

Survivin Expression in Colorectal Adenocarcinoma Using Tissue Microarray

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ABSTRACT

Background: The additional prognostic information closely related to tumor cell biology is essential for the identification of patients with poor prognosis. Survivin, an identified inhibitor of apoptosis, is unique for its expression in human malignancies but not in normal adult cells. This study examined the expression, and potential prognostic value of survivin in colorectal adenocarcinoma (CRC) on tissue microarray (TMA) sections. Analysis of large numbers of tissue samples, improved tissue salvage, cost reduction, ease of interpretation, and significant time saving were realized by using the arrays.

Material and Methods: Two-hundred and eighty cases of colorectal adenocarcinoma were arrayed. Immunohistochemical stains of TMA sections were performed for survivin, bcl-2, and p53. Cases were followed up for 5 years.

Results: Survivin was detected in 147 of 230 cases (63.9%). No expression of survivin was observed in normal tissues. There was no correlation between survivin immunoreactivity and age, sex, tumor site, tumor size, histopathologic subtype, tumor grade and clinical stage ($p > 0.05$). Prevalence of survivin expression was significantly higher in bcl-2 positive than in bcl-2 negative cases (88.1% versus 42.1%, $p < 0.0001$), but was not associated with p53 ($p = 0.09$). The 5-year disease free survival (DFS) for patients with survivin positive colorectal adenocarcinoma was significantly lower than that for patients with survivin negative tumors (46% versus 68.7%, $p = 0.001$).

Conclusion: Survivin expression in colorectal adenocarcinoma provides an important prognostic parameter and targeted antagonists of survivin may be beneficial as apoptosis-based therapy for colon cancer.

Key Words: Survivin - Colorectal adenocarcinoma - Tissue microarray - Prognosis.

INTRODUCTION

Abnormalities in the control of programmed cell death (apoptosis) play an important role in tumorigenesis [1,2]. This process involves an

evolutionary preserved multistep cascade and is regulated by proteins that promote or counteract apoptotic cell death [3]. Bcl-2 was the first protein shown to lead to prolonged survival of cells by preventing apoptosis [4]. Several inhibitors of apoptosis (IAP) related to the baculovirus iap gene have been identified in humans, mice and *Drosophila* [5,6]. Highly evolutionarily conserved IAP proteins contain two to three cysteine/histidine rich [baculovirus IAP repeat (BIR)] motif and a COOH-terminal RING finger [4]. Recombinant expression of IAP proteins counteracted various forms of apoptosis in vivo [7] and in vitro [6]. These molecules are thought to block an evolutionarily conserved step in apoptosis. At least in the case of XIAP, this may involve direct inhibition of the terminal effectors caspase-3 and caspase-7 through a BIR-dependent recognition [8]. A novel gene encoding a structurally unique IAP apoptosis inhibitor, designated as survivin, has been identified by hybridization screening of a human P1 genomic library with the cDNA of effector cell protease receptor-1. It is located at chromosome 17q25, approximately 3% of the distance from the telomere [9], and it comprises three introns and four exons [10], encoding 16.5 kDa protein with 142 amino acids, including one copy of BIR and no RING finger [11,12].

Unlike bcl-2 [13] or other IAP proteins [5,6,7], survivin is expressed during embryonic and fetal development, becomes undetectable in normal adult tissues, and is prominently overexpressed in a variety of human cancers in vivo. Accordingly, a recent analysis of 3.5 million human transcriptomes identified survivin among the top 4 transcripts uniformly up-regulated in cancer but not in normal tissues [14]. At a mo-

lecular level, survivin is expressed during mitosis in a strict cell-cycle-dependent manner, and it localizes to mitotic spindle microtubules in a reaction required for apoptosis inhibition [15]. This pathway may provide a selective cytoprotective mechanism at cell division, because targeting endogenous survivin with antisense or a dominant negative mutant caused spontaneous apoptosis and a profound dysregulation of mitotic progression with supernumerary centrosomes, multipolar mitotic spindles, and generation of multinucleated cells [16].

In 1998, Kononen and colleagues [17] in the lab of Ollie Kallioniemi invented a mechanism for examining several histologic sections at one time by arraying them in a paraffin block. These tissue microarrays (TMA) are assembled by taking core needle biopsies of pre-existing paraffin-embedded tissues and re-embedding them in an arrayed master block. In this way, tissue from hundreds of specimens can be represented on a single paraffin block that can be analyzed using a variety of techniques including immunohistochemistry. In contrast to traditional techniques, which require the processing and staining of hundreds of slides, microarray technology enables the study of an entire cohort of cases by analyzing just one (or a few) master slide (s). Microarray analysis has the added advantage that all specimens are processed at one time using identical conditions. Furthermore, it markedly reduces the amount of archival tissue required for a particular study, thus preserving ample remaining tissue for other research or diagnostic needs [18]. TMA format also greatly increases the number of targets, that can be analyzed from the same set of tumors. Calculations indicate that tens of thousands of TMA sections can be generated from one paraffin block containing 10x10mm of tumor area with a depth of 3mm. This is hundreds of times more than could be accomplished using traditional techniques based on sectioning entire tumor blocks, where less than 200 sections can be generated before the blocks are exhausted [19].

By using TMA, this study was undertaken to investigate the value of survivin as a biologic parameter in colorectal adenocarcinoma (CRC) in order to refine our prognostic capabilities and to identify cohorts that need new treatment modalities.

MATERIAL AND METHODS

Tissue Samples:

Of 280 cases of primary colorectal adenocarcinoma diagnosed between 1995 and 2003 at the National Cancer Institute, Cairo University, Private Practice, and Tanta Cancer Center, 230 cases were evaluable after array construction. All patients underwent potentially curative tumor resection and none of them had received chemotherapy or radiotherapy before surgery.

Histological types and grading were reviewed and classified according to the World Health Organization classification criteria [20], and the disease stage was determined according to Dukes' staging system [21].

Array Construction:

Donor tissue blocks were histologically representative and at least 3mm thick. Hematoxylin and eosin (H&E) tissue sections of formalin-fixed paraffin-embedded tissue blocks were used as a guide to select the regions for sampling. TMA was assembled using a commercially available manual tissue puncher/arrayer (Beecher Instruments, Silver Spring, MD). First, a hole in the recipient TMA block was made, then a cylindrical 0.6mm core sample from the donor tissue block was obtained and deposited onto TMA block at a distance of 1mm between each core. Four cores were punched from each donor block to minimize the number of cases inevaluable due to tissue loss and to increase concordance rates among different cores, following previous reports on the value of multiple core analysis [22,23,24].

As a control, tissue cores taken from paraffin blocks of non-neoplastic mucosa of 10 patients undergoing surgery for non-malignant conditions were additionally arrayed on each block.

The large number of small disks & the value of each section have resulted in a refined strategy for cutting array sections. A tape-based tissue transfer system (Instrumedics, Hackensack) was used. An adhesive tape was placed on the face of the section before cutting. The tape was removed with the section on it and placed on special slides with adhesive-coated surfaces. The section was then ultravioletly cross-linked to the slide before the tape was removed with a special degreasing reagent included in the package. For each block, H & E slides were cut to verify tumor cell content (Fig. 1).

Immunohistochemistry:

Antibodies used were directed against survivin (FL-142; Santa Cruz Biotechnology, USA, dilution 1:100), bcl-2 (clone 124; Dako, Denmark, dilution 1:50) and p53 (D07; Dako, dilution 1:50). Ventana (Tucson, AZ, USA) automated immunohistochemical stainer was used according to the manufacturer's guidelines after microwave heating in citrate buffer (pH 6.0, 0.01mol/L concentration) to maximize antigen retrieval. Ventana/View DAB detection kit for streptavidin horseradish peroxidase was used.

Criteria for Marker Evaluation:

Immunoreactivity for bcl-2 and survivin expressed in the cytoplasm was quantified according to the classification of Sinicrope et al. [25] to evaluate the staining intensity and frequency of stained cells. The staining intensity was scored as follows: weak = 1+; moderate = 2+; intense = 3+. For frequency: 1 = 5-25%; 2 = 25-50%; 3 = 50-75%; 4 = >75%. The percentage of positive tumor cells and staining intensity were multiplied to produce a weighted score for each case. Tumors with a weighted score = 0 were designated as negative; all others were considered positive. Sections were scored as positive for p53 when >10% of tumor cells displayed nuclear immunostaining.

Statistical Analysis:

Disease free survival (DFS) was calculated as the interval between diagnosis and relapse, death, or last follow up. Follow-up duration ranged from 3 to 60 months with a median of 10 months.

The computer software StatView 5 (SAS Institute Inc., Cary, NC) was used for analysis. The relationship between survivin and clinicopathologic data, bcl-2, and p53 was analyzed using the chi-square and Fisher exact tests whenever appropriate. Disease free survival was determined using the Kaplan-Meier product limit method, and log-rank test was performed for comparison between survival curves. All *p* values were two tailed and a *p* value of ≤ 0.05 was considered significant [26,27].

RESULTS

Clinical and Pathological Characteristics:

The mean age patients was 47.4 years \pm 14 (range 18-83 years). One-hundred and fourteen

cases (49.6%) were males, and 116 cases (50.4%) were females. There were 113 (49.1%) colonic, and 117 (50.9%) rectal cancers. The mean tumor size was 7.3 \pm 2.7cm (range 2.5-16cm). One-hundred and eighty-three cases (79.6%) were of conventional adenocarcinoma (79.8% grade 2, and 20.2% grade 3), 40 cases (17.4%) were mucinous, and 7 cases (3%) were signet-ring adenocarcinoma. According to Dukes' staging system, 5 cases (2.2%) were of stage A, 116 cases (50.4%) stage B, 104 cases (45.2%) stage C, and 5 cases (2.2%) stage D.

Expression of Survivin:

Immunohistochemistry for survivin revealed cytoplasmic staining of colorectal carcinoma cells, few tumors showed nuclear staining (Fig. 2-A,B). One-hundred and forty-seven cases out of 230 (63.9%) were survivin positive. No expression of survivin was detected in normal control mucosa.

Relationship between Expression of Survivin and Clinicopathological Factors:

Table (1) shows the correlation between expression of survivin and clinicopathological factors. None of the parameters analyzed including age, gender, tumor site, tumor size, histological type, degree of differentiation, or clinical stage was significantly correlated with survivin expression.

Relationship between Expression of Survivin and bcl-2 or p53:

Among colorectal adenocarcinomas studied, 109 cases (47.4%) showed positive cytoplasmic immunoreactivity for bcl-2 (Fig. 3). Expression of survivin showed a significant correlation with expression of bcl-2, $p < 0.0001$ (Table 2). In contrast, nuclear accumulation of p53 was demonstrated in 133 of 230 cases (57.8%) (Fig. 4-A,B). There was no correlation between survivin and p53 expression ($p = 0.09$).

Prognostic Analysis:

Follow-up data were available for 120 patients, none of whom had died in the post-operative (60 days) period. Follow-up was taken to the end of 2003. Fig. (5) shows the Kaplan-Meier curves categorized according to survivin expression. The 5-year disease free survival for patients with survivin positive carcinomas (46%) was significantly lower than that for patients with survivin negative tumors (68.7%), $p = 0.001$.

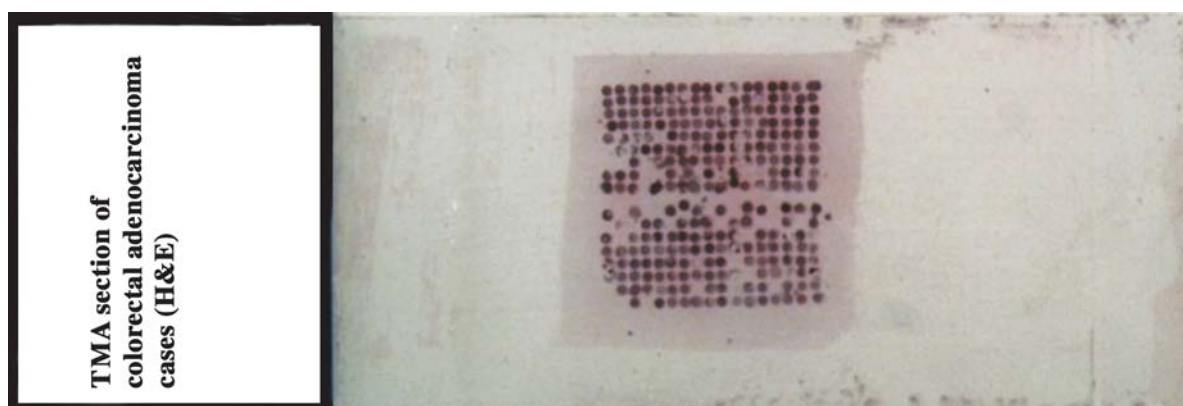


Fig. (1): Overview of TMA section containing samples of the tumor stained with H&E. Each core has a cross-sectioned diameter of 0.6mm.

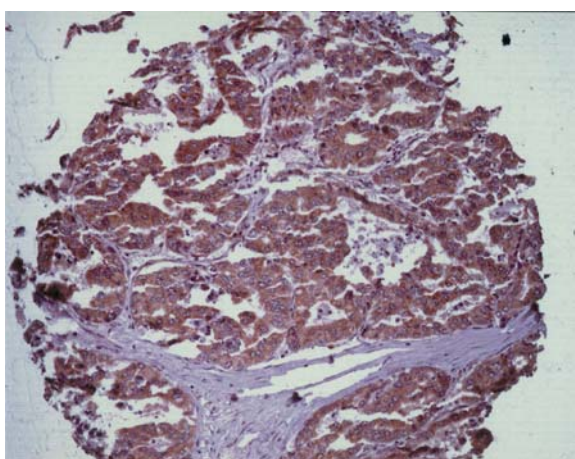


Fig. (2-A): TMA core of colonic adenocarcinoma grade 2 showing positive staining for survivin (x 200).

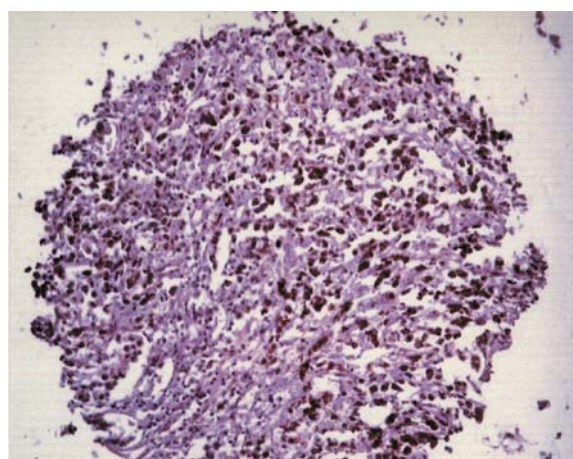


Fig. (2-B): TMA core of colonic signet-ring adenocarcinoma positive for survivin (x 200).

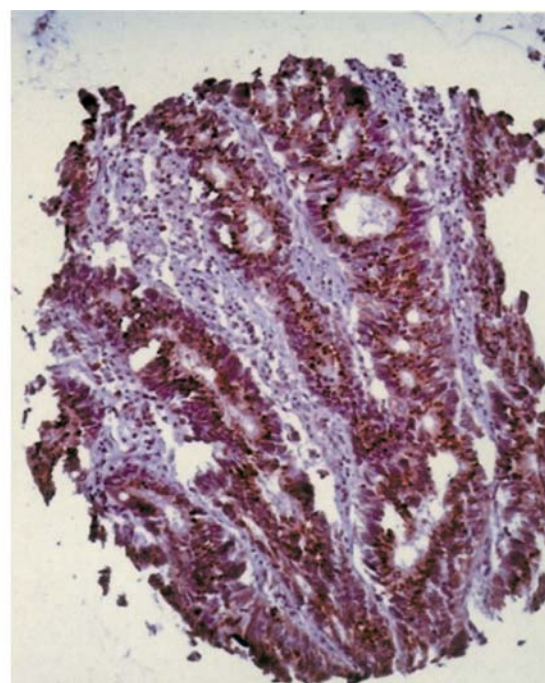
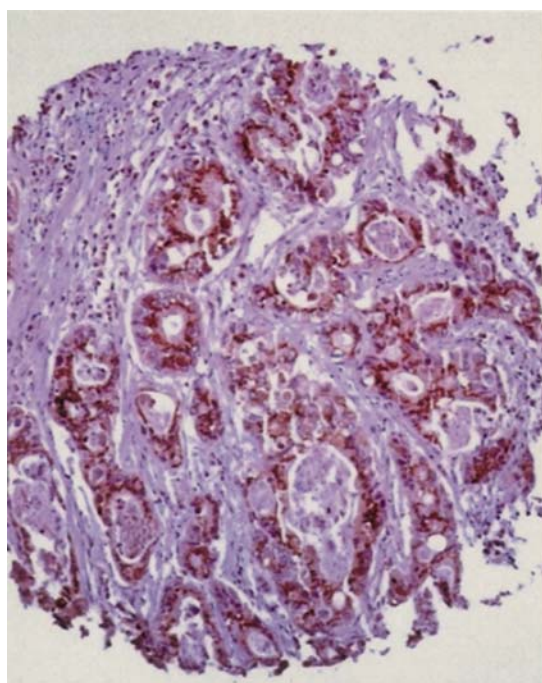


Fig. (3): Two TMA cores of rectal adenocarcinoma showing positive staining for bcl-2 (x 200).

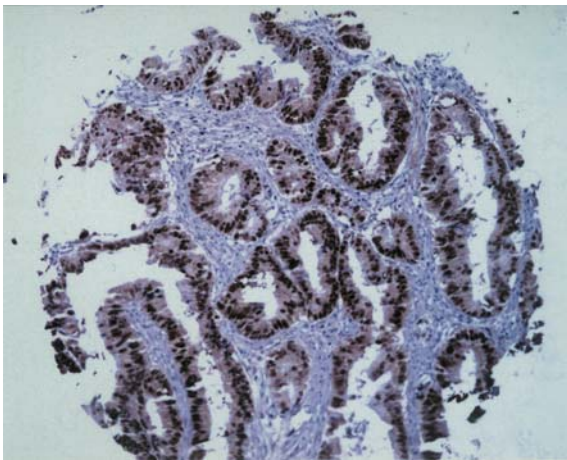


Fig. (4-A): TMA core of rectal adenocarcinoma grade 2 positive for p53 (x 200).

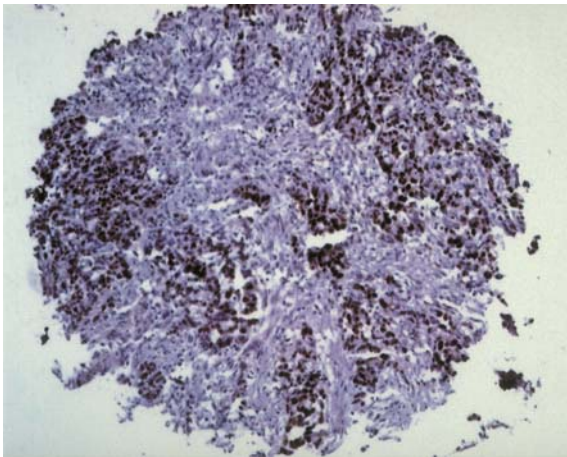


Fig. (4-B): TMA core of colonic adenocarcinoma grade 3 positive for p53 (x 200).

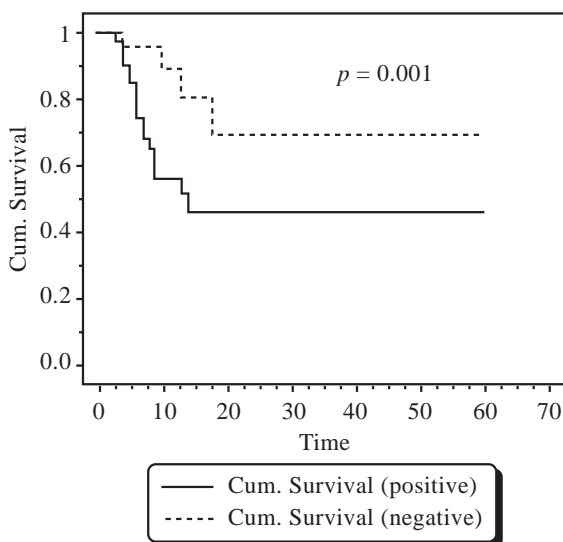


Fig. (5): Disease free survival of 120 cases of colorectal adenocarcinoma according to survivin expression.

Table (1): Survivin expression in relation to clinico-pathologic parameters.

	Survivin positive		Survivin negative		P value
	No.	%	No.	%	
<i>Mean age (years):</i>					
≤ 47.4	80	69.6	35	30.4	0.09
> 47.4	67	58.3	48	41.7	
<i>Sex:</i>					
Male	72	63.2	42	36.8	0.89
Female	75	64.7	41	35.3	
<i>Mean tumor site:</i>					
Colon	70	61.9	43	38.1	0.58
Rectum	77	65.8	40	34.2	
<i>Tumor size:</i>					
≤ 7.3	95	65.1	51	34.9	0.67
> 7.3	52	61.9	32	38.1	
<i>Histopathologic subtype:</i>					
Non-mucin producing	116	63.4	67	36.6	0.87
Mucin producing	31	66	16	34	
<i>Tumor grade:</i>					
Grade 2	94	64.4	52	35.6	0.57
Grade 3	22	59.5	15	40.5	
<i>Tumor stage:</i>					
Non-metastasizing	78	64.5	43	35.5	0.89
Metastasizing	69	63.3	40	36.7	

Table (2): Relationship between survivin expression, bcl-2 and p53.

	Survivin positive		Survivin negative		P value
	No.	%	No.	%	
<i>Bcl-2 expression:</i>					
Positive	96	88.1	13	11.9	< 0.0001
Negative	51	42.1	70	57.9	
<i>p53 expression:</i>					
Positive	79	59.4	54	40.6	0.09
Negative	68	70.1	29	29.9	

DISCUSSION

The tumor tissue array technique has been shown to be a powerful and rapid tool for the simultaneous analysis of hundreds of tumor specimens. A commonly expressed concern is whether the small core samples used in TMA analysis give meaningful information on large tumor specimens. One should keep in mind, however, that the basic principle of TMA analysis is fundamentally different from conventional histological analysis. This technology is a

population level research tool. It is not intended for making clinical diagnoses of individual cases.

To assess this criticism, consider the issue of representation in current practice. Sampling tissue at the rate of one section per cubic centimeter of tumor is generally considered the standard of care for minimal representative sampling of a lesion. It is exceedingly rare to find a study that has analyzed more than one section for protein expression, regardless of the initial tumor size. However, given a tumor 1cc^3 in volume, the standard 5 micron thick tissue section represents 0.05% of the tumor. With the current Beecher Instruments system for array construction, each disk of tissue represents about 0.3% of the tissue currently considered representative [28].

Some investigators have used core samples that are larger in diameter ($> 2\text{-}4\text{mm}$). This does not substantially increase the information content of TMA analysis, since the likelihood of finding heterogeneity within such a small area is often quite low [29]. In contrast, punching multiple small cores from different regions captures the heterogeneity of the tumors more effectively. Recent studies in fibroblastic tumors, prostate cancer, and bladder cancer have shown that the expression of Ki-67 by immunohistochemistry is highly correlated with the results in whole-slide preparations if triplicate or quadruplicate arrays are used [22,24,30]. Three studies on breast cancer report $> 90\text{-}95\%$ concordance when they directly compared biomarker expression such as estrogen, progesterone and HER-2 using TMA and regular sections [31-33].

Several technical issues apparently compensate for some loss of information due to the small tissue size. The staining of a single TMA slide provides a much greater degree of consistency and standardization than the immunostaining of hundreds of individual slides. Furthermore, quantitation of immunostaining is markedly easier on arrayed samples than on large sections. For example, it is possible to directly compare staining intensities of the different specimens on the same TMA slides, thereby improving the subjective interpretation of staining intensities. Most of all, the interpretation is by default, limited to a small predefined area in arrayed samples. This facilitates a reproducible application of the selected scoring cri-

teria because the entire tissue is always used for interpretation and the subjective selection of one tumor area for decision making is avoided [19].

Moreover, the impact of data discrepancies between array and full-section with regard to patient outcome was also evaluated. No significant change in clinicopathologic correlations could be detected between the two methods, indicating that TMA may be a reliable tool for high-throughput clinicopathological analysis of cancer specimens [22]. High numbers of tumors that can be included in a TMA study compensate for some false negative results.

There is a need for informative molecular markers that provide prognostic information over and above that given by conventional pathologic parameters in colorectal adenocarcinoma. Survivin, involved in proper duplication of the centrosomes during cell division and an inhibitor of apoptosis was investigated in this study and was detected by immunohistochemistry in 63.9% of the cases. Immunopositivity for survivin was observed in 53.2% of a series of colorectal cancers at stages I-IV in the study of Kawasaki et al. [34] and in 61.2% of stage II colorectal carcinomas by Sarela et al. [35]. A 95% concordance between survivin protein mRNA expression by RT-PCR and survivin protein expression by immunohistochemistry was reported [35]. These and results in this work suggest that survivin expression in colorectal adenocarcinoma is a common event and may play an important role in the oncogenesis of CRC.

Interestingly, immunohistochemical detection of survivin protein varies substantially in different human malignancies with only 34% of gastric carcinomas reported to be survivin positive [36], as compared with 78% of transitional cell carcinomas of the bladder [37] and 93% of melanomas [38]. This organ-dependent differential expression may be explained by intrinsic differences of tumor biology and higher affinity of the polyclonal antibody used in some studies as compared to its monoclonal counterpart.

In the current study, survivin was not expressed in normal control colorectal mucosa. These results are supported by a previous study [39]. Approximately half the number of survivin

positive tumors, but none of the survivin negative tumors, expressed survivin mRNA in mucosa adjacent to CRC in the series of Sarela et al. [40]. They suggested that survivin expression may represent an intermediate biological change identifying histologically normal mucosa at risk of neoplastic transformation.

There was no correlation between survivin expression and clinical or pathological characteristics of CRC. A similar absence of correlation has also been noted by Kawasaki et al. [34] and Sarela et al. [35], although survivin expression is associated with a histologically more aggressive phenotype of neuroblastomas [41] and transitional cell carcinomas of the bladder [37].

The presence of survivin was associated with expression of bcl-2. The results in this work agree with the findings of previous investigations [34]. The survivin gene is encoded at chromosome 17q25 [42], while the bcl-2 gene is located at chromosome 18q21 [13] which imply that other transcriptional factors may contribute to the coregulation of both gene products in the progression of cancer. In this context, both survivin and bcl-2 genes are regulated by TATA-less, GC-rich promoter sequence in a similar manner, and both are markedly transcribed in actively proliferating cell types [9], suggesting common mechanisms of transcriptional activation. However, regardless of the pathway of simultaneous coexpression, it appears that survivin and bcl-2 proteins may mediate non overlapping, antiapoptosis mechanisms. Although bcl-2 has been implicated in counteracting the upstream initiation of the caspase activation cascade by interfering with cytochrome C release from the mitochondria, IAP molecules, potentially including survivin, prevent apoptosis by targeting the terminal effectors caspase-3 and caspase-7 [43-45]. Survivin is expressed in the G2-M phase of the cell cycle in a cell cycle regulated manner and associates with microtubules of the mitotic spindle. Disruption of survivin-microtubule interactions results in loss of survivin antiapoptosis function and increased caspase-3 activity during mitosis. The overexpression of survivin in cancer may obliterate this apoptotic checkpoint and allow aberrant progression of transformed cells through mitosis [15].

In this work, no significant correlation between expression of survivin and p53. Although

survivin and p53 are both critical modulators of the opposing cellular processes of proliferation and apoptosis, there is currently no evidence of interaction between mechanisms regulating the expression of each of these proteins [46]. Apart from gastric and pancreatic carcinomas which show a strong positive correlation between survivin and p53 expression [36,47], these proteins appear to be expressed independently in human malignancies [37,41].

Survivin expression has been associated with increased aggressiveness and decreased patient survival in a number of different malignancies [40,41,48,49]. The survival analysis in this work revealed that survivin overexpression was significantly associated with worse outcome. Sarela et al. [40] found that expression of survivin mRNA was associated with greater risk of death due to recurrent cancer in patients with stage II CRC. Impaired apoptosis is recognized to enhance tumor progression and to promote metastasis by enabling tumor cells to survive transit in the blood stream and to grow in ectopic tissue sites lacking the otherwise essential survival factors [50]. Inhibition of tumor cell apoptosis due to expression of survivin may contribute to increased aggressiveness of such tumors resulting in a poor prognosis.

In a previous study, expression of survivin protein was not associated with significantly altered survival characteristics of an entire cohort of CRC patients with stages I-IV disease [34]; however, stage wise survival analyses were not reported. Survivin positive cancers did, however, display substantially lower apoptotic indices that correlated with a significantly shorter 5-year survival rates [34].

In conclusion, the expression of survivin in colorectal adenocarcinoma results in unfavourable clinical outcome, and therapeutic targeting of survivin may be beneficial in the management of this cancer.

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