parameters: i) Proportion of residual histologically viable tumour and ii) the degree of fibrosis and inflammation associated with the residual viable tumour. These parameters were incorporated into a four point scale to make an overall assessment of the response of the tumour to pre-operative therapy (complete response, good partial response, partial response and minimal response). This histopathological response classification shows a strong correlation with survival in this cohort (figure 1).

Tumour tissue samples were obtained from 248 primary tumours and 76 lymph node metastases. The rectal cancer tissue microarray was constructed as previously described and contained two 1mm cores of tissue from each primary tumour. [12,13] Samples from complete pathological responders to therapy (n=73) were treated as examples of normal mucosa because they were obtained from the site of the original primary tumour but showed no evidence of malignancy upon histopathological examination. Lymph node metastases were also sampled from tumours with metastatic disease to permit comparison between primary and metastatic tumours.

Survival information (all cause mortality) was available for all patients and at the time of censoring patient outcome data there had been 56 (17.4%) deaths. The mean patient survival was 72 months (95% CI 68-75 months).

Immunohistochemistry

Immunohistochemistry for BCLAF1 was performed with the biotin-free Dako Envision™ system (Dako, Ely, UK) using a Dako autostainer (Dako) as previously described. [12-14] The sections were evaluated by light microscopic examination and the intensity of immunostaining in each core assessed independently by two investigators (GTB and GIM) using a scoring system previously described for the assessment of protein expression in tumour microarrays. [12-14] The intensity of immunostaining in each core was scored as negative, weak, moderate or strong. The sub-cellular localisation (nuclear, cytoplasmic or membranous) of the immunostaining was also recorded. Variation in immunostaining between cores of each case was not identified. Any discrepancies in the immunohistochemical assessment of the tissue cores between the two observers were resolved by simultaneous microscopic re-evaluation.

Statistics

Statistical analysis of the data including the Mann-Whitney U test, Wilcoxon signed rank test, chi-squared test, Kaplan-Meier survival analysis, log-rank test and Cox multi-variate analysis (variables entered as categorical variables) including the calculation of hazard ratios and 95% confidence intervals was performed using IBM SPSS version 21 for Windows 7™ (IBM, Portsmouth, UK). The log rank test was used to determine survival differences between individual groups. A probability value of $p \leq 0.05$ was regarded as significant. The influence of different cut-off points in relation to survival was investigated

by dichotomizing the intensity score for BCLAF1. The groups that were analysed were negative staining versus any positive staining, negative and weak staining versus moderate and strong staining and negative, weak and moderate staining versus strong staining.

Ethics

The project had the approval of The North of Scotland research ethics committee (ref. nos. 08/S0801/81 and 11/NS/0015). The research ethics committee did not require written patient consent for the retrospective tissue samples that were included in the rectal cancer tissue microarray.

Results

Monoclonal antibody

The specificity of the monoclonal antibody to BCLAF1 was determined by ELISA using the immunogenic peptide and also by immunoblotting using BCLAF1 overexpressed cell lysate. A band migrating at the expected molecular weight (110kDa) was observed in the lane containing the BCLAF1 overexpressed cell lysates and no band was observed in the lane containing vector only (figure 2).

BCLAF1 expression in rectal cancer

BCLAF1 showed a nuclear localisation in non-neoplastic rectal epithelium while in rectal cancer there was both nuclear and cytoplasmic immunoreactivity (figure 3). Nuclear BCLAF1 expression was increased in primary rectal cancers compared with normal rectal mucosa (table 2 and figure 4). There was also a difference between expression of nuclear BCLAF1 in primary rectal cancer and metastatic rectal cancer ($p=0.05$). However, when paired primary and metastatic tumours were compared there was no significant difference between nuclear BCLAF1 expression. Cytoplasmic BCLAF1 expression showed a significant decrease in expression between primary and metastatic tumours ($p=0.008$) and also in paired primary and metastatic rectal cancers ($p=0.033$).

Relationship of BCLAF1 with pathological parameters

Nuclear BCLAF1 showed a strong relationship with Dukes stage ($\chi^2=19.134$, $p=0.004$). There were no other significant relationships between BCLAF1 and pathological parameters including tumour stage, lymph node stage and extramural vascular invasion (table 3).

Survival analysis

There was a trend towards significance for overall nuclear BCLAF1 expression (χ^2=6.334, p=0.096). When the nuclear BCLAF1 immunohistochemical scores were dichotomised then there was a significant relationship between nuclear BCLAF1 expression and overall survival when negative and weak BCLAF expression was compared with moderate and strong BCLAF1 expression (table 4 and figure 5). Comparing nuclear BCLAF negative and weakly positive tumours with nuclear BCLAF1 moderate and strong expressing tumours showed that there was a highly significant association with survival (HR=0.502, 95%CI=0.269-0.939, χ^2=4.876, p=0.027). Mean survival for the negative/weak group of tumours (n=55) was 57 months (95%CI=48-66 months) while the mean survival for the moderate/strong group of tumours (n=125) was 70 months (95%CI=63-72 months). Nuclear BCLAF1 was independently prognostic in two multivariate models (table 5A and table 5B); one which included Dukes stage (HR=0.431, 95%CI=0.221-0.840, p=0.013) and the other which contained ypTstage and ypNstage (HR=0.451, 95%CI=0.229-0.891, p=0.022) as the parameters to assess tumour stage.

There were no significant relationships between cytoplasmic BCLAF1 expression and survival.

Discussion

This study of a large series of post therapy rectal cancers with good follow-up treated by neoadjuvant chemo-radio therapy has shown that strong nuclear expression of BCLAF1 in post-treatment rectal cancer cells is associated with increased patient survival. Neoadjuvant chemo-radio therapy is now the standard treatment for rectal cancer judged by thin slice MRI to be at high risk of an involved circumferential resection margin and thus local and systemic recurrence and there is a clear requirement to identify biomarkers of not only prognosis following neoadjuvant chemo-radiotherapy but also the patients who may benefit from post-operative adjuvant chemotherapy. [4] However, there have been relatively few previous studies of post treatment rectal cancers to identify biomarkers of outcome following neoadjuvant therapy. Those studies have generally been limited as they have been performed on relatively small numbers of cases and often with only short term follow-up.

BCLAF1 is a nuclear protein that was originally identified in yeast and was shown to interact with Bcl2 family of proteins and to promote apoptosis. [5,15] It was also hypothesised that BCLAF may repress the transcription of survival genes through P53 inhibition suggesting that BCLAF1 plays critical role in determining cell fate. [5,15] Furthermore, it was demonstrated that BCLAF1 regulates apoptosis related proteins such as Mdm2, p53, BAX and Bcl-2 in HCT116 human colon adenocarcinoma cells. [16] BCLAF1 has also been proposed to have a variety of other cellular functions. [5-7, 17,18]

Our data has shown that BCLAF1 localisation was almost exclusive nuclear localisation in non-neoplastic rectal epithelium while in rectal cancer there was both nuclear and cytoplasmic BCLAF1 immunoreactivity. Nuclear localisation of BCLAF1 is expected. [6] What is interesting and not previously described is the cytoplasmic localisation of BCLAF1 in rectal cancers. The aberrant sub-cellular localisation in tumours of proteins that show nuclear expression in normal cells has also been observed for other putative tumour

biomarkers. For example hnRNPK showed aberrant cytoplasmic expression in colorectal cancer indicative of abnormal protein processing in tumour cells. [12,19,20] Similarly cellular apoptosis susceptibility protein (CSE1L) which is involved in the control of cell proliferation has also been shown to have aberrant expression in colorectal cancer cells. [21] This is indicative of altered protein processing and signalling in tumour cells.

BCLAF1 has not previously been studied in tumours although it has recently been identified as being induced in radiation exposed cells and to promote apoptosis. [18] Moreover, our study also revealed that strong nuclear BCLAF1 expression independently correlated with better survival outcome among rectal cancer patients. Such observations are consistent with current knowledge. BCLAF1 has been proven to induce apoptosis in highly irradiated cell lines that have been deemed irreparable by disrupting inhibition of a p21-mediated apoptotic pathway, which is commonly dysfunctional in tumour cells. Furthermore, tumour cells were found to suppress BCLAF1 triggering a cascade of anti-apoptotic cellular events that contribute to tumour radiation resistance, defective DNA repair pathways and increased capacity for tumour cell survival. [18] An increased level of nuclear BCLAF1 in rectal cancer will induce apoptosis and subsequently lead to improved survival outcome.

In spite of substantial efforts to identify novel prognostic biomarkers in rectal cancer patients, only few putative biomarkers have been identified. [22] The putative stem cell marker aldehyde dehydrogenase 1 (ALDH1) has been studied by immunohistochemistry in post treatment tumour samples from 46 patients with rectal cancer who had received neoadjuvant chemo-radiotherapy. High cytoplasmic ALDH1 in post-operative tumour samples was independently associated with a shorter disease free interval and disease specific survival. [23] In a separate study of 64 patients ALDH1expresion as assessed by immunohistochemistry in post therapy rectal cancers was also shown to be associated with increased risk of recurrence and poor survival. [24]

Another putative stem cell marker, CD 133, has been identified in post therapy rectal tumour samples from 40 patients who had received neoadjuvant chemo-radiotherapy. High CD133 as assessed by immunohistochemistry was associated with a higher rate of recurrence and decreased disease-free survival and was proposed to be a marker of a treatment resistance phenotype in post therapy tumour cells. [25] One more further biomarker showing prognostic potential and was associated with shorter relapse free survival and survival is the chemokine receptor CXCR4. [26] This receptor has recently been studied in the post resection samples of 68 patients who had received neoadjuvant therapy for rectal cancer and high CXCR4 was associated with shorter relapse free survival and survival. [26]

In conclusion this study has shown that BCLAF1 is overexpressed in post therapy rectal cancer cells and its subcellular localisation is aberrant; in normal rectal mucosa BCLAF1 is exclusively nuclear whereas in tumour cells BCLAF1 was present both in the cytoplasm and the nucleus. The study has also shown that strong nuclear BCLAF1 immunoreactivity in post therapy rectal cancer is associated with increased patient survival and is an independent prognostic factor.

Contributions

The study was conceived by GIM, experimental work was performed by GB, BC and AA, data was analysed by GB, BC, AA, LMS and GIM and all the authors contributed to writing the manuscript.

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Table 1.

Clinicopathological characteristics of the patients and their tumours included in the rectal cancer tissue microarray

		Percent (number)	Mean survival (months, 95% CI)	Relationship with survival, hazard ratio and 95% CI
Sex				
	Male	61% (196)	71 (66-76)	χ^2 =0.083, p=0.774, HR=1.081 (0.635-1.814)
	Female	39% (125)	71 (67-75)	
Age (mean:range)		(66:29-91)		
	< 70	62% (200)	76 (73-80)	χ^2=14.758, p=<0.001, HR=2.730 (1.597-4.669)
	≥ 70	38% (121)	63 (57-69)	
Tumour differentiation				
	Well/moderate	73.8% (237)	69 (65-72)	Well/moderate v poor χ^2=29.789, p=<0.001, HR=2.500 (1.712-3.652)
	Poor	3.4% (11)	27 (6-48)	Well/moderate v no residual tumour χ^2=13.651, p=<0.001, HR=15.765 (2.175-114.282)
	No residual tumour	22.7% (73)	83 (80-87)	
ypT stage				
	yT0	24.9% (80)	83 (79-86)	T0 *v* T1 χ^2=0.137, p=0. 711 HR=0.715 (0.119-4.285)
	yT1	11.2% (36)	70 (66-74)	T1 *v* T2 χ^2=2.899, p=0.089, HR=0.153 (0.003-6.744)
	yT2	21.5% (69)	71 (64-77)	T2 *v* T3 χ^2=3.391, p=0.066, HR=1.436 (0.897-2.297)
	yT3	39.6% (127)	63 (57-69)	T3 *v* T4 χ^2=10.022, p=0.002, HR=0.490 (0.303-0.794)
	yT4	2.8% (9)	30 (17-43)	
ypN stage				
	yN0	76.3% (245)	75 (72-79)	N0 *v* N1 χ^2=7.393, p=0.007, HR= 0.401(0.216-0.744)
	yN1	17.1% (55)	62 (53-71)	N1 *v* N2 χ^2= 5.984, p=0.014, HR=0.367 (0.159-0.850)
	yN2	6.5% (21)	37 (25-48)	
EMVI				

	Present	8.7% (28)	35 (25-46)	present *v* absent χ^2=56.209, p=<0.001, HR=0.146 (0.081-0.262)
	Absent	91.3% (293)	75 (71-78)	
Dukes stage				
	A	28% (90)	76 (71-81)	Dukes A *v* Dukes B χ^2=9.673, p=0.002, HR=0.557 (0.376-0.824)
	B	25.5% (82)	59 (53-65)	Dukes B *v* Dukes C χ^2=1.383, p=0.240, HR=0.837 (0.626-1.119)
	C	23.7% (74)	57 (49-66)	
	na[1]	22.7% (73)	84 (83-87)	Path CR v Dukes A χ^2=0.137, p=0. 711 HR=0.715 (0.119-4.285)
Response to neoadjuvant therapy				
	Complete	22.7% (73)	84 (81-87)	Complete *v* good partial χ^2=5.995, p=0.014, HR=0.493 (0.233-1.042)
	Good Partial	35.5% (114)	75 (71-80)	Good partial v partial χ^2=11.908, p=0.001, HR=0.548 (0.392-0.767)
	Partial	30.5% (98)	60 (54-67)	Minimal v partial χ^2=0.364, HR=1.108 (0.792-1.549)
	Minimal	11.2% (36)	50 (41-59)	
Excision				
	No	11.5% (37)	41 (34-49)	χ^2=19.983, p=<0.001, HR= 4.54 (2.529-8.150)
	Yes	88.5% (284)	74 (71-78)	

[1]cases with a pathological complete response (path CR) where Dukes staging is not appropriate

Table 2. Comparison of cytoplasmic and nuclear BCLAF expression in rectal mucosa, primary rectal cancer and lymph node metastasis.

	Immunoreactivity (p value, normal *v* primary tumour)	Change in expression in tumour	Immunoreactivity (p-value primary tumour v lymph node metastasis)	Change in expression in lymph node metastasis	Immunoreactivity (p value, paired primary Dukes C tumour *v* lymph node metastasis)	Change in expression in lymph node metastasis
BCLAFc	<0.001	↑	0.008	↓	0.033	↓
BCLAFn	0.097	-	0.05	↓	0.336	-

Table 3. The relationship of cytoplasmic and nuclear BCLAF1 with pathological parameters.

	Tumour differentiation		EMVI		ypT stage		ypN stage		Dukes stage		Response to neoadjuvant therapy	
	χ^2	p value	χ^2	p value	χ^2	p value	χ^2	p value	χ^2	p value	χ^2	p value
Cytoplasmic BCLAF1	2.633	0.268	2.688	0.261	3.736	0.712	1.852	0.763	1.860	0.761	6.873	0.143
Nuclear BCLAF1	0.695	0.874	5.330	0.149	14.850	0.095	10.235	0.115	19.134	0.004	5.699	0.458

Table 4. The relationship of nuclear and cytoplasmic BCLAF1 with survival.

	Cut-off point							
	Overall		Negative v weak, moderate and strong		Negative and weak v moderate and strong		Negative, weak and moderate *v* strong	
	χ^2	p value	χ^2	p value	χ^2	p value	χ^2	p value
Cytoplasmic BCLAF1	0.065	0.968	0.004	0.952	3.642	0.056	-	-
Nuclear BCLAF1	6.334	0.096	0.389	0.533	4.876	0.027	0.426	0.514

Table 5. The significance of nuclear BCLAF1 expression in multivariate analysis in patients with residual tumour. Two models are shown including either Dukes stage (model A) or yTstage and yNstage (model B) as the parameters for assessing tumour stage.

A. Model including Dukes stage

Variable (categories)	Wald value	p-value	Hazard ratio (95%CI)
Age (< 70 v ≥ 70)	2.262	0.133	1.025 (0.993-1.058)
Gender (male v female)	0.999	0.318	1.410 (0.719-2.764)
Tumour differentiation (well/moderate v poor)	9.060	0.003	0.178 (0.058-0.548)
EMVI (present v absent)	5.844	0.016	0.360 (0.157-0.824)
Dukes stage (A v B v C)	2.663	0.264	0.492 (0.164-2.444)
Response to neoadjuvant therapy (good partial v partial v minimal)	2.032	0.362	0.554 (0.233-2.428)
Adjuvant therapy (yes v no)	0.983	0.321	1.567 (0.645-3.809)
Nuclear BCLAF1 (negative/weak v moderate/strong)	6.104	0.013	0.431 (0.221-0840)

B. Model including tumour stage and lymph node stage

Variable (categories)	Wald value	p-value	Hazard ratio (95%CI)
Age (< 70 v ≥ 70)	3.943	0.047	1.034 (1.000-1.068)
Gender (male v female)	0.700	0.403	1.348 (0.669-2.715)
Tumour differentiation (well/moderate v poor)	5.850	0.016	0.236 (0.073-0.761)
Tumour (ypT) stage (yT1 v yT2 v yT3 v yT4)	3.815	0.432	0.382 (0.010-5.490)
Nodal (ypN) stage (yN0 v yN1 v yN2)	3.185	0.203	0.440 (0.127-1.260)
EMVI (present v absent)	4.835	0.028	0.367 (0.150-0.897)
Response to neoadjuvant therapy (good partial v partial v minimal)	3.381	0.184	0.437 (0.180-2.128)
Adjuvant therapy (yes v no)	0.872	0.350	1.601 (0.596-4.301)
Nuclear BCLAF1 (negative/weak v moderate/strong)	5.259	0.022	0.451 (0.229-0.891)

Figure legends

Figure 1.

The relationship of histopathological response grade and overall survival in rectal cancer.

Figure 2.

Immunoblot of BCLAF1. A band migrating at the expected molecular weight (110kDa) is observed in the lane containing the BCLAF1 overexpressed cell lysates (+) and no band is observed in the lane containing vector only (-).

Figure 3.

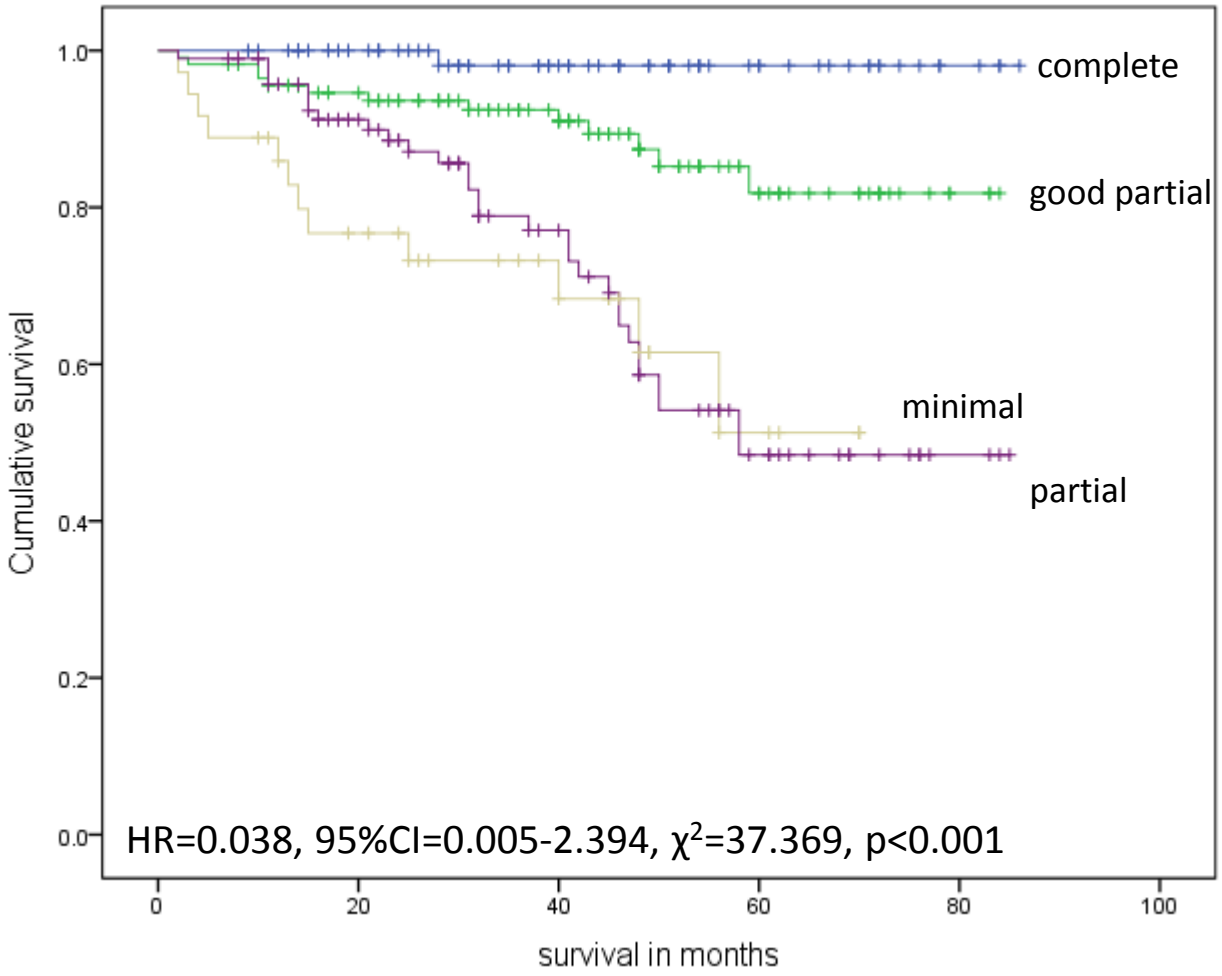
Immunohistochemical localisation of BCLAF1 in non-neoplastic rectal mucosa (A and B), primary rectal cancer following neoadjuvant therapy (C and D) and lymph node metastasis (E and F). A, C and E are low power photomicrographs and the area within each rectangle is shown at high magnification in panels B, D and F respectively.

Figure 4.

The frequency of nuclear and cytoplasmic BCLAF1 in non-neoplastic rectal mucosa, primary rectal cancer following neoadjuvant therapy and lymph node metastasis.

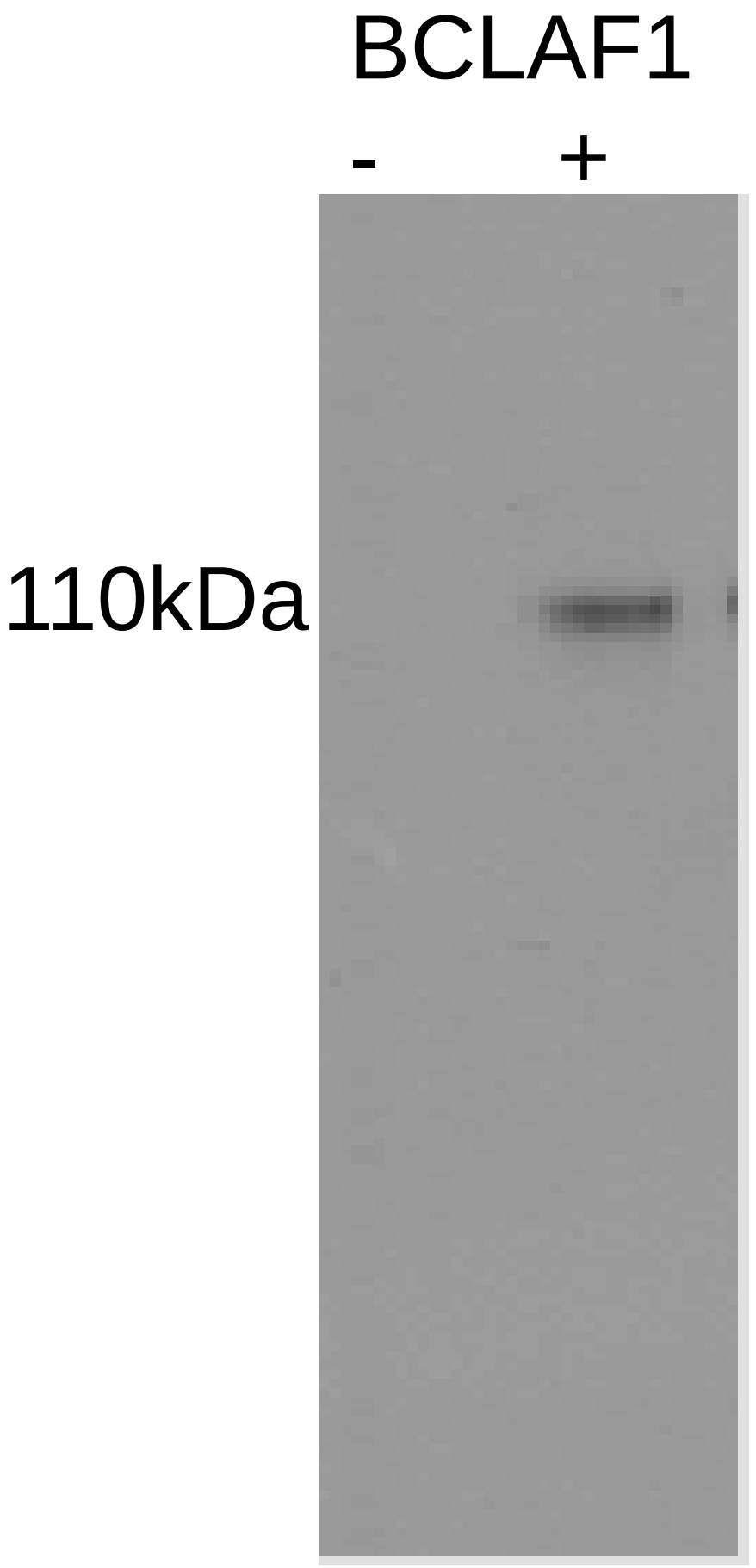
Figure 5.

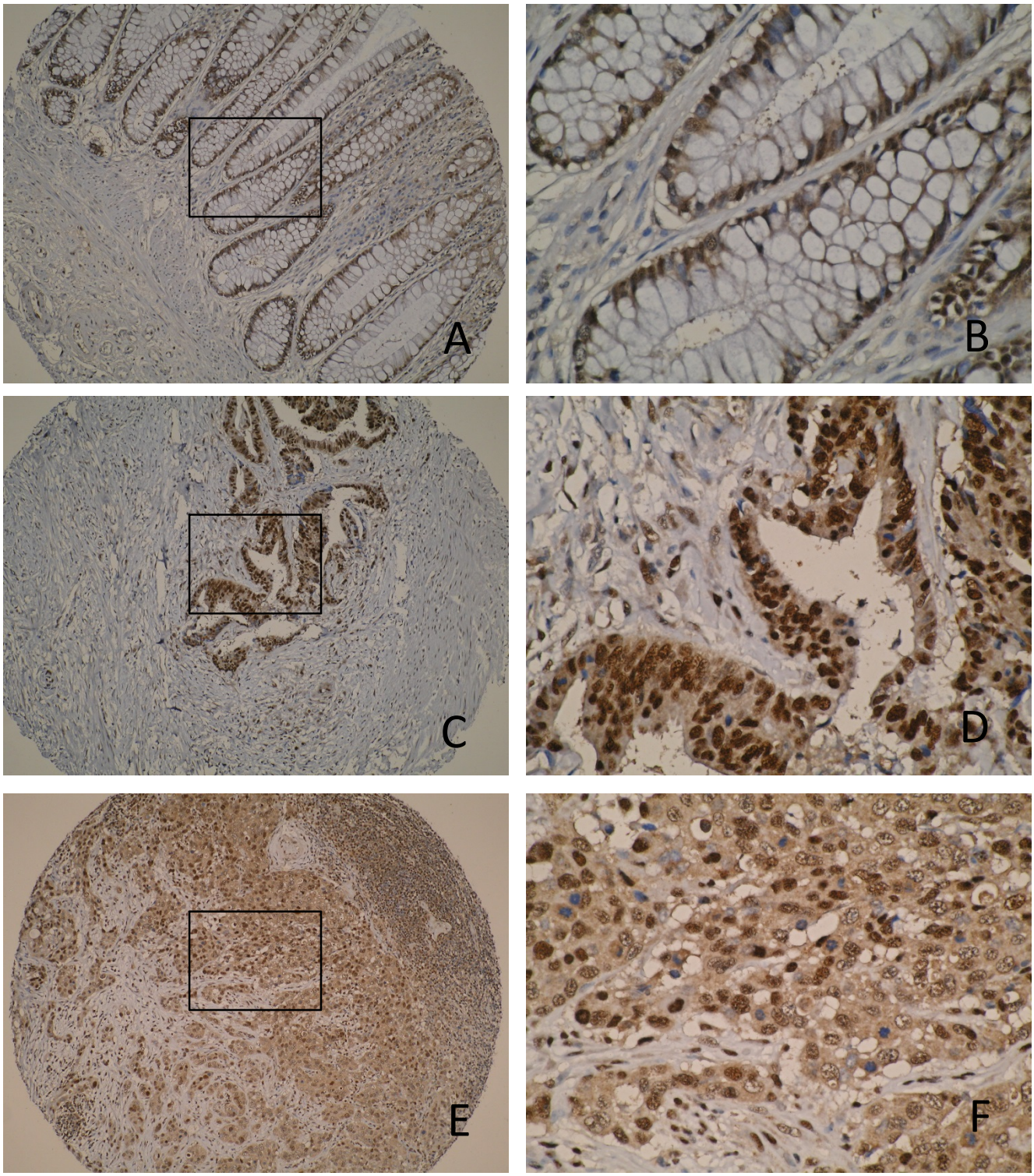
The relationship of nuclear BCLAF1 (BCLAF1n) expression and overall survival in rectal cancer.

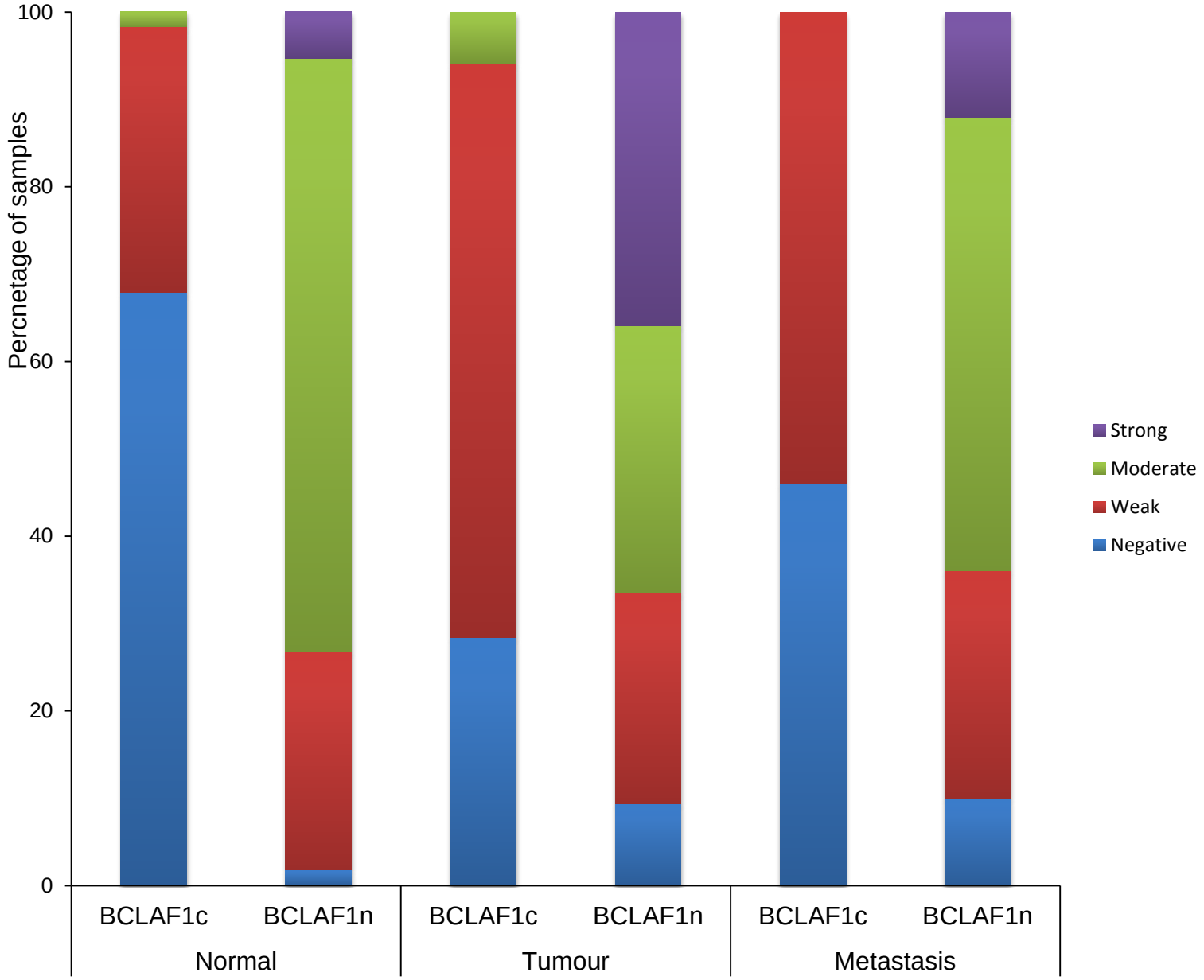


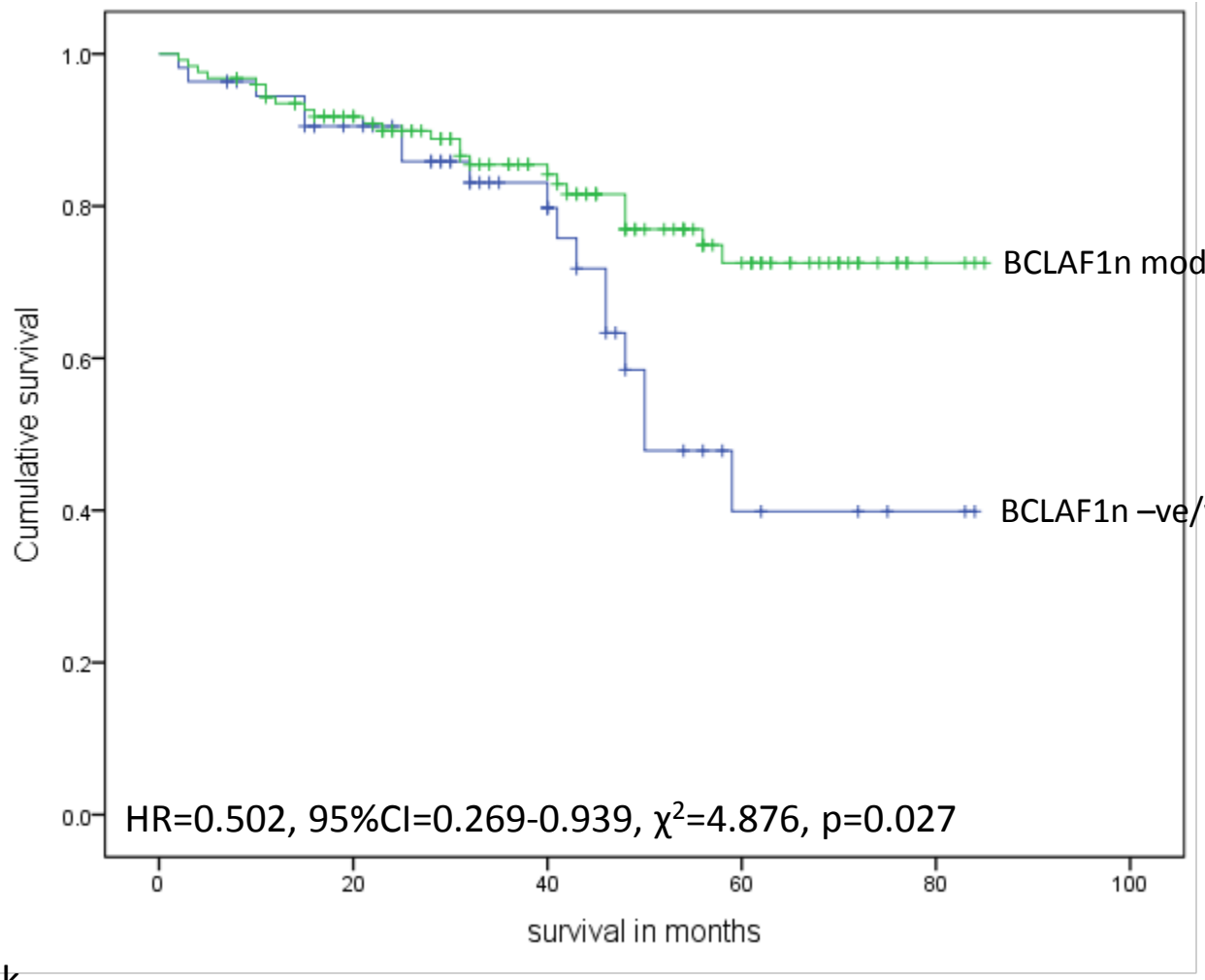
Number at risk

Complete	73	60	36	16	4
Good partial	114	95	60	22	3
Partial	98	70	39	16	3
Minimal	36	24	13	4	0


BCLAF1
-
+
110kDa


A
B
C
D
E
F


Percnetage of samples
100
80
60
40
20
0
BCLAF1c
BCLAF1n
BCLAF1c
BCLAF1n
BCLAF1c
BCLAF1n
Normal
Tumour
Metastasis
Strong
Moderate
Weak
Negative



Number at risk

BCLAF1n mod/strong	125	105	85	65	45	25
BCLAF1n –ve/weak	55	35	15	5	2	0