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Loss of special AT-rich sequence-binding protein 1 (SATB1) predicts poor survival in patients with colorectal cancer

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Aim: Special AT-rich sequence-binding protein 1 (SATB1) is a cell type-specific matrix attachment region binding protein, functioning as a global genome organizer. This study aims to investigate the expression pattern and the prognostic value of SATB1 in colorectal cancer.

Methods and results: Prospectively collected data were obtained and tissue microarrays were constructed from a cohort of 352 patients. SATB1 protein expression was evaluated by immunohistochemistry and scored by two independent investigators. SATB1 expression was predominantly nuclear in both normal and cancer tissues. Loss of SATB1 nuclear expression was seen in 22% of colorectal cancers compared to 1.5% of adjacent normal colorectal tissue, and was associated with worse overall survival ($P = 0.02$) independent of age and stage of disease (HR 2.48 with 95% CI 1.31–4.70). Loss of SATB1 expression was more evident in younger patients ($P = 0.03$), tumours with mucinous or signet ring histology ($P = 0.0001$) and poor differentiation ($P = 0.005$). SATB1 expression was associated with a survival advantage in patients with Dukes C tumours who received adjuvant chemotherapy.

Conclusion: Loss of SATB1 nuclear expression correlates with poor survival and a less favourable response to adjuvant chemotherapy in colorectal cancer. The value of SATB1 in individualized colorectal cancer therapy warrants further evaluation.

Keywords: chemotherapy, colorectal cancer, prognosis, SATB1, survival

Introduction

Colorectal cancer (CRC) is the fourth most common cancer in men and the third most common cancer in women.[1] The incidence continues to increase in economically transitioning countries.[2] In the United States, approximately 142 000 new diagnoses and 50 000 deaths are reported annually from the disease.[3] During the last two decades, major progress has been achieved in the field of molecular genetics and the management strategies of CRC. Despite the advances in surgical technique, the use of newer chemotherapeutic agents, the more frequent use of radiotherapy in rectal cancer and the introduction of targeted biological agents, the prognosis of advanced disease remains poor. While molecular markers (tissue or serum) can be used as an adjunct to determine the prognosis and predict response to different forms of cancer therapy in

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patients with CRC, current treatment decisions are based primarily on clinical and pathological staging of the disease. Examples of molecular markers of prognostic value in CRC are microsatellite instability markers, which are proposed to predict improved survival, and CpG island methylator phenotype markers, which predict adverse outcomes. The molecular profiles of individual cancers could be potentially beneficial in determining appropriate treatment for that particular cancer. A more comprehensive understanding of the genetic changes that drive disease progression in CRCs is required to achieve such an objective.

Special AT-rich sequence-binding protein 1 (SATB1) is a nuclear matrix attachment region binding protein identified initially in thymocytes.[4] It selectively binds AT-rich DNA sequences, where one strand has well-mixed As, Ts and Cs, excluding Gs (ATC sequence).[4] SATB1 is a cell type-specific genome organizer, regulating gene expression by folding chromatin into loop domains. It functions as a 'landing platform' for several chromatin remodelling/modifying enzymes and thus regulates epigenetically the expression of multiple genes.[5] The role of SATB1 in solid organ malignancies has attracted considerable attention during the last few years. In breast cancer, SATB1 was reported initially to promote growth and metastasis and an increase in SATB1 nuclear expression was associated with a poor prognosis.[6] However, the adverse prognostic effect in breast cancer has been disputed by another study.[7] SATB1 overexpression is also a negative prognostic marker in gastric cancer[8,9] and squamous cell laryngeal cancer.[10] However, in squamous cell lung cancer it is loss of expression rather than overexpression of SATB1 that is associated with poor survival.[11] This supports the tissue-specific organizational role of SATB1 and indicates that SATB1 has a different regulatory role in different tissues.

Colorectal cancer evolves through a stepwise accumulation of genetic and epigenetic alterations leading to the transformation of normal colonic mucosa into invasive cancer. Dysregulation of β-catenin-directed transcription is an important step in colorectal carcinogenesis.[12] Notani *et al.* have demonstrated that SATB1 can interact with β-catenin and recruit it into its genomic binding sites, hence mediating Wnt/β-catenin signalling in T helper type 2 cells.[13] In addition, reduced expression of special AT-rich sequence-binding protein 2 (SATB2), a homologue of SATB1, was found to correlate with tumour invasion, metastasis and poor survival in CRC.[14]

Taken together, the importance of SATB1 as a driver of tumorigenesis in several types of cancer as well as its putative β-catenin regulatory activity prompted us to assess further its expression profile and prognostic value in CRC. In this study, we report for the first time that loss of SATB1 expression has prognostic significance in CRC.

Materials and methods

PATIENT COHORT AND SAMPLE COLLECTION

Tissue microarrays were constructed (manual tissue arrayer; Beecher Instruments Inc, Wisconsin, USA) from archival formalin- fixed paraffin-embedded tissues of 352 patients diagnosed with CRC at any Sydney South West Area Health Service (SSWAHS) facility between 2000 and 2003. Slides were reviewed and areas of cancer and normal tissue were marked by a specialist gastrointestinal histopathologist prior to microarray construction, to ensure that representative areas of cancer and normal tissue were collected accurately. Five 1-mm cores were collected for each case: three from cancer tissue and two from resected normal tissue distant to the tumour. Prospectively collected clinical, pathological and follow-up data for all patients were obtained from the South Western Sydney Clinical Cancer Registry (SWSCCR).

SATB1 IMMUNOHISTOCHEMISTRY (IHC)

Monoclonal purified mouse anti-SATB1 antibody (catalogue 611182; BD Biosciences, North Carolina, USA) was used for IHC. Tissue microarrays were cut into 4-μm-thick sections and staining was performed using a Dako autostainer (Dako, Carpentaria, CA, USA). Briefly, after de-paraffinization, heat antigen retrieval was performed manually at 125°C for 2 min in a Pascal pressure chamber (Dako) with Dako buffer pH 9.0 (s2367; Dako). Peroxidase activity was blocked by incubation in H_2O_2 for 5 min. Primary antibody was diluted to 1:75 and incubated for 60 min. The Envision FLEX system (Dako) was used as a secondary antibody. Slides were incubated for 5 min in diaminobenzidine (Dako) as a substrate before counterstaining in Shannon's haematoxylin. All incubations were performed at room temperature. Staining was evaluated initially using tissue sections of CRC and normal colon tissue. Staining was homogeneous in most cases, therefore using tissue cores for this protein was considered appropriate. Staining optimization was achieved with the help of a specialist histopathologist. Mouse IgG1 was included as a negative control and normal spleen was used as a positive control: a subset of lymphocytes in the red and white pulp of the spleen stained positively for SATB1. Spleen was also used as a

positive control for SATB1 staining in a previous study.[11] Scoring was performed by two independent investigators; one of them is a specialist gastrointestinal histopathologist. Both investigators were blinded to the outcome data at the time of scoring. The percentage of tumour cells or normal colonic epithelial cells with nuclear expression was recorded for each core and the mean percentage was calculated for each patient. The average of percentages recorded by both investigators was used in the final analysis. Any discrepant scores were settled by reaching a consensus score. SATB1 expression was considered positive if mean nuclear expression was ≥5% at any intensity. The same method of scoring SATB1 IHC was described in a previous publication.[11]

SATB1 MRNA EXPRESSION

To validate the IHC findings, 54 cases with available fresh frozen tissue samples were chosen randomly from the South Western Sydney Colorectal Tumor Bank. RNA was extracted using TRI Reagent (Ambion, Austin, TX, USA) as per the manufacturer's protocol and cDNA was synthesized using the Superscript III first-strand synthesis system for RT–PCR (catalogue number 18080-051; Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR (qPCR) was performed in triplicate using ABI 7900 (Applied Biosystems, Foster City, CA, USA). SATB1 (Accession no. NM 002971.3) primers used for qPCR were forward: 5′-CAT GTT CCA GCA GAG CAG ATT CAG-3′ (3491-3514), reverse: 5′-CAC CGT GGG TTG CCG TGG-3′ (3628-3611) and probe 5′-d FAM-CCA CAG CAG CAG CCA CAG ACA GGC CC-BHQ-1-3′. TaqMan assays were used for the GAPDH reference gene (Applied Biosystems).

STATISTICAL ANALYSES

SPSS version 17.0 software (IBM, Chicago, IL, USA) was used in all statistical analyses. The association of SATB1 expression with different clinicopathological variables was analysed using the two-tailed Pearson's χ^2 test. Kaplan–Meier log-rank and Cox regression methods were used to analyse the univariate and multivariate survival associations, respectively. *P*-values of <0.05 were considered statistically significant.

Results

COHORT CHARACTERISTICS

Representative tissue from resection specimens as well as clinical and pathological data were obtained for 352 patients with a median follow-up of 66 months. Nine duplicate specimens (recurrent or metachronous tumour samples) were excluded. Surgery was undertaken by colorectal and general surgeons in different hospitals in the area health service (Sydney South West Area Health Service). The majority of procedures were performed by five specialist colorectal surgeons. Total mesorectal excision (TME) was the standard practice for rectal excision. Patient characteristics are outlined in Table 1.

Table 1. Patient characteristics

Characteristics	*n*	%
Sex		
Male	191	55.7
Female	152	44.3
Age (years)		
<50	27	7.9
50–70	170	49.7
>70	145	42.4
Site of primary tumour		
Right colon	107	31.3
Left colon	89	26
Rectum and rectosigmoid	146	42.7
Histology		
Adenocarcinoma	298	87.4
Mucinous adenocarcinoma	38	11.1
Signet ring carcinoma	5	1.5
TNM stage		
I	72	21.2
II	96	28.2
III	133	39.1
IV	39	11.5
CpG island methylator phenotype (CIMP) status		
CIMP high	47	16.5
CIMP low or negative	238	83.5
Microsatellite instability (MSI) status		
MSI-H	49	14.4
MSS or MSI-L	291	85.6
Postoperative chemotherapy given	140	40.9

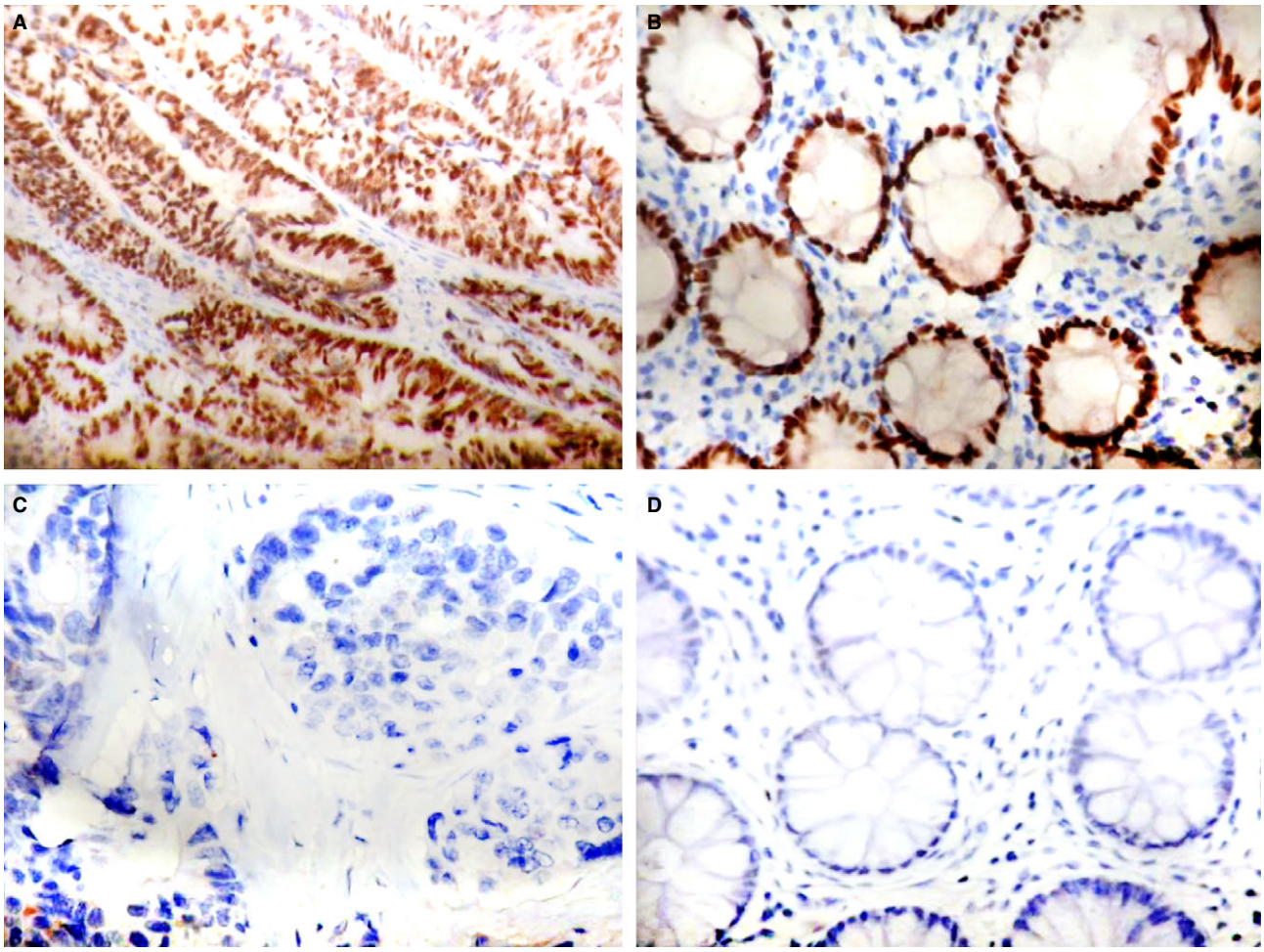

Figure 1. SATB1 immunohistochemistry: **A**, positive SATB1 nuclear expression in colon cancer tissue; **B**, positive SATB1 nuclear expression in normal colon tissue; **C**, negative SATB1 expression in colon cancer tissue; **D**, negative SATB1 expression in normal colon tissue.

SATB1 EXPRESSION IN COLORECTAL CANCER TISSUE

SATB1 protein expression was assessed by IHC (Figure 1). SATB1 expression was predominantly nuclear in both cancer (78% nuclear and 18.7% cytoplasmic) and normal (98.5% nuclear and 2% cytoplasmic) tissues, with some tissues showing combined nuclear and cytoplasmic expression. Figure 2 details the intracellular expression pattern observed with IHC. Complete loss of SATB1 nuclear expression was seen in tumour specimens from 75 patients (22%, including 5.3% with cytoplasmic expression only) and in adjacent normal specimens from five patients (1.5%). The mean percentage of cells with positive nuclear expression of SATB1 was significantly lower in cancer tissue compared to adjacent normal tissue (48.6% and 83.1%, respectively, $P < 0.0001$). The intensity of nuclear SATB1 expression was also higher in normal

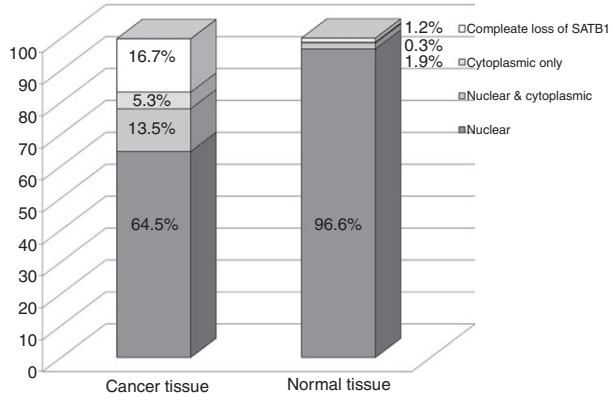

Figure 2. Intracellular localization of SATB1 in colorectal cancer and normal tissue.

compared to cancer specimens, with the majority of normal tissues exhibiting moderate or strong IHC staining. Although some cores showed some

Table 2. Association of SATB1 nuclear expression with clinicopathological variables

	SATB1 nuclear expression (n = 341)		
Characteristic	Positive n = 266 (78%)	Negative n = 75 (22%)	P-value (two-sided)
Gender			
Male	146 (55%)	44 (59%)	0.60
Female	120 (45%)	31 (41%)	
Age group (years)			
<50	16 (6%)	11 (15%)	0.03
50–70	132 (50%)	38 (50%)	
>70	117 (44%)	26 (35%)	
Site			
Right colon	78 (30%)	29 (39%)	0.31
Left colon	72 (27%)	17 (22%)	
Rectum	115 (43%)	29 (39%)	
TNM stage			
I	60 (23%)	12 (16%)	0.44
II	75 (29%)	19 (25%)	
III	101 (38%)	32 (43%)	
IV	27 (10%)	12 (16%)	
Histology			
Adenocarcinoma	241 (91%)	55 (73%)	0.0001
Mucinous adenocarcinoma	20 (8%)	18 (24%)	
Signet ring carcinoma	3 (1%)	2 (3%)	
Grade			
Well differentiated	6 (2%)	1 (1%)	0.005
Moderately differentiated	225 (88%)	54 (74%)	
Poorly differentiated	26 (10%)	18 (25%)	
Tumour size			
<20 mm	11 (4%)	5 (7%)	0.13
20–50 mm	181 (70%)	43 (57%)	
>50 mm	68 (26%)	27 (36%)	
Microsatellite instability (MSI) status			
MSI: high	33 (12%)	16 (22%)	0.059
MSS or MSI low	232 (88%)	57 (78%)	
CpG island methylator phenotype (CIMP) status			
CIMP: high	31 (14%)	16 (25%)	0.05
CIMP: low or negative	190 (86%)	47 (75%)	

heterogeneity of staining intensity, the majority of cases exhibit uniform staining in both normal and cancer tissue. In addition, a subpopulation of lymphocytes stained positively for SATB1. Other stromal cells and fibroblasts were negative for SATB1. Scoring agreement between the two investigators (based on positive versus negative staining) was high, with a kappa score of 0.90.

Quantitative real-time PCR was used to assess the correlation between SATB1 mRNA level and IHC protein expression in 54 colorectal cancer cases. High SATB1 mRNA (RT–PCR) expression was detected in 86% of cancers with nuclear or cytoplasmic SATB1 protein expression (IHC). Only seven specimens with positive mRNA had negative IHC; these included four cancers with an increased inflammatory host response, and the high expression of SATB1 in these specimens may have reflected the presence of T cells.

CLINICOPATHOLOGICAL ASSOCIATIONS OF LOSS OF SATB1 NUCLEAR EXPRESSION

The associations between loss of SATB1 nuclear expression and different clinical and pathological variables are summarized in Table 2. Loss of SATB1 expression was associated with younger age at diagnosis ($P = 0.03$), mucinous or signet ring histology ($P = 0.0001$), higher tumour grade ($P = 0.005$) and CpG island methylator phenotype (CIMP) status ($P = 0.05$). No significant associations were observed with gender ($P = 0.60$), stage of disease ($P = 0.44$), site of primary tumour ($P = 0.31$), tumour size ($P = 0.13$) or microsatellite instability (MSI) status ($P = 0.059$).

ASSOCIATION WITH SURVIVAL

Loss of SATB1 nuclear expression was a negative prognostic marker, with 5-year survival of 57% in patients with loss of nuclear SATB1 expression compared to 66% in patients with positive SATB1 expression. This survival difference was statistically significant ($P = 0.02$) (Figure 3A). This was independent of age at diagnosis and TNM disease stage but not independent, however, of histology and grade (hazard ratio 1.60 and 95% confidence interval 1.09–2.36, $P = 0.016$). On subgroup analysis, poor survival was observed mainly in patients with primary tumours of the right colon (defined as tumours of the caecum, ascending colon or transverse colon), with 5-year survival of 45% in patients with loss of SATB1 nuclear expression compared to 65% in patients expressing nuclear SATB1 ($P = 0.004$) (Figure 3B). This was independent of age, TNM stage or MSI status (hazard ratio 2.48 with 95% confidence interval 1.31–4.70, $P = 0.005$). The time-table detailing cumulative survival is illustrated in Table 3.

To assess the predictive value of SATB1 expression as a response marker to chemotherapy, we performed survival analysis in patients with Dukes C cancers ($n = 132$) comparing patients who received post-operative chemotherapy with those who did not receive chemotherapy. Patients with positive nuclear SATB1 expression had a survival advantage if given adjuvant chemotherapy ($P = 0.0001$), whereas patients with loss of SATB1 expression did not ($P = 0.32$) (Figure 3C,D). This suggests a possible role for SATB1 in determining chemotherapy responsiveness. There was no significant correlation between cytoplasmic SATB1 expression and clinicopathological variables or survival.

Discussion

Colorectal cancer is a major global health problem. There are considerable differences in tumour behaviour among patients of similar pathological stage. Identifying biomarkers of prognosis and therapy response will help in identifying high-risk patients and provide personalized therapy for cancer sufferers. Many biomarkers have been studied over the last three decades; however, their use in clinical practice is limited. This is due probably to differences in study design, small sample size and inconsistent or inconclusive results. In this study, we identify a new marker with prognostic and therapy predictive value in a large cohort of CRC patients using a simple and reliable method.

SATB1 acts as a genome organizer which orchestrates overexpression of some genes and repression of others.[6,15] In this study we report for the first time that SATB1 is expressed in normal colon epithelial cells and that loss of SATB1 expression is associated with poor survival in colorectal cancer. We studied SATB1 expression by IHC, which enables the investigator to identify the cell types expressing the protein and its cellular localization. We found that the majority of SATB1 expression was nuclear, which is consistent with its role as an epigenetic regulator. This nuclear expression was lost in 22% of colorectal cancer specimens, while adjacent normal tissue retained nuclear expression in the majority of samples (98.5%). SATB1 expression is a feature of many normal tissues, including T cells,[4] lung bronchial epithelium[11] and colon epithelial cells, whereas some other tissues, such as breast,[6] do not normally express

Table 3. Yearly survival percentage based on SATB1 nuclear expression, all sites colorectal cancers and right-sided colon cancers

	Colorectal cancer n = 340				
	SATB1 positive n = 265		SATB1 negative n = 75		
Time from diagnosis	No. of deaths	Cumulative proportion surviving (%)	No. of deaths	Cumulative proportion surviving (%)	*P*-value
1 year	26	90.2	18	76	0.001
2 years	49	81.5	24	68	0.005
3 years	68	74.2	28	62.7	0.02
4 years	81	69.2	30	60	0.05
5 years	91	64.5	32	54.9	0.05
Overall survival	97	63.4	37	50.7	0.02
	Right-sided colon cancers n = 107				
	SATB1-positive n = 78		SATB1-negative n = 29		
Time from diagnosis	No. of deaths	Cumulative proportion surviving (%)	No. of deaths	Cumulative proportion surviving (%)	*P*-value
1 year	10	87.2	10	65.5	0.01
2 years	16	79.5	13	55.2	0.008
3 years	22	71.8	16	44.8	0.005
4 years	26	66.2	16	44.8	0.01
5 years	27	64.9	16	44.8	0.02
Overall survival	29	55.1	19	34.5	0.004

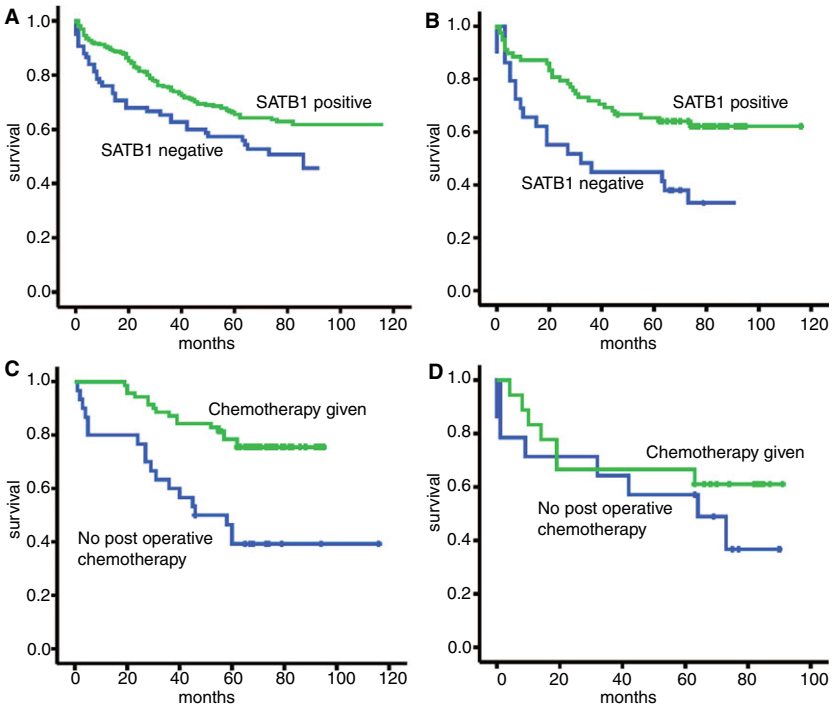

Figure 3. **A**, Loss of SATB1 nuclear expression was associated with worse overall survival ($P = 0.02$) in patients with colorectal cancer. **B**, This association was stronger in patients with right-sided colon cancers ($P = 0.004$). Patients with Dukes C colorectal cancer with positive SATB1 nuclear expression had a survival advantage when given adjuvant chemotherapy ($P = 0.0001$) **C**, whereas patients with Dukes C colorectal cancer with loss of SATB1 expression did not ($P = 0.32$) **D**.

SATB1. Loss of SATB1 expression in CRC specimens was associated with reduced overall survival; this was more evident in proximal colon cancer. The association of SATB1 loss with poor survival was independent of age at diagnosis and TNM disease stage, but not independent of histology and grade. There was also a correlation with poor differentiation and mucinous or signet ring histology, which are associated with worse prognosis of CRC.[16,17]

In addition to colon cancer, loss of SATB1 expression is associated with poor survival in squamous cell lung cancer.[11] However, opposite findings have been made in other solid organ malignancies, where SATB1 overexpression was reported to be associated with worse outcome. This may reflect the different organizational role of SATB1 in different tissues. Loss of SATB1 function may also be critical because it causes widespread disruption of histone modifications and loss of its regulatory role on many genes. This may lead to promotion of tumorigenesis. A recent study on the relationship between SATB1 expression and rectal cancer found that SATB1 overexpression was associated with adverse pathological features and increased metastatic potential.[18] This is in contrast to our study, but highlights the importance of tumour location in genetic and epigenetic regulation of colorectal cancer. Colorectal cancer is viewed traditionally as a single entity, but stark differences in right-sided, left-sided and rectal cancers in both phenotypic and genotypic characteristics are now more apparent. Our study shows that SATB1 loss is associated with poor prognosis in colorectal cancers, predominantly in right-sided colon cancers. A number of studies[18–21] have analysed the expression of SATB1 in CRC, with all but one (Nodin *et al.*[21]) involving small sample sizes ($n < 100$). These smaller studies have described an increase of SATB1 expression in patient tumour samples relative to normal tissue but have not, however, analysed the prognostic significance of tumours expressing SATB1. The largest study (Nodin *et al.*[21]), which analysed 529 CRC tumours but only a small subset of benign colon mucosa samples ($n = 20$), describe no prognostic significance of SATB1 expression, except in the absence of SATB2 expression. Our research does not confirm this finding; however, the discrepancy may be a result of the use of different antibodies.

Further studies are required to define the genes dysregulated following loss of SATB1 in the colon and rectum. Loss of SATB1 correlates with high

methylator (CIMP) phenotype. This association lends credence to the possibility of an interaction between DNA methylation and SATB1. Further investigations are required to ascertain whether promoter methylation results in loss of SATB1 or loss of SATB1 augments methylation of other genes.

Surgery remains the cornerstone for cure in localized colorectal cancers. Current treatment guidelines recommend the use of adjuvant chemotherapy for resectable node-positive (TNM stage III) and some node-negative stage II cancers with high-risk features. Despite the use of chemotherapeutic agents, estimated 5-year survival post-surgery is still variable, ranging from 17 to 77% depending on the depth of tumour invasion and involvement of lymph nodes.[22] This indicates that a large proportion of patients receiving adjuvant chemotherapy have little benefit from it. Identifying molecular markers predicting response to adjuvant therapy may help to stratify therapy and improve candidate selection. In this study we found that patients with loss of SATB1 expression responded less favourably to adjuvant chemotherapy, indicating a possible role for SATB1 in determining chemotherapy responsiveness. As SATB1 potentially regulates multiple genes, identifying the target genes of SATB1 in colorectal cancer cells may reveal new markers of chemotherapeutic responsiveness or new drug targets. The interaction between gene expression and chemotherapy response was highlighted recently by the relationship between Kras mutations and the use of EGFR inhibitors. This vital discovery has led to a change in prescription practice for EGFR inhibitors and Kras mutation testing is now a vital part of any pre-treatment investigation. Similarly, biomarker-based therapy can be potentially important in both choice of chemotherapy and development of targeted drugs.

Our study highlights the potential complex interactions of SATB1 and its role as both an indicator of prognosis and response to chemotherapy. While further studies are required to ascertain the exact mechanisms of action of SATB1 and its role in chemotherapy response, this study highlights the importance of SATB1 in colorectal cancer.

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