Expression of the antiapoptosis gene, *Survivin*, predicts death from recurrent colorectal carcinoma

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Abstract

***Background/aims*—Inhibition of programmed cell death (apoptosis) is associated with increased tumour aggressiveness, and expression of *Survivin*, an antiapoptosis gene, in colorectal carcinomas may provide important prognostic information.**

***Patients/methods*—Expression of *Survivin* messenger RNA was evaluated by reverse transcription-polymerase chain reaction in 144 colorectal carcinomas and 86 adjacent histologically normal mucosa samples from patients for whom long term follow up data were available.**

***Results*—*Survivin* transcripts were detected in a significantly greater proportion of carcinomas (63.5%) than normal mucosa samples (29.1%; p<0.001). The prevalence of *Survivin* expression was independent of advancing pathological stage. Death due to recurrent cancer following curative resection was predicted independently by tumour expression of *Survivin* (hazard ratio (HR) 2.60; 95% confidence interval (95% CI) 1.17–5.75) and lymph node metastases (HR 2.38; 95% CI 1.21–4.70). On stage wise analysis, the predictive value of *Survivin* expression was limited to patients with stage II colorectal carcinomas; those with *Survivin* negative tumours had a five year survival rate of 94.4% compared with 44.8% for patients with *Survivin* positive tumours (p=0.004, log rank test).**

***Conclusion*—In patients with stage II colorectal carcinomas, *Survivin* expression provides prognostic information that may have important therapeutic implications.**

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Keywords: colorectal neoplasia; messenger RNA; polymerase chain reaction; prognosis; survival

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The development and progression of colorectal carcinoma (CRC) involves unregulated epithelial cell proliferation associated with a series of accumulated genetic alterations.[1] There is evidence that prolonged survival of such genetically unstable colorectal epithelial cells, with their ultimate malignant transformation, is associated with progressive inhibition of apoptosis.[2] Apoptosis is a morphologically distinct form of cell death which is genetically regulated and, in addition to other roles, provides a vital protective mechanism against the development of neoplasia by removing cells with DNA damage. Inhibition of apoptosis thus confers a survival advantage on cells harbouring genetic alterations and may promote acquisition of further mutations to cause neoplastic progression and also contribute to the development of resistance to chemotherapy.[3 4]

Apoptosis plays an important role in maintaining homeostasis in a continually regenerating population of cells, such as the colonic epithelium.[5] Normally, mitotic activity of large intestinal stem cells located in the basal region of colonic crypts produces a continuous supply of new cells. These cells migrate up the colonic crypt where they differentiate before eventually undergoing apoptosis and exfoliation at the luminal surface. Occurrence of apoptotic activity correlates with the topographically restricted distribution of BCL-2 in basally located, but not more superficial, cells of normal colonic crypts.[6] BCL-2 is one of the most biologically relevant inhibitors of apoptosis[7] and its localisation in crypt bases of normal colonic epithelium suggests that it may be physiologically important for the viability of regenerating stem cells. In contrast, overexpression of BCL-2 is observed in colorectal adenomas and carcinomas, implying a role for this oncoprotein in apoptotic inhibition in colorectal neoplasia.[8] Furthermore, inactivation of inducers of apoptosis, such as wild-type P53[9] and APC,[10] in colonic epithelium has also been clearly implicated in neoplastic transformation.

A novel antiapoptosis gene, designated "*Survivin*",[11] which is also implicated in the control of cell cycle progression,[12] has been recently identified. In contrast with BCL-2, *Survivin* does not appear to be involved in the physiological regulation of apoptosis in adult colonic epithelium but is prominently expressed in CRC[13] and several other malignancies.[11 14 15] The mechanisms governing expression of *Survivin* in malignant cells are presently unclear but a complex response to dedifferentiation of normal epithelium appears likely.[16] A recent report indicates that profound inhibition of apoptosis in CRC, mediated by simultaneous co-expression of *Survivin* and BCL-2, is associated with poor survival.[13] However, a direct correlation between *Survivin* expression and death due to recurrent CRC has not been demonstrated. Consequently, the

Abbreviations used in this paper: CRC, colorectal carcinoma(s); mRNA, messenger RNA; RT-PCR, reverse transcription-polymerase chain reaction; HR, hazard ratio.

present study aimed to examine expression of *Survivin* messenger RNA (mRNA) in CRC at different pathological stages and to evaluate any association between expression of this gene and disease specific survival following curative resection of the primary tumour.

Patients and methods

CANCER CELL LINES

Human CRC cell lines LoVo, COLO 205, COLO 320, SW 480, SW 948, and HT29 were obtained from the European Cell Culture and maintained in appropriate culture media at 37°C in 5% carbon dioxide. Cell pellets obtained at passage were stored in liquid nitrogen until assay.

PATIENTS AND SPECIMENS

The study group comprised patients who had undergone a histopathologically confirmed curative resection of primary sporadic CRC (TNM stages I, II, and III). All patients had single tumours. Lung metastases were not detected by preoperative chest radiography in any case. Liver metastases were excluded by preoperative computerised tomography scanning and intraoperative palpation of the liver. Additionally, to evaluate any association between gene expression in the primary tumour and presence of distant metastases, patients with liver metastases (stage IV) who had undergone palliative bowel resection were included. Patients who died of postoperative complications within 30 days were excluded. All operations were performed in one surgical unit during a period when adjuvant therapy was not routinely administered.

Biopsies of tumour edge and normal colonic mucosa at the proximal margin of a freshly resected specimen were obtained and immediately embedded in tissue freezing medium in liquid nitrogen. Cryosections were stained with haematoxylin-eosin to histologically confirm that tumour biopsies contained more than 70% malignant cells and, conversely, that malignant cells were absent from biopsies of normal mucosa. Consecutive sections were collected and immediately stored at −80°C for subsequent assay. The remainder of each resected specimen was fixed in 10% formaldehyde solution. Pathological assessment was conducted according to the original guidelines of the United Kingdom Co-ordinating Committee on Cancer Research (UKCCR)[17] or its subsequent modifications by one of two consultant histopathologists with a specialist interest in gastrointestinal pathology or by a consultant histopathologist with a tumour interest. It was confirmed that circumferential resection margins were not infiltrated by cancer cells for all rectal tumours. To allocate a TNM stage, a median of 13 (range 6–33) lymph nodes were counted.

Following discharge from hospital, patients attended regular follow up clinics at which a detailed clinical examination and serial measurement of carcinoembryonic antigen serum levels were performed. Regular colonoscopic surveillance was conducted and chest *x* rays and computerised tomography scans of the abdomen were performed when clinically indicated. Death due to recurrent colorectal carcinoma was determined by reviewing medical records and the database of the Yorkshire Regional Cancer Registry. Survival was measured from date of operation to date of latest follow up visit or death due to recurrent CRC.

The studies were approved by the clinical ethics committee of St James's and Seacroft University Hospitals NHS Trust.

RNA EXTRACTION

Thawed cell pellets or powdered tissue sections (10 mg) were suspended in 1 ml Catrimox-14 cationic surfactant solution (Iowa Biotechnology Corp, Oakdale, Iowa, USA) and centrifuged for five minutes at 1000 *g* to form a detergent bound RNA pellet. This was subjected to three cycles of washing and sedimentation at 10 000 *g* for five minutes in 1 ml of 2 mol/l lithium chloride, followed by a final wash with 70% ethanol at 4°C, according to the manufacturer's protocol. The RNA pellet was vacuum dried and resuspended in 20 µl sterile distilled water containing 1 mmol/l dithiothreitol and 1 U/µl rRNasin (Promega, Southampton, UK).

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR) AND GEL ELECTROPHORESIS

RNA (1 µg) was reverse transcribed in a 20 µl reaction using 120 U M-MLV RT enzyme (Promega), 1 mmol/l of each dNTP (Pharmacia Biotech, St Albans, UK), 0.5 µg oligo$(dT)_{15}$, and 20 U rRNasin in an RT buffer with 3 mmol/l magnesium chloride. The reaction mixture was incubated at 42°C for one hour followed by incubation at 95°C for five minutes. To ensure the fidelity of mRNA extraction and reverse transcription, all samples were subjected to PCR amplification with oligonucleotide primers specific for the constitutively expressed gene glyceraldehyde-3-phosphate dehydrogenase. PCR conditions were identical to those described below. Primers were designed to amplify a 338 base pair product of *Survivin* complementary DNA (cDNA; accession number U75285). The oligonucleotide sequences were GGA CCA CCG CAT CTC TAC AT (forward) at positions 2855–2874 in exon 1 and GCA CTT TCT TCG CAG TTT CC (reverse) at positions 11990–12009 in exon 4. A BLAST search was performed to confirm that these primers were specific in the current sequence databases for *Survivin*. PCR was performed in a final volume of 25 µl containing 2 µg cDNA, 20 pmol of each oligonucleotide primer, 2 mmol/l magnesium chloride, 0.625 U *Taq* DNA polymerase, 20 mmol/l $(NH_4)_2SO_4$, 75 mmol/l Tris HCl, and 0.2 mmol/l of each dNTP in prealiquoted tubes (Advanced Biotechnologies, Epsom, UK). Thirty cycles of PCR amplification were performed in a DNA thermal cycler (MJ Research Inc) with denaturing at 95°C for one minute, annealing at 55°C for one minute and extension at 72°C for one minute. PCR products were visualised on 2% agarose gels with ethidium bromide

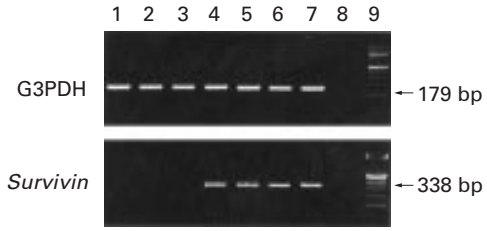


Figure 1 RT-PCR products of paired samples of normal colonic mucosa (lanes 1, 3, and 5) and colorectal carcinoma (lanes 2, 4, and 6) obtained from three different resection specimens. Lane 7 is a positive control (SW 480 colon cancer cell line) and lane 8 a negative control. Expression of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) confirmed the fidelity of the reverse transcription. In the first pair, neither normal mucosa nor cancer samples expressed Survivin (lanes 1 and 2); in the second pair, only the cancer sample (lane 4) expressed Survivin but not normal mucosa (lane 3); and in the third pair, both normal mucosa and cancer samples (lanes 5 and 6) expressed Survivin.

Table 1 Correlation between tumour expression of Survivin mRNA and clinicopathological characteristics of patients with colorectal carcinoma

Variable	*Category*	*Total No*	*Survivin positive (No (%))*	*p Value*
Sex	Male	78	54 (69.2)	0.05
	Female	66	35 (53.0)	
Site	Colon	83	53 (63.9)	0.48
	Rectum	61	36 (59.0)	
Stage	I	22	11 (50.0)	0.50*
	II	59	37 (62.7)	
	III	45	31 (68.9)	
	IV	18	10 (55.6)	
Vital status†	Alive/censored	93	53 (56.4)	0.05
	Dead	33	25 (75.8)	

*χ^2 test for trend.
†Patients with stages I, II, and III colorectal cancer.

staining under ultraviolet transillumination. The cell line SW 480 and water were used as positive and negative controls, respectively. A 100 base pair DNA ladder (Gibco BRL, Paisley, UK) was used as a molecular weight marker on each gel. Samples which exhibited a 338 base pair PCR product, subsequently verified to be homologous with the *Survivin* cDNA sequence, were designated *Survivin* positive. Samples which exhibited no PCR product were designated *Survivin* negative.

VERIFICATION OF PCR PRODUCTS BY SEQUENCING

To verify that PCR amplification was specific for *Survivin*, PCR products were excised from agarose gels and purified using the QIAquick gel extraction kit (Qiagen, Crawley, UK). Sequencing of both strands was performed by the chain termination method using oligonucleotide primers designed for PCR amplification and α^{32}P-dATP with the Sequenase Version 2.0 DNA sequencing kit (Amersham, St Albans, UK), according to the manufacturer's protocol.

STATISTICAL ANALYSIS

The statistical software package SPSS 6.0 was used. The prevalence of *Survivin* mRNA expression in cancers and normal tissues was compared by the Wilcoxon matched pairs test. Association between *Survivin* expression in tumours and various clinicopathological variables was examined by either the χ^2 method or Student's *t* test. Survival analyses were conducted according to the Kaplan-Meier method and survival characteristics were compared using log rank tests. The Cox proportional hazards regression model was used to compare the relative influences of different prognostic factors. A p value less than 0.05 was considered to indicate statistical significance.

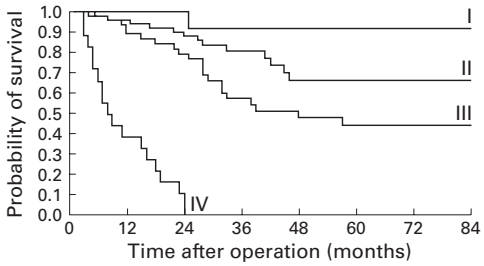
Results

SURVIVIN mRNA EXPRESSION

Expression of *Survivin* mRNA was detected by RT-PCR in all cancer cell lines (data not shown) and the cell line SW 480 was used as a positive control in subsequent experiments.

Eighty six pairs of CRC and normal mucosa samples were available for analyses. *Survivin* mRNA expression was detected in a significantly greater proportion of CRC than in normal mucosa samples (63.5% *v* 29.1%, respectively; p<0.001). In no case was *Survivin* mRNA detected in normal tissue when the associated cancer was *Survivin* negative (fig 1). There was no association between prevalence of *Survivin* expression in normal mucosa and pathological stage of the associated cancer (data not shown).

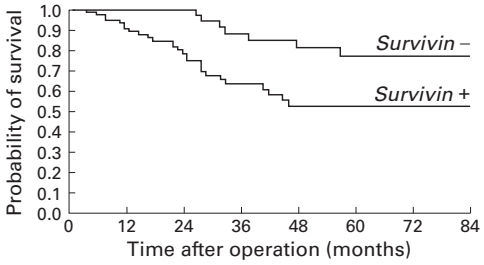
Biopsies of CRC were obtained from another 58 patients, providing a total of 144 can-



No of patients at risk:

I	22	20	13	9	7	5	4	2
II	59	51	41	28	17	16	12	8
III	45	39	30	19	15	12	9	6
IV	18	7	1					

Figure 2 Kaplan-Meier survival plot for patients with colorectal carcinoma stratified by TNM stage (I–IV) and displaying standard survival characteristics (p=0.01, log rank test for trend).



No of patients at risk:

–	47	44	38	28	21	18	14	8
+	79	66	46	28	18	15	11	8

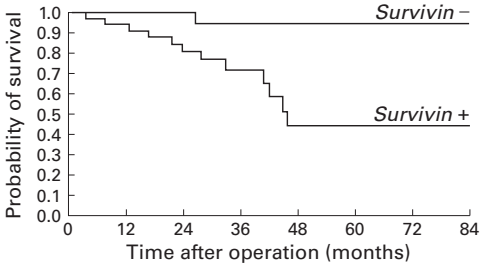
Figure 3 Kaplan-Meier survival plot for patients with curatively resected colorectal carcinomas (stages I, II, and III) stratified according to tumour expression of Survivin. The five year survival rate of patients with Survivin positive tumours was 53.0% compared with that of 77.5% for patients with Survivin negative tumours (p=0.007, log rank test).

Table 2 Univariate and multivariate analyses of prognostic factors following curative resection of colorectal carcinomas (stages I, II, and III)

Variable	*Category*	*HR*	*95% CI*	*p Value*
Univariate analysis				
Lymph node metastases	Present *v* absent	2.61	1.33–5.11	0.004
Survivin expression	Present *v* absent	2.82	1.27–6.23	0.01
Age	>70 y *v* <70 y	1.30	0.57–2.92	0.49
Sex	Male *v* female	0.67	0.32–1.41	0.19
Site	Rectum *v* colon	0.97	0.46–2.03	0.91
Multivariate analysis				
Lymph node metastases	Present *v* absent	2.38	1.21–4.70	0.01
Survivin expression	Present *v* absent	2.60	1.17–5.75	0.02

HR, hazard ratio.

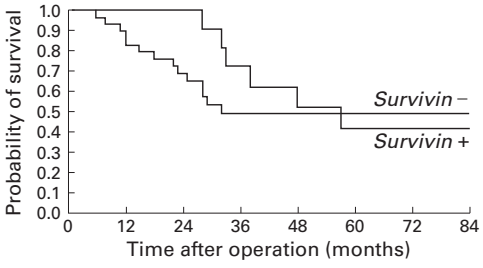
cer samples from an equivalent number of patients. The pathological staging of these cancers is listed in table 1. Stage I cancers comprised 14 colonic and eight rectal cancers. Stage II cancers comprised 35 colonic and 24 rectal cancers with 52 T3 and seven T4 tumours. Stage III cancers comprised 26 colonic and 19 rectal cancers with 40 T3 and five T4 tumours. *Survivin* mRNA was expressed in 89 (61.8%) CRC. Mean (SD) age of patients with *Survivin* positive and *Survivin* negative tumours was similar (70.2 (11.1) *v* 72.5 (10.1) years, respectively; p=0.40). The association of various clinical and pathological characteristics with *Survivin* mRNA expression is shown in table 1. *Survivin* positive tumours appeared more frequent in male than in female patients (p=0.05). The proportion of *Survivin* positive tumours was similar in the colon and rectum (p=0.48) and at advancing pathological stages (p=0.50). For patients who had undergone curative resections (stages I, II, and III) there was a significantly greater incidence of cancer related deaths associated with *Survivin* positive than with *Survivin* negative tumours (p=0.05).



No of patients at risk:

–	22	20	19	14	11	10	7	4
+	37	31	22	14	6	6	5	4

Figure 4 Kaplan-Meier survival plot for patients with curatively resected stage II colorectal carcinoma stratified according to tumour expression of Survivin. The five year survival rate of patients with Survivin positive tumours was 48.3% compared with that of 94.1% for patients with Survivin negative tumours (p=0.001, log rank test).



No of patients at risk:

–	14	14	12	8	5	4	4	4
+	31	25	18	11	10	8	5	4

Figure 5 Kaplan-Meier survival plot for patients with curatively resected stage III colorectal carcinoma stratified according to tumour expression of Survivin. The five year survival rate of patients with Survivin positive tumours was 49.0% compared with that of 43.4% for patients with Survivin negative tumours (p=0.66, log rank test).

SURVIVIN EXPRESSION AND PROGNOSIS

Actuarial survival analysis of the entire cohort of 144 CRC patients, stratified by pathological stage, confirmed that these patients displayed standard survival characteristics (p=0.01, log rank test for trend) (fig 2). The cumulative five year survival rate for patients with stage I disease was 92.3%; for stage II, 67.0%; and for stage III, 46.2%. All patients with stage IV disease died within two years of operation. Association between *Survivin* mRNA expression and survival characteristics of patients with stages I, II, and III CRC was examined next. The five year survival rate of patients with *Survivin* positive tumours was significantly lower compared with that of patients with *Survivin* negative tumours (53.0 *v* 77.5%, respectively; p=0.007) (fig 3). In this cohort of patients, the presence or absence of *Survivin* mRNA transcripts and lymph node metastases were the only two significant predictors of survival on univariate analysis. These factors were confirmed to retain their significance independently on multivariate analysis (table 2). As pathological staging is the conventionally accepted method for assessing prognosis of patients with CRC, it was decided to study the association of *Survivin* expression with survival characteristics of each stage individually. The presence of *Survivin* transcripts was associated with a significantly worse outcome only in the group with stage II CRC; patients with *Survivin* positive tumours displayed a five year survival rate of 48.3% compared with that of 94.1% for patients with *Survivin* negative tumours (p= 0.001) (fig 4). Patients with stage II *Survivin* positive CRC had a hazard ratio for death due to recurrent cancer of 11.2 (95% confidence interval 1.4–86.7; p=0.02) compared with those with *Survivin* negative tumours of the same stage. In contrast, expression of *Survivin* was not associated with significantly altered survival characteristics in patients with stage III CRC (five year survival rates of 49.0% and 43.4% for patients with *Survivin* positive and *Survivin* negative tumours, respectively; p=0.66) (fig 5). In the relatively small group with stage I disease, the only death occurred in a patient with a *Survivin* positive cancer.

Discussion

The aim of our study was to investigate expression of *Survivin*, which encodes a novel inhibitor of apoptosis,[11] in CRC. Expression of *Survivin* mRNA was detected by RT-PCR in 62% of CRC and was independent of advancing pathological stage. It has been previously demonstrated by in situ hybridisation analyses that *Survivin* mRNA is expressed only in the

cytoplasm of malignant cells, but not in surrounding stromal tissues, in colonic carcinomas.[11] Consequently, the present data are consistent with the hypothesis that *Survivin* plays a role in inhibiting apoptosis in malignant colonocytes in the majority of cancers in this series. These results are supported by a recent study demonstrating expression of Survivin protein by immunohistochemistry in 53% of CRC and significantly reduced apoptotic indices in Survivin positive tumours.[13] As opposed to immunohistochemistry, the PCR based approach used in our study is exquisitely sensitive and may detect gene transcripts even in a single cell or cell cluster with a comparable specificity.[18] Furthermore, the prevalence of *Survivin* mRNA expression in the present study correlates well with expression of the Survivin protein (unpublished data). Post-translational modifications, which might render the Survivin protein functionless, have not been reported and mRNA expression may, therefore, be taken to imply a functioning biological pathway. Similar arguments have been previously applied for use of mRNA detection in other gastrointestinal malignancies, such as gastric carcinoma.[19]

Approximately half the number of *Survivin* positive tumours, but none of the *Survivin* negative tumours, were associated with normal mucosa which also expressed this gene. These data appear at variance with previous studies which did not detect expression of either *Survivin* mRNA by in situ hybridisation[11] or Survivin protein by immunohistochemistry[13] in normal epithelium adjacent to CRC. Detection of mRNA transcripts by the more sensitive technique of RT-PCR[20] used in the present series suggests that *Survivin* expression may represent an "intermediate" biological change[21] identifying histologically normal mucosa at risk of neoplastic transformation. Histologically normal colorectal epithelial cells from patients with a history of colorectal carcinoma are subject to as yet unidentified influences that result in significantly reduced apoptotic activity compared with that in similar cells from patients with no neoplasia.[22] Comparative studies of apoptotic rates and *Survivin* expression are in progress to determine whether *Survivin* contributes to this phenomenon.

Expression of *Survivin* mRNA in the primary tumour was associated with a significantly greater risk of death due to recurrent cancer in patients with stage II CRC. Metastasis of cancer cells from the primary tumour, which ultimately results in recurrence of disease, involves detachment of cells from the matrix. This event triggers apoptosis in most adherent cells; however, cells that express antiapoptosis genes may survive and continue to divide.[23] These cells migrate and re-attach in secondary sites. Such micrometastases remain dormant when tumour cell proliferation is balanced by an equivalent rate of apoptosis but exhibit rapid growth when apoptosis is inhibited.[24] Apoptotic inhibition due to expression of *Survivin* may contribute to increased aggressiveness of *Survivin* positive stage II cancers. In contrast, patients with lymph node metastases (stage III CRC) have cancers which have undergone complex genetic alterations to generate the metastatic phenotype.[25] This may confer adverse biological properties which override those of *Survivin* expression.

Patients with stage II *Survivin* positive tumours had a five year survival rate similar to that of patients with stage III disease. In contrast, patients with stage II *Survivin* negative tumours displayed a very favourable five year survival rate similar to that of patients with stage I disease. In a recent study, expression of Survivin protein was not associated with significantly altered survival characteristics of the entire cohort of patients with stages I–IV disease[13]; however, stage wise survival analyses were not reported. This latter approach was adopted in the present study because patients with stage II CRC pose a dilemma regarding administration of adjuvant chemotherapy and, based on clinical and pathological criteria alone, it is difficult to identify those 20–30% patients who will go on to suffer recurrence.[26] Molecular markers which may be used to stratify patients with stage II disease into two separate groups whose prognosis and, by extension, indications for chemotherapy are significantly different have generated much interest. However, few molecular events, such as allelic deletion on chromosome 18q,[27] loss of expression of the deleted in colon cancer protein,[28] and Ki-*ras* mutations,[29] are of prognostic value specifically in stage II disease. Furthermore, conflicting results from similar studies has led to much confusion in the literature. For example, one recent report demonstrated that allelic deletion on chromosome 18q was associated with poor survival of patients with stage II CRC[30] while another study concluded that this phenomenon does not provide any prognostic information.[31] Consequently, it has been suggested that construction of composite genetic profiles of tumour tissues, with inclusion of several prognostic markers, may be a way forward.[32]

The present data provide a compelling case for inclusion of measures of *Survivin* expression in any such prognostic panel used for the purpose of selecting patients for administration of adjuvant chemotherapy.

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