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Protein expression of p53, p21 (WAF1/CIP1), bcl-2, Bax, cyclin D1 and pRb in human colon carcinomas

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Abstract Tumour growth is regulated by a balance between proliferation, growth arrest and programmed cell death (apoptosis). Until recently, the majority of the studies dealing with oncogenesis has been focused on the regulation of cell proliferation. There is now growing understanding that control of growth arrest and apoptosis play key roles in the development of human cancer and in cancer treatment. Some of the more heavily studied proteins of importance for the control of growth arrest and apoptosis are p53, p21, bcl-2 and bax. Alterations in the p53 protein may lead to malignant transformation and defect therapy response, most likely as a result of defective p53-dependent apoptosis. In addition, p21 (WAF1/CIP1) is involved in cell-cycle arrest and probably in induction of p53-dependent apoptosis. Proteins belonging to the bcl-2 family are also important for normal apoptosis. Overexpression of bcl-2 protein is thought to reduce the apoptotic capacity, while bax protein seems to be necessary for induction of apoptosis. In this study, we have immunostained tissues from 93 primary colon carcinomas and have examined the expression of p53, p21 (WAF1/CIP1), bcl-2 bax, pRb and cyclin D1 for evaluation of their roles in colon-cancer progression. A highly significant association between p53 accumulation and downregulation of p21 (WAF1/CIP1) was seen. We also found a strong association between reduced/absent p21 and the development of metastases and death due to cancer disease. Cyclin D1, bcl-2 and bax protein failed to have independent prognostic impacts. Bcl-2 and bax protein levels showed an inverse relationship. The results of the present study indicate that reduced p21 protein levels play an important role in progression of colon cancer. We concluded that evaluation of p21 expression in primary colon carcinomas at the time of surgery might be a valuable tool in defining patients with a high risk of developing metastases.

Key words Apoptosis · bax · bcl-2 · Colon carcinoma · CyclinD1 · Immunohistochemistry · p21 · p53 · pRb

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Introduction

Apoptosis of tumour cells is necessary and desirable when chemotherapy treatment is given, and resistance to apoptosis plays an important role in tumours that are refractory to chemotherapy and ionising radiation. Factors affecting the apoptotic function by any mechanism may also interfere with the prognosis of cancer patients.

Many of the genes involved in the induction and inhibition of apoptosis have been mapped [16]. Both p53-dependent and p53-independent pathways seem to be of importance [4]. One of the main functions of p53 after DNA damage is the induction of transcription of effector genes, such as p21 (WAF1/CIP1) [20]. p21 binds cyclin-dependent kinases in the G1 phase of cell cycle and inhibits their ability to phosphorylate other proteins like pRb (which is necessary for cell-cycle progression) [10]. The temporary growth arrest gives sufficient time for DNA repair prior to DNA synthesis. p53-Protein abnormalities (alone or in combination with defects in critical downstream effector proteins) can lead to altered cell-cycle growth arrest, deficient DNA repair and apoptosis.

Two other proteins that seem to play a significant role in apoptosis are bcl-2 and bax. Bcl-2 has been shown to block both p53-dependent and -independent cell-death pathways [6, 18], while bax increases the apoptotic capacity of the tumour cells [17]. Another family of proteins that interfere with cell survival and cell division are D-type cyclins. The D-type cyclins (D1, D2 and D3) are important in regulating the G1 checkpoint [11]. The human cyclin-D1 gene (CCND1) is located on chromosome band 11q13; the cyclin-D2 gene (CCND2) is on 12p13, and the cyclin-D3 gene (CCND3) is on 6p21 [12]. Cyclins D1, D2 and D3 promote progression

through the G1 phase of the cell cycle by regulating the activity of the cyclin-dependent protein kinases (CDKs) Cdk4 and Cdk6. In their activated forms, these kinases are capable of phosphorylating the retinoblastoma protein pRb, a critical target of G1 CDKs that is also thought to be of importance for the stability of D-cyclin kinases [23].

The role of these proteins in the evaluation of patient prognosis has been elucidated in some studies. Caffo et al. [3] demonstrated that the presence of p21 protein in tumour cells is a marker for chemotherapy response in a study of breast-cancer patients. In a previous study of breast-cancer patients, we demonstrated that overexpression of bcl-2 is associated with downregulation of p21 in tumours with normal p53 protein [1]. Recently, Zhan et al. [24] also showed that increased expression of bcl-2 in human Burkitts-lymphoma WMN cell line is capable of p21 suppression. Abnormal expression of the retinoblastoma protein (pRb) and cyclin D1 have been reported in a variety of malignancies, but their frequency and prognostic impact in colorectal cancer have been evaluated in only a few studies [15, 22].

In this study, we analysed the protein expression of p53, p21, cyclin D1 pRb, bcl-2 and bax in human colon carcinomas. Thus, the aim of the study was to explore apoptotic (p53, p21, bax) and anti-apoptotic (bcl-2, cyclin D1) potentials in colon-carcinoma patients.

Materials and methods

Material for this study was obtained from 93 patients with primary colon carcinoma admitted to Central Hospital of Akershus between 1988 and 1990. The mean age at diagnosis was 76.7 years (range: 45–89 years). Forty-three patients were classified as Dukes B, 30 as Dukes C and 20 as Dukes D. Mean observation time was 4.2 years (range: 0.5–5 years). Thirty-six (39.8%) patients had recurrence or distant metastases. Forty-one (44.1%) patients were dead of colon cancer and 20 (21.5%) of other causes. For 12 of the patients, the exact cause of death was not available.

All the tumours were classified as adenocarcinomas. One was well differentiated, 73 were moderately differentiated, and 18 were poorly differentiated. All samples included in this study were histologically evaluated and judged to contain more than 50% tumour tissue.

Four- to six-millimetre-thick sections of formalin-fixed, paraffin-embedded tumour tissue obtained at the time of surgery were placed on coated slides. After antigen retrieval by either the microwave technique or pressure heat, immunostaining was performed in an Optimax Plus Automated Cell Stainer model 1.5 (BioGenix, USA) according to the instructions in the operating manual. The antibodies used for the detection of different proteins and the sources and dilutions of the antibodies are shown in Table 1. The amounts of immunopositive cells were estimated semiquantitatively: grade "+" corresponds to 5–10%, grade "++" to 10–50%, and grade "+++" to more than 50% positive cells. All series included positive and negative controls. The results of control staining were satisfactory.

Statistical methods

Statistical analysis was performed using the χ^2 test. The level of statistical significance was defined as $P<0.05$.

Results

The immunostaining results are presented in Table 2. p53-Protein expression was detected in 72 tumours (77.4%; Fig. 1A). Sixteen tumours showed protein expression in 5–10% of the cells (+), three tumours showed protein expression in 10–50% of the cells (++) and 53 tumours showed protein expression in more than 50% of the cells (+++).

p21 (WAF1/CIP1) immunoreactivity was detected in 15 (16.1%) of the tumours (Fig. 1B). Only nine of these showed strong immunoreactivity (++/+++), and six were immunoreactive in 5–10% of the tumour cells (+).

When bcl-2 protein immunoreactivity was evaluated, we detected protein expression in 23 (24.7%) of the tumours (Fig. 1E). Ten of these 23 cases showed immunoreactivity in 5–10% of the cells, 11 in 10–50% of the cells and two in more than 50% of the cells.

Immunoreactivity for bax protein was detected in 87 (93.5%) of the tumour samples (Fig. 1F). The immunostaining was abundant in 80 of these (++/+++) while, in seven of the samples, bax-protein immunoreactivity was detected in 5–10% of the tumour cells (+). In some samples immunostaining was also seen in tumour cell nuclei.

Only eight samples (8.6%) showed immunoreactivity for cyclin D1 (Fig. 1C). Four of these were strongly immunoreactive (++/+++), while the other four showed immunoreactivity in only few of the tumour cells (+). Strong pRb-protein reactivity was detected in all samples (Fig. 1D).

Table 1 Different antibodies and dilutions

Antibody	Catalogue no.	Clone	Producer	Dilution	Pretreatment
E-cadherin	13-1700	HECD-1	Zymed	1:500	2×5-min microwave
α-Catenin	C21620	5	Transduction Laboratories	1:100	2×5-min microwave
β-Catenin	C19220	14	Transduction Laboratories	1:4000	2×5-min microwave
γ-Catenin	C26220	15	Transduction Laboratories	1:300	2×5-min microwave
Bcl-2	Code no. Mo887	124	DAKO	1:20	5×5-min microwave
Bax	13666E		Pharmingen	1:500	2-min pressure heat
WAF1	OP64	EA10 (3)	Oncogene Research Products	1:60	10-min pressure heat
p53 (DO1)	sc-126		Santa Cruze Biotec	1:800	2×5-min microwave
Cyclin D1	CC12	DCS-6	Oncogene Research Products	1:300	2×5-min microwave
pRb (C-15)	sc-50		Santa Cruz Biotec	1:700	2×5-min microwave

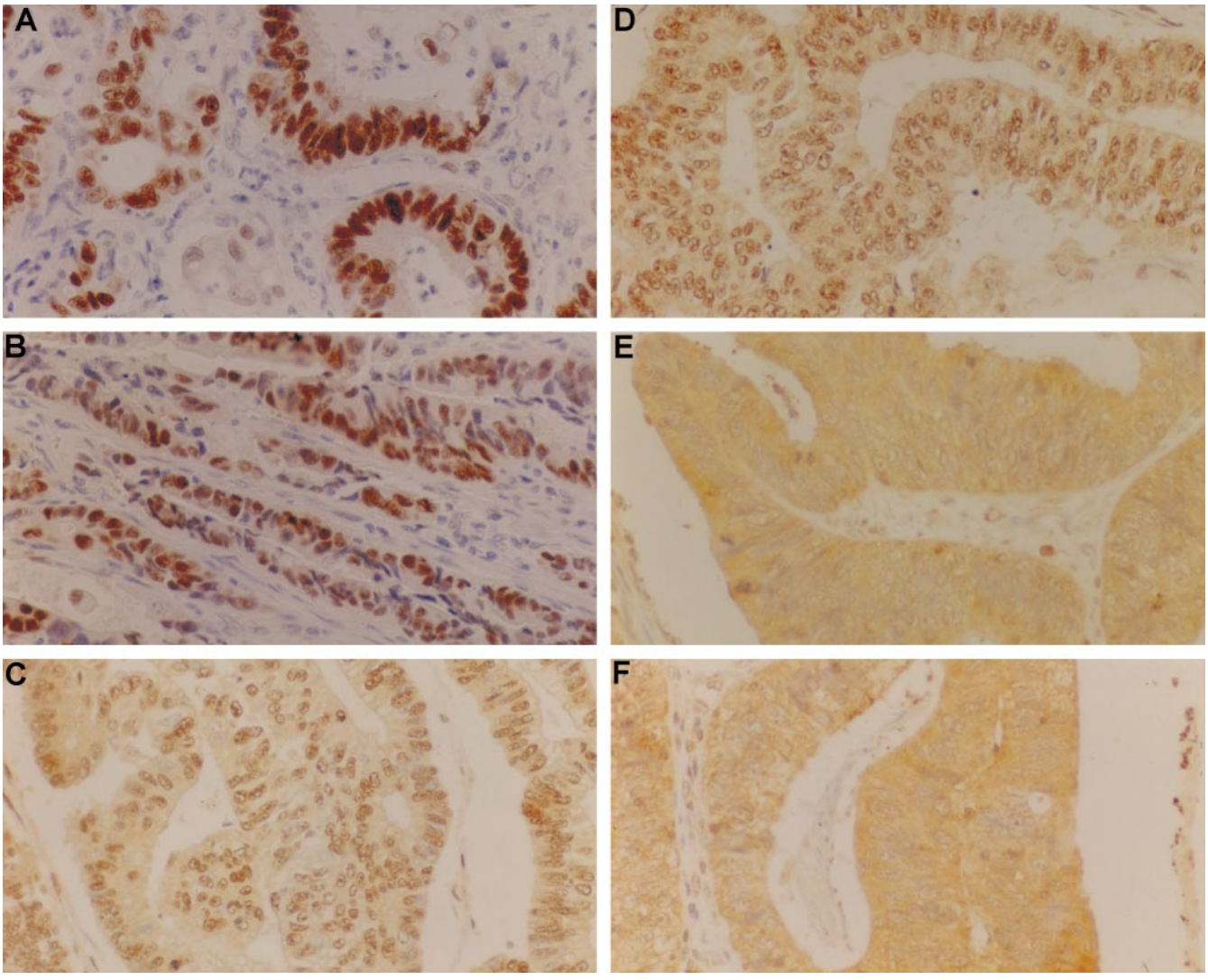

Fig. 1 Tumour tissue from adenocarcinomas of the colon showing immunoreactivity for various proteins. **A** p53-Protein immunoreactivity was seen in cell nuclei. **B** p21-Protein immunoreactivity. When positive, p21 was detected in tumour cell nuclei. **C** Cyclin D1 and pRb (**D**) immunoreactivity was seen in the nuclei of the tumour cells. **E** bcl-2-Protein expression was detected in the cytoplasm. **F** bax Protein was detected in the cytoplasm. In one case, nuclear staining was seen

Table 2 Protein accumulation of p53 relative to other proteins and clinico-pathological parameters. + 5–10% immunopositive cells; ++ 10–50% immunopositive cells; +++ more than 50% immunopositive cells

Protein or clinico-pathological parameter		Number of patients exhibiting p53-protein accumulation		*P* value
		0/+	++/+++	
p21	0/+	29	55	–
	++/+++	8	1	*P*<0.001
Bcl-2	0/+	30	50	–
	++/+++	7	6	Not significant
Bax	0/+	7	6	–
	++/+++	30	50	Not significant
Cyclin D1	0	33	51	–
	+	4	5	Not significant
Dukes stadium	A+B	18	25	–
	C+D	18	31	Not significant
Distant metastases?	No	22	34	–
	Yes	15	22	Not significant
Patient alive?	No	14	27	–
	Yes[a]	11	9	Not significant

[a] Only patients who died of cancer disease

Table 3 Protein expression of p21 (WAF1) relative to clinico-pathological parameters. + 5–10% immunopositive cells; ++ 10–50% immunopositive cells; +++ more than 50% immunopositive cells

Clinico-pathological parameter		Number of patients exhibiting p21-protein expression		*P* value
		0/+	++/+++	
Dukes stadium	A+B	36	8	
	C+D	48	1	$P<0.001$
Distant metastases	No	47	9	
	Yes	37	0	$P<0.001$
Patient alive?	No	41	0	
	Yes[a]	18	2	$P<0.001$

[a] Only patients who died of cancer disease

When comparing p53 protein accumulation with p21 protein expression, a significant association was found between high p53 protein accumulation and downregulation of p21 expression ($P<0.001$). In the group with no or low p53-protein accumulation, eight samples showed strong p21 expression, compared with only one in the group with high p53-protein expression (Table 3). No association was seen between p53-protein accumulation and Dukes classification, relapse or colon-cancer-related death.

However, when we analysed expression of p21, we found a significant association between p21 expression and Dukes stadium, metastases and survival. All patients with later development of metastases had low or no expression of p21 in their primary tumours. Bcl-2 and bax protein did not show any correlation to clinical parameters (Dukes stadium, relapse and cancer death). An inverse relationship between bcl-2 protein expression and expression of bax protein was seen.

When Dukes stadium B and C were compared with development of metastases during the follow-up period, no difference between these groups was seen. The relationship between p53 and p21 and different parameters is shown in Table 2 and Table 3.

Discussion

p21 Is a crucial protein in cell-cycle control and is one of the downstream effector proteins of p53. p21 Is not directly involved in the DNA-repair process but is believed to be involved in proliferating-cell-nuclear-protein redistribution, suggesting an indirect role of p21 in DNA repair. The most important function of p21 is probably inhibition of protein kinases known as cyclin-dependent kinases, causing growth arrest during G1 of the cell cycle. This growth arrest may be an important signal for the apoptotic process. Impaired p21-protein expression leading to defective growth arrest of the dividing cell may cause impaired apoptosis.

In this study, a strong association was seen between p53 accumulation and p21 (WAF1/CIP1) protein expression; highly significant correlations between p21 (WAF1/CIP1) protein expression and Dukes stadium, the development of metastases and death from cancer in colon carcinoma patients were also seen. An association between p53 protein and p21 was demonstrated previously in experimental systems [13, 21] and in tumour tissue samples [2, 5, 7, 8, 9]. However, the relationship between p53 accumulation and p21 and the prognostic value of p21 immunostaining of colon carcinoma tissue have not been investigated. Despite this, we found a strong association between p21 downregulation and p53 protein accumulation and we had some cases with no expression of p21 and without detectable p53 protein. This is most likely due to a silent mutation (producing no protein) in the *TP53* gene or due to an alteration in p21 protein itself.

Cyclin-D1 overexpression does not seem to play any significant role in colon cancer tumorigenesis. Only eight samples revealed protein expression, which is lower than the portion showing protein expression in a study by Palmqvist et al. [14, 15]. Palmqvist and co-workers observed nuclear expression of cyclin D1 in 12% of the cases. The different results may be caused by different compositions of the patient groups.

Bcl-2 and bax-protein expressions were not associated with any prognostic effects in our study. However, Sinicrope et al. [19] demonstrated that overexpression of bcl-2 oncoprotein is associated with a favourable prognosis in stage-II colon-cancer patients.

In summary, in this study, we found a strong association between downregulation of p21-protein expression and the development of metastases and cancer death in patients with colon cancer. Bcl-2, bax and p53-protein accumulation were not of prognostic value.

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