c-Myb and Bcl-x Overexpression Predicts Poor Prognosis in Colorectal Cancer

Clinical and Experimental Findings

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The aim of this study was twofold: to assess the relationship between c-Myb and Bcl-x expression and to evaluate the prognostic significance of their expression in colorectal carcinoma (CRC) patients. Analysis of tumors from 91 CRC patients for expression of c-Myb and Bcl-x revealed a significant relationship between these two proteins. Kaplan-Meier's analysis showed an increased risk of relapse and death in patients whose tumor specimens displayed high c-Myb levels and Bcl-x positivity. Similar results were also observed excluding Dukes' D patients. Molecular analysis using three c-Myb-overexpressing LoVo clones indicated that c-Myb overexpression was accompanied by up-regulation of Bcl-x_L protein and mRNA. Tumors originating from these clones injected in nude mice were significantly larger than those formed in mice injected with parental or vector-transfected LoVo cells. Moreover, tumors derived from parental and control vector-transfected but not from c-Myb-overexpressing LoVo cells showed high frequency of apoptotic cells. These results provide direct evidence of an association between c-Myb and Bcl-x expression and suggest that expression of both molecules might be a useful prognostic marker in CRC. (Am J Pathol 2001, 158:1289-1299)

Colorectal carcinoma (CRC) is one of the most common malignancies in the western world. Several clinical, biological, and genetic parameters have been used to as-

sess the prognosis and to help the clinician in optimizing therapies for CRC patients. Studies indicate that the most important prognostic variable is the tumor stage.¹ However, patients who are apparently at the same pathological stage often have different outcomes. Alteration in DNA content and high tumor proliferative activity seem to predict adverse outcome in CRC,^{2,3} although a lack of correlation has been also reported.⁴ The role of some cellular oncogenes and tumor suppressor genes in clinical aggressiveness of CRC has been also studied. Point mutations of the p53 and K-ras tumor genes occur in ~50% of CRCs and have been associated with a poor prognosis.⁵⁻⁸ However, available data are again controversial.^{9,10} Thus, recent efforts have focused on identifying new prognostic factors that accurately predict the clinical outcome of CRC patients with the goal of providing a rational approach for planning specific therapies.

c-Myb is a 75- to 80-kd transcription factor^{11,12} expressed in immature and transformed hematopoietic cells. Hematopoiesis depends on c-Myb expression for the expansion of most cell lineages,¹³ whereas a decline in c-Myb expression can be a prelude to differentiation.¹² c-Myb is also expressed in nonhematopoietic tissues of chickens, mice, and humans, with high levels of transcript and protein in the gastrointestinal tract.¹⁴⁻¹⁷ c-Myb expression increases from colonic normal mucosa through premalignant polyps up to colonic tumors.¹⁸ Cell lines derived from colon tumors also express c-Myb and seem to depend on c-Myb expression for growth.^{15,19,20}

Deregulated expression of c-Myb inhibits growth arrest and accelerates apoptosis of myeloid cells, consistent with the involvement of c-Myb in the regulation of apoptotic process.²¹ Indeed, overexpression of c-Myb protects T lymphocytes from apoptosis induced by growth

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factor deprivation or dexamethasone treatment, and it is accompanied by enhanced Bcl-2 expression, dependent on activation of the Bcl-2 promoter.²² In colon cells, decreased c-Myb expression during the commitment to differentiation and apoptosis is accompanied by a decrease in Bcl-2 levels.²³ Moreover, Bcl-2 expression is reduced and apoptosis is increased in colonic epithelium of embryos with a disrupted *c-myb* gene.²⁴ The role of the anti-apoptotic Bcl-2 in CRC patients remains unclear. Several authors have found a lack of Bcl-2 expression in CRC²⁵ and we recently found that only 30% of CRC patients studied were positive for Bcl-2 and that Bcl-2 expression predicted a better clinical outcome.²⁶ Other authors found no prognostic significance of Bcl-2 expression in CRC.^{27,28} On the other hand, the anti-apoptotic protein Bcl-x_L seems to play a major role in colorectal tumorigenesis and progression. A shift from expression of Bcl-2 to Bcl-x_L has been demonstrated during progression of colorectal tumors,²⁹ and significant Bcl-x_L overexpression has been found in the majority of CRC patients when compared with the corresponding normal colonic tissue.³⁰

Here we show that the expression of c-Myb correlates with that of Bcl-x and that the levels of these two proteins provide a reliable predictor of survival in CRC patients. Moreover, analysis of a human colon carcinoma cell line, transfected with the human *c-myb* cDNA, demonstrated that overexpression of c-Myb up-regulates Bcl-x_L and increases tumorigenesis of colon carcinoma cells by inhibiting the apoptotic process.

Materials and Methods

Patient Characteristics

Ninety-one patients surgically treated for CRC (70 colon and 21 rectum cancers) at the Regina Elena Cancer Institute between 1990 and 1998 have been included in this study. Multiple representative samples were collected from the tumor.

Tumor tissues were pathologically staged according to the Dukes' classification as follows: 5 stage A (5.5%), 40 stage B (43.9%), 19 stage C (20.9%), and 27 stage D (29.7%). Tumors were categorized according to the World Health Organization classification, as well differentiated (G1; four cases, 4.4%), moderately-differentiated (G2; 65 cases, 71.4%), and poorly-differentiated (G3; 22 cases, 24.2%).

Flow Cytometry (FCM)

Frozen tissue specimens were mechanically desegregated and cell suspensions were fixed at 4°C with a solution of acetone-methanol (1:4, v/v). Indirect immunofluorescence was performed using the anti-c-Myb primary monoclonal antibody (mAb) 1.1 (diluted 1:20).³¹ A fluorescein-isothiocyanate rabbit anti-mouse (DAKO, Glostrup, Denmark) was used as secondary antibody and the negative control was obtained by omitting the primary antibody. Specificity for the c-Myb protein of the

Figure 1. Two-parameter FCM analysis in four representative samples of normal mucosa (NM) and primary tumor (T). The number reported represents the percentage of c-Myb-positive cells. The threshold (▼) was set to the negative control of each sample. Windows 1 and 2 indicate the diploid and aneuploid cell populations, respectively.

mAb 1.1 in FCM was tested using resting and phytohemagglutinin-activated (Sigma Aldrich, Milano, Italy) lymphocytes. Resting lymphocytes were negative for c-Myb protein expression, whereas ~50% of lymphocytes exposed to 2 µg/ml of phytohemagglutinin for 72 hours were c-Myb-positive. Suspended cells were incubated with antibodies, stained for DNA content determination in a solution containing 75 kU/ml RNase A and 50 µg/ml propidium iodide, and then analyzed using a FACScan cytofluorimeter (Becton Dickinson, San Jose, CA). Linear propidium-iodide red fluorescence was monitored through a LP 620 filter and logarithmic green fluorescence from fluorescein isothiocyanate-labeled anti-c-Myb antibody was measured through a BP 530/15 filter. Debris, damaged cells, and doublets were excluded by gating on a forward and side-scatter dot plot. At least 30,000 events per sample were stored as list mode data and the monodimensional or bidimensional FCM distributions were analyzed using Lysis II C32 Becton Dickinson software. Each tumor was analyzed in parallel with normal mucosa of the same patients. In each sample the percentage of c-Myb-positive cells was calculated by

Table 1. Relationship between c-Myb and Bcl-x Expression and Biopathological Variables

Variables	No. of cases	c-Myb >58% (%)	P value*	Bcl-x-positive (%)	P value*
G1 G2	69	63.8	0.4401	59.4	0.0500
G3	22	72.7		81.8	
Dukes' A [†]	5	40.0		20.0	
Dukes' B	40	67.5		60.0	
Dukes' C	19	63.2	0.6057	63.2	0.0435
Dukes' D	27	70.4		81.5	
Diploidy	23	34.7		34.8	
Aneuploidy	68	76.5		75.0	
% S ≤ 20.1%	37	62.2	0.4534	56.7	0.3450
% S > 20.1%	34	70.6		67.6	
Bcl-2-negative	60	70.0		70.0	
Bcl-2-positive	31	58.1		54.8	
Bcl-x-negative	37	13.3	0.0001		0.1511
Bcl-x-positive	54	86.7			

*P value: Pearson's chi-square test.

[†]Dukes' stage was dichotomized as follows: CD versus AB.

subtracting a background of 2 to 5% from the positive cell distribution. The cut-off of c-Myb and percent S-phase, calculated on the median value of tumor specimens, were 58 and 20.1, respectively.

Primary Antibodies and Immunohistochemistry

Immunoreactivity for Bcl-x protein was detected by using affinity-purified polyclonal antibody S-18 (Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:100, raised against a peptide mapping at the N terminus of human *bcl-x* gene (identical to corresponding mouse sequence). Bcl-2 protein expression was evaluated using mAb 124 (Dakopatts, Copenhagen, Denmark), diluted 1:20. Immunohistochemical staining was performed on 5- μ m thick sections on silane-treated (APES; Sigma, St. Louis, MO) slides from routinely fixed paraffin-embedded blocks. The deparaffinized and rehydrated sections were pre-treated twice in a microwave oven at 750 W for 5 minutes in citrate buffer and incubated for 60 minutes at room temperature with primary antibodies. The reaction was visualized using a streptavidin-biotin immunoperoxidase system (LSAB 2 kit; DAKO, Milan, Italy) and 3-amino-9-ethyl carbazole solution (DAKO) as chromogenic substrate. Sections were then slightly counterstained with Mayer's hematoxylin and mounted in aqueous mounting medium (Glycergel, DAKO). Sections without primary antibodies served as negative controls.

Bcl-x and Bcl-2 expression was determined semiquantitatively by assessing the whole tumor section by two investigators (MM and SB), who were blinded to the clinical data at the time of interpretation. Tumors were classified, according to Sinicrope and colleagues³² with minor modifications, into four categories and assigned to one of the four: 0, <5%; 1, 5 to 25%; 2, 25 to 50%; 3, >50%. The staining intensity was scored as follows: 0, completely negative; 1+, weak; 2+, moderate; and 3+, strong. For tumors showing heterogeneous staining, the predominant pattern was taken into account for scoring.

The percentage of positive tumor cells and staining intensity were multiplied to produce a weighted score for each case. Cases with weighted scores ≤ 1 were defined as negative; otherwise they were defined as positive.

Cells and Transfection Experiments

Human colon carcinoma LoVo cells were grown in HAM'S-F12 (Bio-Whittaker, Walkersville, MD) supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, and antibiotics.

LoVo cells ($1 \times 10^6/200 \mu$ l) were transfected by electroporation (960 μ F, 200 V, Gene Pulser; Bio-Rad, Milano, Italy) with the expression vector pcDNA3c-*myb* carrying the *c-myb* gene and the gene for the resistance to neomycin (G418). c-Myb full-length cDNA was obtained by digesting the plasmid LXSnc-*myb*²² with *EcoRV*, by end-blunting and by cloning it into pcDNA3 vector (CMV promoter). pcDNA3c-*myb* constructs were screened by sequencing and plasmids with *c-myb* gene in sense orientation were chosen for the experiments. Transfected LoVo cells were grown in G418-containing medium (0.8 mg/ml; Life Technologies, Inc., Gaithersburg, MD) to obtain individual clones. Two weeks later, clones were expanded and screened for c-Myb expression by Western blot analysis.

Western Blotting

Western blot and detection were performed as previously reported³³ using 40 μ g of total proteins loaded from each sample on denaturing 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Anti-c-Myb mAb 1.1³¹ was used at 1:1,000 dilution; anti-Bcl-x (S-18, Santa Cruz) polyclonal antibody and nonspecific purified IgG were used at 1:500 dilution. To check the amount of proteins transferred to nitrocellulose membrane, heat shock protein (HSP) was used as control and detected by an anti-human HSP 72/73 mAb (Ab-1, clone W27; Calbiochem, Cambridge, MA). The relative amounts of the

Figure 2. Expression of Bcl-x protein, as revealed by streptavidin-biotin immunoperoxidase staining, in two representative colon adenocarcinomas displaying positive (score 9, see MM) (a) and negative (score 1, see MM) (b) cytoplasmic reactivity. Original magnification, $\times 400$.

transferred proteins were quantified by scanning the autoradiographic films with a gel densitometer scanner (Bio-Rad) and then normalized to the related HSP 72/73 amounts.

Northern Blotting

Total RNA was isolated by Trizol (Life Technologies, Inc.) following a standard protocol, and 30 μg was size-fraction-

Figure 3. DFS and OS (81 cases) for patients with CRC categorized according to the c-Myb (**a** and **b**) and Bcl-x expression (**c** and **d**). Survival curves were generated according to the Kaplan-Meier method; statistical comparisons were made using the log-rank method.

tionated on denaturing formaldehyde agarose gel, blotted onto nylon filter, and hybridized with a 920-bp fragment cut from a human *bcl-x_L* plasmid. The filter was exposed to an autoradiographic film for 3 days. After stripping (0.1% sodium dodecyl sulfate for 5 minutes at 100°C) the filter was reprobated with the glyceraldehyde-3-phosphate dehydrogenase probe to assess equivalence of RNA loading and transfer.

In Vitro and In Vivo Cell Growth

The growth of LoVo parental line, one neo clone (LoVo/neo) and three c-Myb-overexpressing clones (M11, M18, and M30) was assessed by seeding 8×10^4 cells in 60-mm Petri dishes (Nunc, Mascia Brunelli, Milano, Italy). Cell counts (Coulter Counter model ZM, Kontron) and viability (trypan blue dye exclusion) were determined daily, from day 1 to day 15 of culture.

CD-1 male nude (nu/nu) mice, 6 to 8 weeks old and 22 to 24 g in body weight, were purchased from Charles River Laboratories, Calco, Italy. All procedures involving animals and their care have been previously described and were in accordance with institutional guidelines in compliance with national and international laws and policies.³⁴ Mice were injected intramuscularly with parental LoVo cells, LoVo/neo

clone or each of c-Myb-overexpressing clones at 2×10^6 of tumor cells/mouse. Eight mice for each group were used and tumor mass was monitored daily.³⁴

Terminal Deoxynucleotide Transferase-Mediated dUTP Nick-End Labeling (TUNEL)

Apoptotic activity on sections of the murine tumors was analyzed by TUNEL with a commercial kit (ApopDetek *in situ* apoptosis detection kit; Enzo Diagnostic, New York, NY), following the manufacturer's instructions. Briefly, formalin-fixed, paraffin-embedded tissue sections (4 μ m) were deparaffinized with xylene and stripped of proteins by incubation with 25 μ g/ml proteinase K for 1 hour at room temperature before the TUNEL reaction. The incorporated Bio-16-dUTP was then stained with streptavidin-biotinylated horseradish peroxidase complex, AEC (3-amino-9-ethyl carbazole) and hematoxylin. Apoptotic cells had red nuclear staining. Omission of the terminal deoxynucleotide transferase enzyme in the TUNEL reaction was used as negative control and resulted in no staining.

Three mice for each group were sacrificed at day 50 after cell injection. The percentage of apoptotic cells

Figure 4. DFS and OS (54 cases obtained by excluding the Dukes' D patients) for patients with CRC categorized according to the c-Myb (**a** and **b**) and Bcl-x expression (**c** and **d**). Survival curves were generated according to the Kaplan-Meier method; statistical comparisons were made using the log-rank method.

was determined by light microscopic examination of sections at $\times 400$. For each slide, five high-power fields of nonnecrotic tumor areas were examined and 300 cells in each field were counted. For each tumor, four different sections were evaluated. Results were expressed as mean $\pm$ SD of the samples analyzed. The apoptotic index was calculated as number of apoptotic cells per 100 cells.

Statistical Analyses

Association between variables was tested by the Pearson's chi-square test. The TUNEL results were expressed as mean $\pm$ SD of the samples analyzed. Student's *t*-test was used to evaluate statistical significance. Disease-free survival (DFS) and overall survival (OS) curves were estimated by the Kaplan-Meier product-limit method; the log-rank test was used to assess differences between subgroups. A *P* value < 0.05 was considered significant. The BMDP statistical package program (BMDP, Los Angeles, CA) was used for analysis.

Results

Expression of c-Myb Oncoprotein in Colorectal Tumor and Its Relationship with Biological and Clinicopathological Variables

c-Myb levels were monitored in 91 colorectal tumor samples using FCM. This methodology permits the simultaneous evaluation of more than one biological parameter and might allow to accurately calculate the percentage of c-Myb-positive cells in heterogeneous cell populations. Thus, c-Myb expression, DNA ploidy, and percent S-phase were determined in each tumor sample and compared to those of the corresponding normal mucosa.

Two-parameter FCM analysis (c-Myb expression and DNA content) in four representative samples of normal mucosa and primary tumor demonstrates that c-Myb is differentially expressed in the same neoplastic sample containing diploid and aneuploid cell populations (Figure 1). The expression of c-Myb was relatively low in cells with a diploid DNA content (window 1), whereas an increase in c-Myb positivity was observed in cells with

Figure 5. DFS and OS (71 cases) for patients with CRC categorized according to the combinations of c-Myb and Bcl-x expression (**a** and **b**) and DFS and OS (49 cases obtained by excluding Dukes' D patients) for patients with CRC categorized according to the combinations of c-Myb and Bcl-x expression (**c** and **d**). Survival curves were generated according to the Kaplan-Meier method; statistical comparisons were made using the log-rank method.

aneuploid DNA content (window 2). Because these results suggested that there was a relationship between aneuploidy and c-Myb overexpression, the association between c-Myb expression, ploidy, and percent S-phase cells was evaluated in each tumor sample.

Table 1 shows the statistical evaluation of the relationship between clinicopathological and biological variables. Tumor cell differentiation and Dukes' stage did not correlate with c-Myb overexpression, whereas a significant relationship between c-Myb overexpression and aneuploidy ($P = 0.0003$) was observed. On the other hand, expression of c-Myb, a cell cycle-related gene, did not correlate with the percentage of cells in S phase ($P = 0.4534$). In fact, the number of cases with more or less 20.1% S-phase cells was similar regardless the degree of c-Myb expression.

On the basis of previous results demonstrating a relationship between Bcl-2 and c-Myb,^{22,23} and data that report an elevated expression of Bcl-x in primary CRC,^{29,30} we studied the correlation between c-Myb, Bcl-2, and Bcl-x in this series of patients. Bcl-2 and Bcl-x immunoreactivity was determined by immunohistochem-

istry on paraffin-embedded sections (Table 1). Although the expression of c-Myb did not correlate with that of Bcl-2 ($P = 0.2549$), it was directly related to that of Bcl-x ($P < 0.0001$). In fact the majority of tumors expressing high c-Myb levels were positive for Bcl-x ($\sim 87\%$, Figure 2a), whereas tumors expressing low c-Myb levels were negative for Bcl-x ($\sim 93\%$, Figure 2b). In addition, tumors positive for Bcl-x staining were associated with aneuploid CRC ($P = 0.0115$) and Dukes' stage. Pearson chi-square test revealed a borderline correlation between Bcl-x positivity and tumor grading.

Prognostic Role of c-Myb and Bcl-x

To evaluate the prognostic impact of c-Myb and Bcl-x, patients who died within 30 days after surgery or those who died from noncancer-related causes were excluded from DFS and OS Kaplan-Meier analysis. This selection permitted us to analyze 81 cases, with a median follow-up of 50 months (range, 24 to 77 months), for DFS and OS, respectively. In this series of patients, 33 re-

Figure 6. Western blot analysis of c-Myb expression in LoVo parental line, LoVo/neo control clone, and several c-Myb transfectants (M11, M15, M18, and M30). HL60 cells was used as positive control. Expression of HSP 72/73 was measured as control of equal proteins loading.

lapses were recorded and 30 patients died from cancer-related causes.

Kaplan-Meier curves, stratified for c-Myb and Bcl-x expression, showed a significantly longer DFS and OS in patients with low c-Myb expression (Figure 3, a and b) and in patients with Bcl-x-negative tumors (Figure 3, c and d). To better investigate the predictive value of c-Myb and Bcl-x in patients not presenting distant metastases, DFS and OS Kaplan-Meier analysis was performed excluding the Dukes' D patients. As shown in Figure 4 c-Myb low-expressing and Bcl-x-negative patients had a significant increased DFS and OS benefit compared to those with high c-Myb expression and Bcl-x-positive.

On the basis of these results, we evaluated the impact of the combination of these two factors on DFS and OS, analyzing the two groups of patients bearing high c-Myb expression/Bcl-x-positive and low c-Myb expression/Bcl-x-negative tumors. As shown in Figure 5, a and b, the results obtained in the entire series of patients (Dukes' A to D) provide statistically significant evidence that the concomitant expression of c-Myb and Bcl-x identifies a group of patients with a higher probability of relapsing and dying. Similar results were observed excluding Dukes' D patients (Figure 5, c and d).

Figure 7. A: Cell lysates from the LoVo/neo clone, and M18, M30, and M11 c-Myb-overexpressing clones were loaded on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted, and incubated with rabbit anti-Bcl-x polyclonal antibody (**top**), nonspecific purified rabbit IgG (**middle**) and mouse anti-HSP mAb (**bottom**). Expression of HSP 72/73 was measured as control of equal proteins loading. **B:** Northern blot analysis of *bcl-xL* expression in LoVo/neo clone, M18, M30, and M11 c-Myb-overexpressing clones. Glyceraldehyde-3-phosphate dehydrogenase was measured as control of equal RNA loading.

Expression of Bcl-x_L Protein and mRNA in LoVo Cells and c-Myb Transfectants

To examine the potential relationship between c-Myb and Bcl-x_L in tumor cells, the human colon cancer cell line LoVo was transfected with a full-length *c-myb* cDNA driven by a constitutively active promoter. LoVo cells were selected as low c-Myb-expressing cells from a panel of five colon carcinoma lines (Colo205, HT29, LoVo, SW, DLD-1) (data not shown). Figure 6 shows different c-Myb protein levels in the parental LoVo cells, LoVo/neo clone (the vector control) and in some of the transfectants. Clones M11, M18, and M30, which show a 5- to 10-fold increase in c-Myb expression compared to the LoVo/neo clone, were used for further experiments.

To verify whether c-Myb modulates Bcl-x_L protein and mRNA levels, Western and Northern blot analysis of the different cell lines were performed. Figure 7A shows Western blot analysis of Bcl-x_L protein in LoVo/neo control clone and three clones overexpressing c-Myb (M11, M18, and M30). Compared to vector-transfected LoVo cells, c-Myb-transfected cells show a twofold to threefold increase in Bcl-x_L protein. Bcl-x_L protein corresponds to the lower band (29 kd) in the top panel of the figure because the top prominent 35 kd is a nonspecific band recognized by a nonspecific rabbit IgG (middle panel). A twofold to threefold increase in *bcl-xL* mRNA was also observed in M11, M18, and M30 clones (Figure 7B). The LoVo parental cells showed the same level of Bcl-x_L expression observed in the LoVo/neo clone (data not shown).

Effect of c-Myb Expression on In Vitro and In Vivo Growth

c-Myb-overexpressing LoVo cells were analyzed for *in vitro* and *in vivo* growth characteristics. The *in vitro* growth curves of the c-Myb overexpressing M11, M18, and M30 transfectants were similar to those of the parental cells and the LoVo/neo clone (Figure 8). Similar results were obtained by performing the growth curves in medium supplemented with 1% FCS (data not shown). Thus, overexpression of c-Myb protein did not affect proliferation of LoVo cells. By contrast, *in vivo* growth of c-Myb-overexpressing LoVo cells was different (Figure 9A). The tumor mass of the c-Myb-overexpressing clones was larger than control tumors, even at a late stage of tumor growth; ie, 60 days after cell injection the weight of tumors originating from LoVo and LoVo/neo cells was ~1,200 mg compared to the 3,000 mg from c-Myb-overexpressing clones. In addition, 75% of mice injected with parental cells or with the LoVo/neo control clone developed tumors, whereas tumors formed in all mice injected with the M11, M18, or M30 c-Myb transfectants.

To assess whether the different tumor growth could be related to differences in apoptosis rate, a TUNEL assay for detection of DNA fragmentation caused by apoptosis, was performed 50 days after cell injection. Figure 9B shows TUNEL staining in tumor section of LoVo/neo control clone (a) and one representative c-Myb-overexpress-

Figure 8. *In vitro* growth curves of LoVo cells, LoVo/neo clone, and three c-Myb-overexpressing clones (M11, M18, and M30).

ing clone (b). Apoptosis was detected in tumors from LoVo/neo cells but not in tumors from the M30 c-Myb-overexpressing clone. In the LoVo/neo clone, the mean apoptotic index was 10.1 ± 2.02 , whereas in the M30 c-Myb-overexpressing clone it was 2.36 ± 0.98 and this difference was significant ($P < 0.0001$). Similar results have been obtained with M11 and M18 clones (data not

shown). These results are consistent with the hypothesis that the increased tumorigenicity observed in c-Myb-overexpressing clones is closely correlated to decreased apoptosis.

Discussion

In the present study, FCM enabled the simultaneous analysis of c-Myb expression, DNA content, and percent S-phase cells, the importance of which in CRC has been documented.^{2,15} We found that c-Myb protein was heterogeneously expressed when cell populations with altered DNA content were present within a tumor, ie, c-Myb levels were higher in aneuploid than in diploid cells. Thus, cells with high levels of c-Myb expression may be selected as colorectal tumors become more aggressive.

The development of colorectal malignancies is known to involve the interplay between genes that drive cell division and regulate cell cycle checkpoints.³⁵ c-Myb protein is required for entry into the cell cycle and its persistent expression is required for cells to remain in the cell cycle.¹⁶ In CRC cells, this continual cell cycling might favor the accumulation of additional genetic alterations²⁸ that, in turn, may enhance survival of tumor cells.³⁶ On the other hand, in our model, the high level of c-Myb expression in tumor cells is not directly proportional to the fraction of S-phase cells. The results using tumor specimens are in agreement with the findings that no changes occur in the proliferation of c-Myb-overexpressing LoVo colon carcinoma cells. Thus, *c-myb* oncogene seems to be involved in the regulation of other aspects of cell behavior underlying colorectal tumorigenicity.

Although the stage of disease usually determines the prognosis of CRC patients, in the 91 CRCs tested no relationship was found between Dukes' stage and c-Myb overexpression. We also found a direct correlation between high c-Myb expression and Bcl-x-positive tumor cells in ~90% of the patients, a finding consistent with

Figure 9. **A:** *In vivo* growth curves of tumors from injected LoVo cells (●), LoVo/neo clone (▽), and M11 (■), M18 (○), M30 (▲) c-Myb-overexpressing clones. **B:** Detection of apoptosis (TUNEL) in LoVo/neo (a) and M30 (b) tumor cells grown in nude mice. Original magnification, $\times 200$.

previous studies suggesting that the apoptosis inhibitor Bcl-x_L protein plays a major role in colorectal tumorigenesis and progression.²⁹ Overexpression of Bcl-x_L as well as a shift from expression of the anti-apoptotic Bcl-2 to Bcl-x_L protein have been detected during progression of colorectal tumors.^{29,30} Kaplan-Meier curves show that c-Myb and Bcl-x are predictors of poor clinical outcome. Of particular clinical interest is that the significant prognostic value of c-Myb and Bcl-x is also retained in the Dukes' A to C patients both for DFS and OS. Moreover, the concomitant high expression of c-Myb and Bcl-x positivity identifies a group of patients at higher risk, probably requiring more aggressive therapies.

The relationship between c-Myb and Bcl-x observed in tumor specimens may reflect the modulation of Bcl-x_L by c-Myb. Indeed, three c-Myb-overexpressing clones showed increased levels of Bcl-x_L protein and mRNA expression. Because several studies have recently shown that genes involved in inhibition of apoptosis can confer growth advantage and increased tumorigenicity,^{37,38} we studied the growth characteristics of three clones of LoVo cells overexpressing c-Myb and with up-regulation of Bcl-x_L. *In vitro*, parental, and c-Myb-overexpressing LoVo cells were indistinguishable. *In vivo*, the behavior of c-Myb-overexpressing LoVo cells was markedly different from the parental line as indicated by the high tumorigenicity of c-Myb-transfected cells. However the degree of c-Myb expression does not seem to directly correlate with the expression of Bcl-x_L and tumorigenicity. In fact, in transfectants, c-Myb overexpression is always associated with increased Bcl-x_L expression and tumorigenicity, but the enhancement of these two parameters does not reflect the different c-Myb overexpression levels. The apparent discrepancy could be explained by the mechanism of negative autoregulation of c-Myb activity by homodimer formation as reported by Nomura and colleagues.³⁹

The increase in tumorigenicity of c-Myb-overexpressing clones was closely related to the inhibition of apoptotic process as determined by TUNEL assay. A positive relationship between enhanced cell survival and tumorigenic phenotype has recently been suggested by our and other studies showing that breast and melanoma cells overexpressing Bcl-2 were more tumorigenic than the nontransfected counterpart.^{40,41} Similar conclusions were drawn from models of experimental tumorigenesis where reduced apoptosis marked a late stage of tumor development.²⁰ Thus, the enhanced tumorigenic potential of c-Myb-overexpressing LoVo cells might be because of a reduced susceptibility to apoptosis.

In conclusion, our results demonstrate that overexpression of c-Myb is associated with enhanced Bcl-x protein levels in primary CRC and the co-expression of these two markers is a predictor of poor prognosis. The increased levels of Bcl-x_L are probably the direct consequence of c-Myb overexpression as observed in LoVo cells transfected with c-Myb. Overexpression of c-Myb in these cells is associated with a lower frequency of apoptotic cells in tumor mass that likely contributes to the enhanced tumorigenicity of c-Myb-overexpressing LoVo cells.

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