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Prognostic significance of p53 and c-erbB-2 immunohistochemical evaluation in colorectal adenocarcinoma

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Summary. Mutant p53 tumour suppressor gene and c-erbB-2 proto-oncogene are involved in human carcinogenesis, and their protein product detection in human malignancies might influence the evolution of many neoplasms. Our aim was to estimate their association with histopathological and clinical parameters of prognostic value in colorectal cancer.

An immunohistochemical assay was undertaken in formalin-fixed sections from tissue specimens of 60 colorectal carcinomas. Nuclear p53 expression was detected in 46.6%, while membranic c-erbB-2 positivity was noticed in 35% of the examined cases. P53 positivity rate significantly correlated with poor differentiation ($p<0.001$), high mitotic activity ($p<0.0001$), tumour stage ($p<0.001$) and 5-year overall survival period ($p<0.01$). C-erbB-2 positivity incidence significantly correlated with advanced Dukes' stage ($p<0.001$) and high mitotic activity ($p<0.05$). Significant association between p53 and c-erbB-2 immunostaining was observed ($p<0.05$) and p53/c-erbB-2 co-expression was related to poor differentiation ($p<0.001$), high mitotic activity ($p<0.001$), advanced Dukes' stage ($p<0.001$), tumour aneuploidy ($p<0.05$) and worse overall survival ($p<0.05$).

P53 and c-erbB-2 immunohistochemical detection in combination with known prognostic indicators may be a useful future tool in determining colorectal cancer prognosis and subsequently in deciding on optimal postoperative treatments.

Key words: p53, c-erbB-2, Colorectal cancer, Survival

Introduction

Among noncutaneous malignant lesions, colorectal cancer is second in incidence after carcinoma of the lung in many Western countries (Trock et al., 1990; Bring et al., 1992). Despite advances in chemotherapy, radiation therapy and surgery, there has been little change in the survival of patients with colorectal carcinoma during the past three decades.

Recent progress in the genetic analysis of human cancer contributed valuable information to our understanding of the mechanisms of carcinogenesis. Two classes of genes that are involved in the neoplastic process have been studied in detail; oncogenes and tumour suppressor genes (Bishop, 1987; Boss, 1989; Cohen and Ellwein, 1991; Harris, 1991). The p53 gene is a tumour suppressor gene mapped in chromosome 17p and is involved in a wide range of human malignancies (Hollstein et al., 1991). This gene encodes a 375-aminoacid nuclear phosphoprotein which is involved in the regulation of cell proliferation (Chang et al., 1993). The wild-type p53 acts as a tumour suppressor gene in normal cells and is lost or inactivated during the development of many neoplasms. Loss of tumour suppressor function requires inactivation of both alleles usually by chromosomal deletion or point mutations, or both (Chang et al., 1993; Harris and Hollstein, 1993). Abnormal expression of this gene leads to the accumulation of mutant proteins in the cell nucleus which can be detected immunohistochemically. Additionally, mutation of p53 gene is a common occurrence associated with allele loss on chromosome 17 in colorectal carcinomas (Campo et al., 1991; Kawasaki et al., 1992; Starzynska et al., 1992; Khine et al., 1994).

On the other hand, changes in structure and expression of oncogenes have increasingly been shown to be involved in both onset and progression of tumours (Spandidos and Anderson, 1987). The proto-oncogene c-erbB-2 (also termed HER 2) is the human homologous of the rat neu oncogene and has been isolated from human cells and mapped on chromosome 17 at q21 (Semba et al., 1985; Fukushige et al., 1986). It encodes a 185-Kilodalton (KD) protein with tyrosine kinase

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Table 1. Relation between p53 expression and examined parameters.

PARAMETERS	No. OF CASES	p53 POSITIVE CASES	p53 NEGATIVE CASES	P VALUE CONCERNING p53 EXPRESSION
Sex				
Male	35	13	22	p>0.10
Female	25	15	10	
Degree of differentiation				
Well differentiated	12	6	6	p>0.10
Moderately differentiated	38	17	21	
Poorly differentiated	10	5	5	
Mitotic activity				
Group I	36	13	23	p<0.001
Group II	18	11	7	
Group III	6	4	2	
Dukes' stage				
A	16	3	13	p<0.001
B	16	5	11	
C	21	15	6	
D	7	5	2	
Tumour ploidy				
ND	38	13	25	p<0.05
AN	22	15	7	
c-erbB-2				
Positive	21	14	7	p<0.05
Negative	39	14	25	
Patients survival				
Alive	29	7	22	p<0.01
Dead	31	21	10	

activity that may share homology with, but is distinct from the epidermal growth factor receptor (Schechter et al., 1985; Akiyama et al., 1986; Yamamoto et al., 1986). Amplification of the c-erbB-2 gene is frequently found in adenocarcinomas (Yokota et al., 1986) and association of gene amplification with immunohistological protein expression has been demonstrated (Slamon et al., 1987; Tsuda et al., 1989).

Our aim in this study was to examine the relationship of p53 protein overexpression and c-erbB-2 gene product expression and especially both oncoprotein synchronous detection with pathological tumour variables and patient survival in a well documented series of colorectal adenocarcinomas.

Materials and methods

Sixty patients with colorectal cancer were included in this study. The mean age of the patients was 68.75 years (range: 25-85 years, mean age±SD: 68.75±14.76, median age: 69) and the male/female ratio was 35/25 (1.4). Most adenocarcinomas of our survey were located in rectum (n=29) and sigmoid (n=10) (48 and 17% of the cases, respectively). All patients received conventional surgical management for colorectal cancer at the Departments of Surgery in Hippokration General Hospital between August 1988 and March 1989. Collection of data was accomplished from hospital records including age, sex, tumour size [max. dia <3.5 cm (n=7), 3.5-5 cm (n=17) and >5 cm (n=36)], tumour location, mitotic activity, degree of differentiation, Dukes' stage and a sixty-months follow-up period. The colorectal cancer was classified as adenocarcinoma in all cases with no signs of any neuroendocrine differentiation. The mean number of mitoses in at least 10 high power fields (x 4000) and the ploidy of each tumour, were also taken into account as malignancy markers, during pathological and flow cytometry assessment. The tumours were classified into three groups according to their mitotic activity. Group 1: 0-3 mitoses per high power field (x 400), group 2: 4-6 and group 3: >6 mitoses. A flow cytometric analysis was undertaken in all cases according to Hedley's classical technique (Hedley et al., 1983) and the tumours were characterized as either near diploid (ND) or aneuploid (AN) according to histogram type (Histogram type 1 for ND tumors and Histogram type 2 for aneuploid and/or tetraploid tumours).

A three-step immunoperoxidase staining technique was used on paraffin-embedded 4 μm thick tissue sections from primary colorectal tumours and adjacent uninvolved mucosa. After deparaffinization through graded alcohols, endogenous peroxidase activity was blocked by incubating the slides in 0.1% hydrogen peroxide in methanol for 20 minutes. Immunostaining was performed using the Avidin-Biotinylated Horseradish Peroxidase (ABC-HRP) method (Dakopatts, Denmark). As primary antibodies we used the Pab 1801 monoclonal antibody (Oncogene Science) at a dilution of 1/30 for mutant p53 protein detection and the Dakopatts Rabbit Anti-Human c-erbB-2 oncoprotein Ab at a dilution of 1/100 for c-erbB-2 detection, with an

overnight incubation. Diaminobenzidine tetrahydrochloride 0.06% in PBS buffer containing 0.03% hydrogen peroxide was used as a chromogen. Tumour sections subjected to the whole procedure except for incubation with the primary antibody were used as negative controls. Previously positive breast cancer tissue sections for p53 and c-erbB-2 were used as positive controls.

All immunostained slides were analysed and scored in blind fashion by two different observers without knowledge of stage or survival data. In each section at least twenty high power fields (x400) were examined under light microscopy and the mean percentage of p53 positive and c-erbB-2 positive neoplastic cells was separately calculated among all malignant cells. Staining intensity was also taken into account and it was subjectively graded as low (+), moderate (++) and intense (+++). As in several of our reference studies, cases with p53 cancer cell percentages lower than 10% were considered as p53 negative and those with c-erbB-2 percentages lower than 10% were considered as c-erbB-2 negative. Nuclear staining and membranic reactivity were considered as positive immunostaining for p53 and c-erbB-2 respectively.

Statistical analysis was undertaken using Student's test and chi-squared test for association (Yates' correction factor). All the results were considered at the 5% level of statistical significance.

Results

Analytical data and statistics concerning our sample are given in Tables 1-3. Of the 60 tumours evaluated for p53 expression, 28 (46.5%) were immunohistochemically positive for p53. C-erbB-2 was overexpressed in 21 out of the 60 carcinomas evaluated for this proto-oncogene product (35%). Only nuclear staining of cells was considered positive for p53 detection (Fig. 1), while positive membranic immunoreactivity was taken into account in order to define c-erbB-2 positivity (Fig. 2). Staining intensity grade was generally analogous to the percentage of immunostained

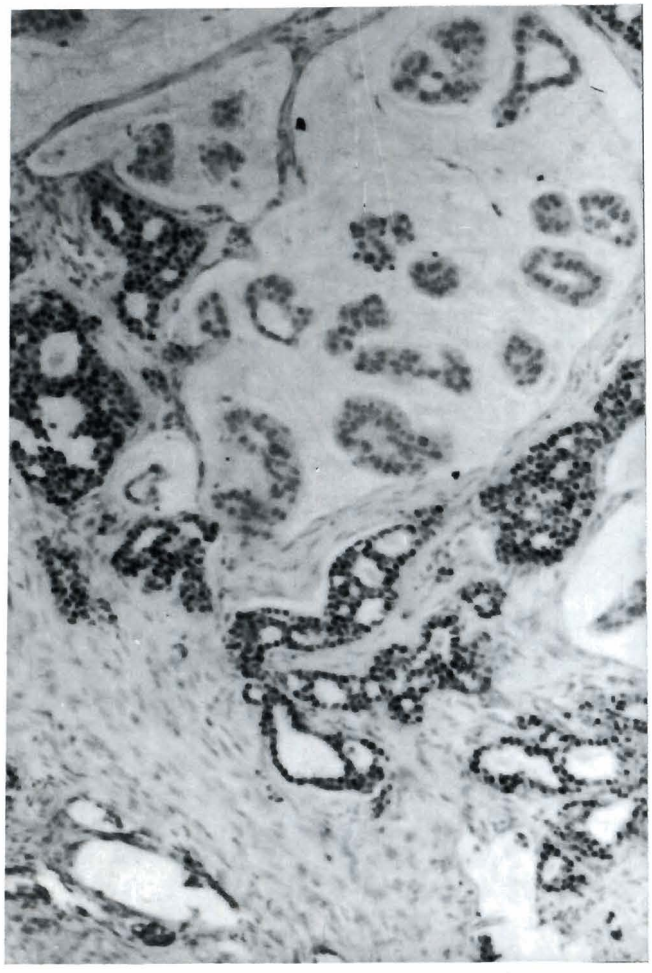

Fig. 1. p53 nuclear positive expression in a colon cancer. ABC-HRP, x 150

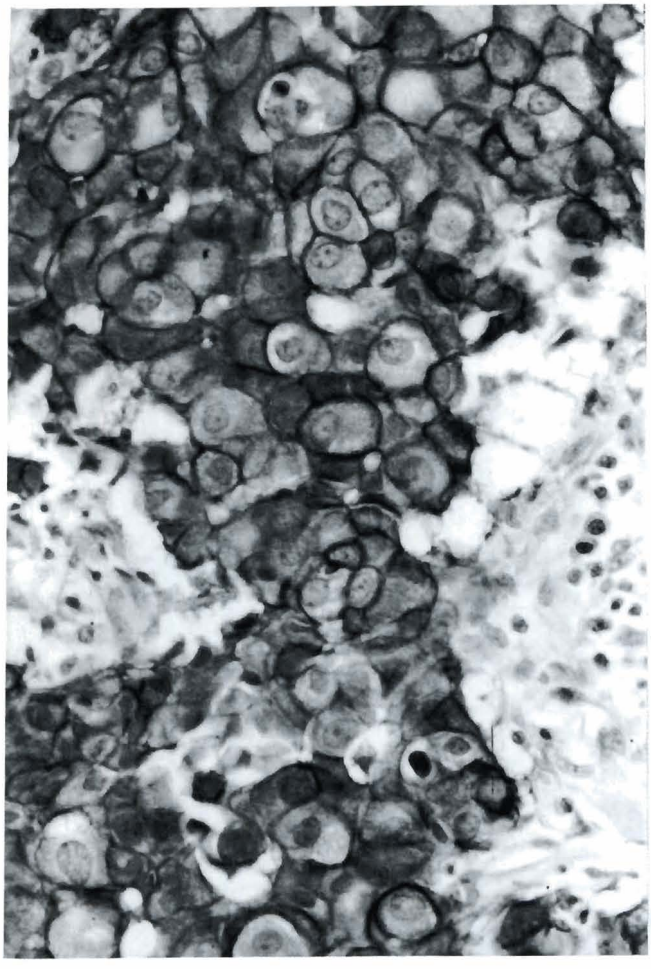

Fig. 2. C-erbB-2 membranic immunostaining in a case of a poorly differentiated rectum adenocarcinoma. ABC-HRP, x 300

Table 2. Relation between c-erbB-2 expression and examined parameters.

PARAMETERS	No. OF CASES	c-erbB-2 POSITIVE CASES	c-erbB-2 NEGATIVE CASES	P VALUE CONCERNING c-erbB-2 EXPRESSION
Sex				
Male	35	14	21	p>0.10
Female	25	7	18	
Degree of differentiation				
Well differentiated	12	4	8	p>0.10
Moderately differentiated	38	13	25	
Poorly differentiated	10	4	6	
Mitotic activity				
Group I	36	11	25	p<0.05
Group II	18	7	11	
Group III	6	3	3	
Dukes' stage				
A	16	4	12	p<0.001
B	16	3	13	
C	21	9	12	
D	7	5	2	
Tumour ploidy				
ND	38	11	27	p>0.10
AN	22	10	12	
Patients survival				
Alive	29	8	21	p>0.10
Dead	31	13	18	

cells; thus, cases with high percentages of immunopositive neoplastic cells (40-70% of the neoplastic cells showing nuclear immunoreactivity) demonstrated intense (+++) staining and those with lower percentages (10-40%) demonstrated low (+) or moderate (++) staining intensity. Stromal cells and adjacent uninvolved non-cancerous mucosa showed neither p53 nor c-erbB-2 expression. Inflammatory cell infiltrates were observed around most p53 positive tumors. By flow cytometric analysis, 38 tumours (63.3%) were proved to be ND (Fig. 3), whereas 22/60 (36.6%) were characterized as AN (Fig. 4).

p53 oncoprotein was overexpressed in 3/16 (18.75%) of Dukes' stage A carcinomas, 5/16 (41.6%) of stage B, 15/21 (71.4%) of C and 5/7 (71.4%) of D (Table 1). A tendency for increased p53 positive neoplastic cells percentages with advancing tumour stage was observed. There was a statistically significant

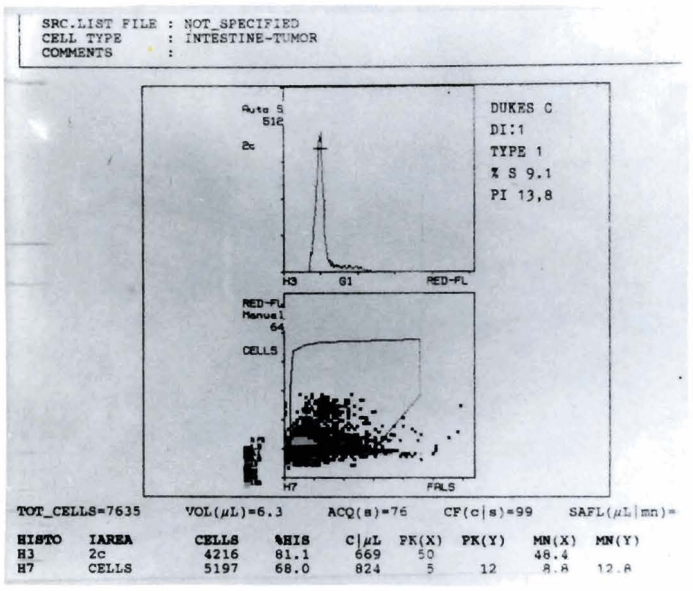

Fig. 3. Histogram of a typical diploid colon cancer (Type 1 Histogram).

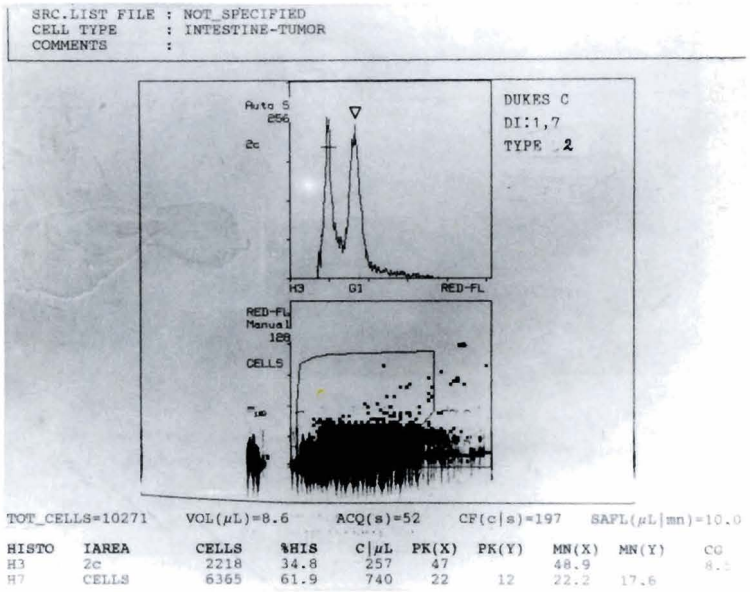

Fig. 4. Type 2 histogram of a neoplasm with a distinct aneuploid population.

Table 3. Relation between p53/c-erbB-2 expression and examined parameters.

PARAMETERS	No. OF CASES	p53/c-erbB-2 POSITIVE CASES	ALL OTHER CASES	P VALUE CONCERNING P53/c-erbB-2 EXPRESSION
Sex				
Male	35	8	27	p>0.10
Female	25	6	19	
Degree of differentiation				
Well differentiated	12	1	11	p<0.01
Moderately differentiated	38	10	28	
Poorly differentiated	10	3	7	
Mitotic activity				
Group I	36	5	31	p<0.001
Group II	18	6	12	
Group III	6	3	3	
Dukes' stage				
A	16	2	14	p<0.001
B	16	2	14	
C	21	5	16	
D	7	5	2	
Tumour ploidy				
ND	38	5	33	p<0.05
AN	22	9	13	
Patients survival				
Alive	29	8	21	p>0.10
Dead	31	13	18	

association between p53 protein expression and high mitotic activity ($p<0.001$), tumour stage ($p<0.001$), tumour aneuploidy ($p<0.05$) and worse 5-year overall survival period ($p<0.01$) (Table 1). Moreover, patients with p53 positive carcinomas had a significantly shorter mean survival period in months [mean±SEM ($\bar{x}$±SEM: 31.2±3.95, 95% confidence interval (95% CI): 23.3-39.1] in comparison with p53 negative ones ($\bar{x}$±SEM: 53.9±1.84, 95% CI: 50.2-57.6) (t value: 5.86, $p<0.01$). In addition, patients with Dukes' stage B and C colorectal adenocarcinomas had a longer mean survival period, if they were stained p53 negative ($\bar{x}$±SEM: 54.5±2.47, 95% CI: 49.55-59.45 and $\bar{x}$±SEM: 48.33±4.02, 95% CI: 40.28-56.38, respectively for B and C stages), compared to the p53 positive ones ($\bar{x}$±SEM: 46.2±19.56, 95% CI: 7.14-85.26 and $\bar{x}$±SEM: 28.4±5.52, 95% CI: 17.36-39.4 respectively for B and C stages); however, these differences were not statistically significant.

C-erbB-2 overexpression was observed in 4/16 (25%) of Dukes' stage A tumours, 5/16 (41.6%) of stage B, 9/21 (42.8%) of C and 5/7 (71.4%) of D (Table 2). C-erbB-2 positivity incidence was significantly correlated only with advanced Dukes' stage ($p<0.001$) and high mitotic activity ($p<0.05$).

p53 expression was associated with c-erbB-2 protein overexpression ($p<0.05$) (Table 1). Concomitant p53 and c-erbB-2 positive expression was evident in 14 out of the 60 cases (23.3%) (Table 3). p53 and c-erbB-2 overexpression was clearly associated with advanced Dukes' stage ($p<0.001$), poor differentiation ($p<0.01$), high mitotic activity ($p<0.001$), DNA aneuploidy ($p<0.05$) and overall survival ($p<0.05$) (Table 3).

Finally, let us point out that none of the examined oncoproteins significantly correlated either with tumour size, patients' ages or with tumour location (rectal or intra-abdominal colon cancer).

Discussion

Normal wild-type p53 gene coding for a nuclear phosphoprotein is critically related to cell-cycle regulation (Chang et al., 1993; Harris and Hollstein, 1993). This nuclear oncoprotein is involved in the negative regulation of cell growth by inhibiting the cell-cycle progression and suppressing the initiation of DNA replication (Barkett et al., 1991). Little is known about the exact function of p53 protein, but recent evidence suggests that its inactivation following either a missense mutation in p53 gene or the allelic loss of the gene is highly involved in the deregulation of cell growth and tumorigenesis (Chang et al., 1993; Harris and Hollstein, 1993). The wild type p53 does not accumulate in amounts detectable by immunohistochemistry due to a short half-life (6-20 min), whereas the mutant p53 protein in tumours is detected in increased concentrations because of its longer half-life (6 h) (Levine et al., 1991). Moreover, DNA sequencing techniques have already demonstrated that mutations are present in cases with overexpressed p53 oncoprotein (Chang et al., 1993; Harris and Hollstein, 1993). Therefore, an immunohistochemically detectable protein usually means mutation; thus, the nuclear detection of p53 overexpression can be used as an indirect indication of p53 mutations.

Gusterson et al. (1988) have demonstrated that c-erbB-2 membrane staining in breast cancer is generally due to the oncogene amplification. Other authors demonstrated that c-erbB-2 overexpression is not so commonly associated with gene amplification in stomach cancer (Jain et al., 1991). There is speculation that expression of the oncogene product may be a more accurate predictor of tumour behaviour than gene copy number alone (Tandon et al., 1989). From this point of view immunohistochemical techniques are complementary to molecular genetic approaches as morphological information is preserved and malignant cells are studied in their appropriate morphological context.

Our results confirm the relevantly high frequency of p53 overexpression in colorectal cancer and the negative immunostaining in non-malignant tissue, which are in line with previous reports (Campo et al., 1991; Scott et al., 1991; Kawasaki et al., 1992; Starzynska et al., 1992). Moreover, recent studies have demonstrated the presence of c-erbB-2 gene product in colon cancer cells (D'Emilia et al., 1989; Barket et al., 1990; Natali et al., 1990; Amaout et al., 1992). Interesting findings were the homogeneous diffuse pattern of p53 and c-erbB-2 immunoreactivity observed thoughout the neoplasm in most positive tumours, and the tendency for increased percentages of p53 positive cells with the progression of tumour stage. These data suggest that p53 and c-erbB-2 alterations in carcinoma cells might provide the cells with a certain growth advantage. Additionally, recent evidence points to clonal expansion of mutant p53 gene containing cells in the progression of neoplastic disease, implying that tumour cells which show p53 gene mutations may have a selective growth advantage in contrast with tumour cells without p53 mutations (Sidransky et al., 1992).

Inflammatory cell infiltrates were occasionally observed around p53 positive tumours, which may be related to a possible immunological reaction against the p53 positive rapidly proliferating tumours (Harris and Hollstein, 1993).

Let us point out the finding that neither p53 nor c-erbB-2 expression was detected in the mucosa adjacent to carcinoma. The latter might lead to the thought that the gene product expression seems to be a rather late event in colorectal cancer histogenesis; a hypothesis reinforced by the fact that the proportion of p53 and c-erbB-2 positively reacting tumours was higher among the far-advanced tumours. This hypothesis is in accordance with the p53 non-detection in previous studies concerning colon adenomas which are considered as precancerous lesions (Kawasaki et al., 1992).

It could be postulated that alterations in the p53 and c-erbB-2 genes may have contributed to one or more of the late steps of carcinogenesis in a percentage of the colorectal cancer patients and that the genetic change may have been inherited through the tumour progression. Higher incidence of p53 and c-erbB-2 expression in advanced colorectal cancer (Dukes' stage C and D) may reflect the fact that these oncoproteins contribute to the metastazing ability of tumour cells (Amaout et al., 1992; Kawasaki et al., 1992). The significant correlation between p53 positivity and advanced stage might also suggest either a relation with a more aggressive behaviour of p53 positive carcinomas or imply than in large tumours with a more prolonged natural history there are greater changes that mutational events at the p53 locus may occur. Apart from tumour stage, higher p53 positivity rates were also associated with other high-risk prognostic factors such as poor differentiation, DNA aneuploidy and also with patients' clinical outcome. Our results are in line with previous authors' reports (Remvikos et al., 1990; Starzynska et al., 1992). Nevertheless, there are discrepancies in results derived from different studies with regard to p53 expression in colorectal cancer and its relation to prognosis. The differences in the role of p53 expression in determining prognosis might reflect differences in the number of tumours examined or the different kind of material, methods and antibodies used. Remvikos et al. (1990) found a significant association between elevated p53 and the presence of DNA aneuploidy, a factor connected with poor prognosis, whereas Scott et al. (1991) and Campo et al. (1991), using monoclonal antibodies and frozen material, reached different conclusions. Furthermore, Starzynska et al. (1992) revealed that p53 overexpression was significantly associated with early relapse and death in a study of a larger series of patients. They used a polyclonal antibody in contrast to our and other authors survey where monoclonal antibodies, recognizing specific antigenic epitopes, were used. According to some researchers the use of monoclonal antibodies might lead to under-estimation of the number of tumours overexpressing p53 which can affect final results on the relationship between p53 expression and prognostic factors (Arai et al., 1986; Barket et al., 1990; Starzynska et al., 1992).

In terms of clinical outcome, patients with p53 negative stage B and C colorectal tumours had a longer mean survival period in comparison to those with tumours which stained positive for p53. The contribution of the oncogene expression to prognosis may provide the possibility to identify subpopulations of colorectal cancer patients having shorter survival and needing more aggressive postoperative treatment.

In considering the c-erbB-2 expression in colorectal cancer of our series of patients, a significant trend towards a higher production of positive cases with advancing clinical stage was observed. Previous studies have indicated the association of c-erbB-2 presence with adverse prognosis in breast cancer (Van de Vijver et al., 1980), while different conclusions have derived from studies in gastric adenocarcinoma (Jain et al., 1991). Yamaguchi and Ohki (1992) consider c-erbB-2 positivity as a likely marker of liver metastases in advanced colon cancer. Generally speaking, the percentage of c-erbB-2

positive cases is influenced by the type of antibody (recognizing the external or the internal domains of c-erbB-2 molecule) used and the type of assay being carried out.

A principal finding of our survey was the high p53 and c-erbB-2 simultaneous overexpression rate in a group of colon carcinomas. Similar significant association of p53 positive status and c-erbB-2 overexpression has been identified in previous series of breast carcinomas (Harris et al., 1990; Chang et al., 1991). Additionally, the cases with p53 positive/c-erbB-2 positive phenotype compared to the rest of the examined cases demonstrated an intimate association with adverse prognostic factors, such as high grade, DNA aneuploidy, advanced stage and overall survival. p53/c-erbB-2 concomitant immunohistochemical overexpression might have higher prognostic relevance that the expression of each one of the proteins alone. P53 and c-erbB-2 overexpression may be either related events or may be triggered by a common event taking place during the earliest neoplastic steps and leading to genetic instability. Moreover, cancer progression may additionally facilitate neoplastic genetic instability, leading to further genetic damage and its subsequent effect on tumour progression. Taking into consideration the evidence that p53 and c-erbB-2 genes are both mapped on chromosome 17 we could assume the existence of a certain biological subgroup of colorectal cancers showing genetic alterations in this chromosome; such information may provide additional insight into the understanding of the colon cancer genetics. The concomitant expression of two or more oncoproteins could be used as a most sensitive method to differentiate tumours with different behaviour and clinical evolution as well as to direct therapeutic choices.

C-erbB-2 protein product is one of the antigens implicated in neoplastic cell growth. Such antigens, which are encoded by activated oncogenes and expressed on the cell membrane, display a restricted tissue distribution and may be of clinical relevance. These molecules may be exploited as tumour markers of various clinical applications and may enhance the development of specific immunotherapeutic approaches (Natali et al., 1990). Recent studies concerning c-erbB-2 inducible membrane protein and its probable relation to antibody mediated targeted cytolysis raise the question as to whether expression of this proto-oncogene product in neoplasias may be of clinical usefulness for future new modes of therapeutic interventions (Natali et al., 1990).

In conclusion, the implication of different groups of oncogenes (protooncogenes, tumour suppressor genes) can be a valuable tool in the elucidation of the complex biological steps of colorectal carcinogenesis as well as in identifying along with established prognostic factors patients with poor short-term prognosis and in deciding on their optimal postoperative treatments.

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