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 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

Abbreviations used in this paper: CRC, colorectal carcinoma(s); mRNA, messenger RNA; RT-PCR, reverse transcription-polymerase chain reaction; HR, hazard ratio.
The 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 preserved in 10% formaldehyde solution. Pathological assessment was conducted according to the original guidelines of the United Kingdom Co-ordinating Committee on Cancer Research (UKCCR) 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 last 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 Cattrimox-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, (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 TAG AT (forward) at positions 2874–2853 in exon 1 and GCA TCT TCT TCG CAT TTT CC (reverse) at positions 11990–12009 in exon 4. A BLAST search was performed to confirm that these primers were specific for 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 (NH4)2SO4, 75 mmol/l Tris HCl, and 0.2 mmol/l of each dNTP in prediluted 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.
Survivin in colorectal carcinoma

†Patients with stages I, II, and III colorectal cancer.

**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% vs 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 cancer samples for analyses. A p value less than 0.05 was considered to indicate statistical significance.

**STATIONARY 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 χ² 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.
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cytoplasm of malignant cells, but not in surrounding stromal tissues, in colonic carcinomas. 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. As opposed to immunohistochemistry, the PCR based approach used in our study is excessively sensitive and may detect gene transcripts even in a single cell or cell cluster with a comparable specificity. 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.

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 or Survivin protein by immunohistochemistry in normal epithelium adjacent to CRC. Detection of mRNA transcripts by the more sensitive technique of RT-PCR used in the present series suggests that Survivin expression may represent an “intermediate” biological change 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. 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. 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. 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. 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. 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. 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, loss of expression of the deleted in colon cancer protein, and Ki-ras mutations, 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 while another study concluded that this phenomenon does not provide any prognostic information. Consequently, it has been suggested that construction of a composite genetic profiles of tumour tissues, with inclusion of several prognostic markers, may be a way forward.

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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