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ORIGINAL PAPER

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DNA Ploidy and S-phase fraction, but not p53 or NM23-H1 expression, predict outcome in colorectal cancer patients. Result of a 5-year prospective study

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Abstract *Purpose*: The aim of this study was to determine TP53 and NM23-H1 immunoreactivity, DNA ploidy, and S-phase fraction (SPF) in a series of 160 patients undergoing resective surgery for primary operable colorectal cancer (CRC) and to establish whether these alterations have any clinical value in predicting CRC patients' prognosis. *Methods*: TP53 and NM23-H1 expressions were evaluated on paraffin-embedded tissue by immunohistochemistry and DNA-ploidy and SPF on frozen tissue by flow-cytometric analysis. *Results*: The median follow-up time in our study group was 71 months (range 34–115 months). P53 protein expression was associated with distal tumors ($P < 0.05$) and DNA aneuploid tumors ($P < 0.05$) tumors. DNA-aneuploidy was associated with distal tumors ($P < 0.01$), histological grade (G3) ($P < 0.05$), advanced Dukes' stage (C and D) ($P < 0.01$), lymph node metastases ($P < 0.01$) and high SPF ($> 18.3\%$) ($P < 0.01$). The major significant predictors for both disease relapse and death were advanced Dukes' stage, DNA-aneuploidy, and high SPF, while lymphohematic invasion was the only independent factor for relapse and non-curative resection for death. *Conclusions*: Our results indicate that DNA aneuploidy and high SPF are associated in CRC with a poor clinical 5-year outcome, while in contrast the prognostic role of TP53 and NM23-H1 expression is still to be clarified.

Keywords Flow-cytometric variables · TP53 expression · NM23-H1 expression · Colorectal cancer · Prognosis

Introduction

The pathological evolution of human colorectal cancer (CRC) has yet to be completely understood and the factors involved in patient survival are still not clear.

What is, however, quite sure, is that proteins such as TP53 play an extremely important role in the neoplastic development of such tumors. Alterations of p53, in fact, are probably responsible for the transition from adenoma to carcinoma in CRCs (Fearon 1998). This nuclear phosphoprotein functions as a transcriptional regulator; in most normal tissues, the wild-type TP53 protein is constitutively expressed at low levels because of a short half-life (5'-40' depending on the cell cycle phase in which it is evaluated) (Brown and Pagano 1997) due to rapid degradation, but it may accumulate in the cell as a result of several stresses, such as DNA damage, hypoxia, loss of normal growth and survival signals, acidity, and inflammatory processes (Fearon 1998; Takashi et al. 2000), which may occur in different physiological or pathological situations, including tumorigenesis. When its intracellular levels increase, the cell cycle at phase G1 – or, less frequently, at phase G2/M – is arrested in order to allow the cell to repair the damage, or, if this is no longer possible, apoptosis occurs. Overexpression of TP53 has been found in most human tumors (Pich 1998); however, its prognostic impact in CRC is still to be clarified (Adrover et al. 1999; Ahnen et al. 1998; Bell et al. 1993; Rew et al. 1996).

Although it has been known for some time that p53 plays an important part in neoplastic progression of CRC, there is still considerable controversy about the role of nm23 in that most complex process called metastatization. Nm23-H1 was first isolated in a murine cell line (K-1735), where its reduced expression was accompanied by high metastatic potential. Subsequently, a second murine gene, nm23-M2, was identified, and also homologous genes in humans (nm23-H1, nm23-H2, DR-nm23, nm23-H4, nm23-H5). Nm23-H1 codifies for the sub-unit A of the dinucleotide diphosphate (NDP) kinases involved in the synthesis of nucleoside triphosphate, not mediated by ATP (Lombardi et al. 2000). In several human neoplasias, such as breast, ovarian, and hepatocellular carcinomas, a reduced expression of NM23 has been observed, together with a higher metastatic potential and a reduction in patient survival (Luo et al. 1993; Nakamura et al. 1998; Shaitoh et al. 1996). Nevertheless, in many other tumors, such as melanomas and thyroid and gastric cancers, the role played by NM23 in metastatic ability and prognosis is still not clear (Gazzeri et al. 1996; Nakamori et al. 1993). In carcinomas of the lung and of the pancreas, increased NM23 expression was associated with advanced stages of the disease and with poor prognosis, while contrasting data have been reported with regard to CRCs (Haut et al. 1991; Martinez et al. 1995; Myeroff and Markowitz 1993; Tannapfel et al. 1995; Yamaguchi et al.1993).

Moreover, data regarding the prognostic significance of DNA-ploidy in CRCs are still controversial (Silvestrini 2000), even though multiclonality would seem to be a frequent indicator of worse clinical outcome (Buglioni et al. 2001; Cosimelli et al. 1998), whereas the prognostic value of the S-phase fraction (SPF) as a measure of the proliferative activity of the cancer cells is more clearly established (Daidone et al. 2001).

The aim of this study was to determine TP53 and NM23-H1 immunoreactivity, DNA ploidy, and SPF in a series of 160 patients undergoing resective surgery for primary operable CRC and to relate TP53 and NM23-H1 status to flow cytometric and traditional clinico-pathological variables. In addition, our purpose was to assess the clinical value of TP53 and NM23-H1, DNA ploidy, and SPF in predicting the outcome of CRC patients.

Methods

Study design

A prospective study was performed on paired tumor and normal tissue samples collected by the Molecular Oncology Section of the University of Palermo from a consecutive series of 160 patients undergoing potentially radical surgical resection for primary operable CRC at a single institution (Department of Oncology, University of Palermo) from January 1988 to December 1992. Written informed consent was obtained from all patients included in this study.

Briefly, the following exclusion criteria were used: a) history of previous neoplasms; b) patients from families with familial adenomatous polyposis or hereditary nonpolyposis CRC with a highly penetrant genetic predisposition to CRC; c) synchronous or metachronous CRC; and d) chemotherapy or radiation therapy prior to surgery.

The inclusion criteria used were: a) histologically proven primary CRCs; b) processing of fresh paired normal mucosa-tumor samples within 30 min after tumor removal; and c) available cells from normal and tumor tissue.

The patients in this series comprised 84 females and 76 males with a median age of 66 years (range 31–88). In order to avoid evaluator variability in the patients, all resection specimens and microscopic slides were meticulously examined by two independent pathologists (RMT and VM) who were not aware of the original diagnosis and of the results of the molecular analysis. In addition, the pathologists assessed tumor site (proximal or distal tumors), tumor size, pathological stage, tumor grade (histological differentiation), presence or absence of lymph node metastases, tumor growth (expansive or infiltrative), tumor type (adenocarcinoma NOS or mucinous), presence or absence of vascular and lymphatic invasion or tumor lymphocytic infiltrate. According to Turnbull's modification of Dukes' system (Turnbull et al. 1967) the tumors were staged from A to D. Patients with Dukes' stage A and B CRC were treated with surgery alone, whereas only ten patients with Dukes' stage C received adjuvant chemotherapy with 5-FU, leucovorin, and levamisole since in the pre-1991 period hardly any of the patients received adjuvant treatment. Patients with non-radical surgery and/or distant metastases were treated by 5-FU and leucovorin. Postoperatively, all patients were checked at 3-monthly intervals for the first 2 years, at 6-monthly intervals for the next 2 years, and annually thereafter. The follow-up program included a clinical examination, blood tests (including CEA assay), annual chest X-ray and endoscopy. Abdominopelvic computed tomography scan was also performed each year for the first 2 years. Disease relapse (local recurrence or distant metastases) was confirmed histologically where possible.

Clinico-pathological and follow-up data of all patients were recorded prospectively in a computerized registry database and are presented in Table 1. A total of 137 patients were potentially cured by means of radical surgical tumor resection with regional en bloc lymphadenectomy proximally up to the origin of the vascular trunks (this group includes five patients who underwent partial hepatic resection for single liver metastasis). Twenty-three patients had either non-radical surgery for locally advanced rectal cancer or non-operable distant synchronous metastases.

Table 1 Patient characteristics ($n = 160$)

Site	No. Patients
Proximal tumor	31
Distal tumor	129
Tumor size (cm)	
≤ 5	60
> 5	100
Dukes' stage	
A	40
B	51
C	41
D	28
Node status	
Negative	101
Positive	59
Tumor growth	
Expansive	20
Infiltrative	140
Tumor grade	
Well-differentiated (G1)	23
Moderately-differentiated (G2)	104
Poorly differentiated (G3)	33
Tumor type	
Adenocarcinoma NOS	137
Mucinous	23
Lymphohematic invasion	
None	45
Present	115
Lymphocytic infiltrate	
Prominent	48
Non-prominent	112
Surgery	
Curative resection	137
Non-curative resection	23

Tissue handling

Multiple samples (6–10) of the primary tumor tissue were taken from different tumor areas (including the core and the invasive edge of the tumor). The tissues were bisected, one half of each sample was processed for pathological examination, and the remaining half of the sample pool was immediately frozen and stored at –80 °C until analyzed. The suitability of the material was checked on frozen tissue sections and only tissue samples with more than 80% tumor content were utilized in subsequent flow-cytometric analysis. Where present, areas with a high content of non-neoplastic cells were removed from the frozen block with a scalpel. Evaluation of each biomolecular variable (TP53 and NM23-H1 expression, DNA-ploidy, and S-phase fraction) was performed independently by researchers who had no knowledge of the clinical data of the samples.

TP53 immunohistochemistry

TP53 immunostaining (Fig. 1) was assessed on 5-μm-thick sections cut from formalin-fixed, paraffin-embedded tissue specimens. After deparaffinization, sections were pre-treated with 3% H_2O_2, to quench endogenous peroxidase activity. Antigene retrieval was performed by microwave heating in 10 mmol citrate buffer (pH 6.0). The sections were then immunostained with the DO-7 monoclonal antibody (dilution 1:60, Dako, Glostrup, Denmark). This antibody reacts with an epitope between monoacids 19 and 26, recognizing both wild type and mutant forms of the TP53 protein. After incubation with the biotinylated anti-mouse IgG secondary antibody, immunohistochemical reaction was performed by a standard peroxidase-labeled streptavidin-biotin procedure (LSAB+, Dako). Detection was performed using the AEC Substrate-Chromogen (Dako-AEC), and the slides were then counterstained with Carazzi hematoxylin/eosin and mounted with a permanent medium. Normal human serum was substituted for primary antibody on some sections, to serve as non-immune controls, while positive controls were sections of other CRCs defined as strongly positive. Positive tumor cells were quantified by the pathologists (RMT and VM) by evaluating at least 5,000 cells from four different specimens of the same tumor and were expressed as the percentage ratio of the total number of tumor cells (Tomasino et al. 1994). A section was scored as positive when at least 5% of tumor cells showed staining. The median cut-off point in our study was fixed at 5% (values ≥ 5% of the stained cells indicated protein overexpression).

All samples were evaluated blind, with no knowledge of either the biomolecular or the clinical pathological variables of patients. The tumors were divided unequivocally into two groups: negative and positive, on the basis of DO-7 immunohistochemistry.

NM23-H1 immunohistochemistry

Tissue sections were dewaxed in xylene and hydrated through graded ethanols to phosphate-buffered saline (PBS) pH 7.4. Endogenous peroxidase activities were blocked in 0.3% (v/v) hydrogen peroxidase in absolute methanol for 30 min. Immunohistochemical analysis was performed using a streptavidin-biotin System kit for primary mouse antibody (Zymed, San Francisco, Calif., USA). Each section was incubated with MoAb NM23/NDPK-A (Novocastra, Laboratories, Newcastle upon Tyne, UK), diluted 1:125 in PBS for 30 min at room temperature. The peroxidase reaction was initiated by the addition of 0.06% diaminobenzidine tetrahydrochloride (Sigma Chemical, St. Louis, Mo., USA) in PBS containing fresh hydrogen peroxidase. The slides were counterstained with Harris Hematoxylin and were permanently mounted. The degree of staining intensity was evaluated by counting DBA-staining tumor cells out of a minimum of 200 cells in five microscopic fields at ×400 magnification. Negative controls were carried out using non-immune sera, instead of the primary antibodies.

Expression of NM23-H1 protein was primary cytoplasmatic and was scored for: a) distribution and assessment of the percentage of stained cancer cells; and b) staining intensity. Evaluation of the cytoplasmatic staining reaction was performed in accordance with the immunoreactive score (IRS) proposed by Remmele and Stegner (Remmele and Stegner 1986). Percentage of positive cells was defined as 0 if negative; 1, ≤ 10% positive cells; 2, 11–50% positive cells; and 3, 51–100% positive cells. Five (×400 magnification) visual fields from different areas of each tumor were used for the IRS evaluation. Tumor slices scoring at least three points in our study were classified as immunoreactive, indicating NM23 expression.

Flow-cytometric analysis

DNA flow cytometry was performed on mechanically disaggregated samples of frozen tumor tissue as previously described (Russo et al. 1994). A FACSort flow cytometer (Becton Dickinson, Calif., USA) was used to obtain data. DNA-ploidy, DNA index, and S-phase fraction (SPF) were determined as previously reported (Russo et al. 1994).

Statistical analysis

Fisher's exact test (StatXact Turbo, Cytel Software, Cambrige, Mass., USA) was used to value the associations between biological variables. The relationship of different prognostic variables to disease-free survival (DFS) and overall survival (OS) was assessed univariately by means of the Kaplan-Meier method. Survival time was calculated from the date of surgery to the date of death (cancer-related causes) or last follow-up, with times censored for patients dying of causes unrelated to CRC and those surviving.

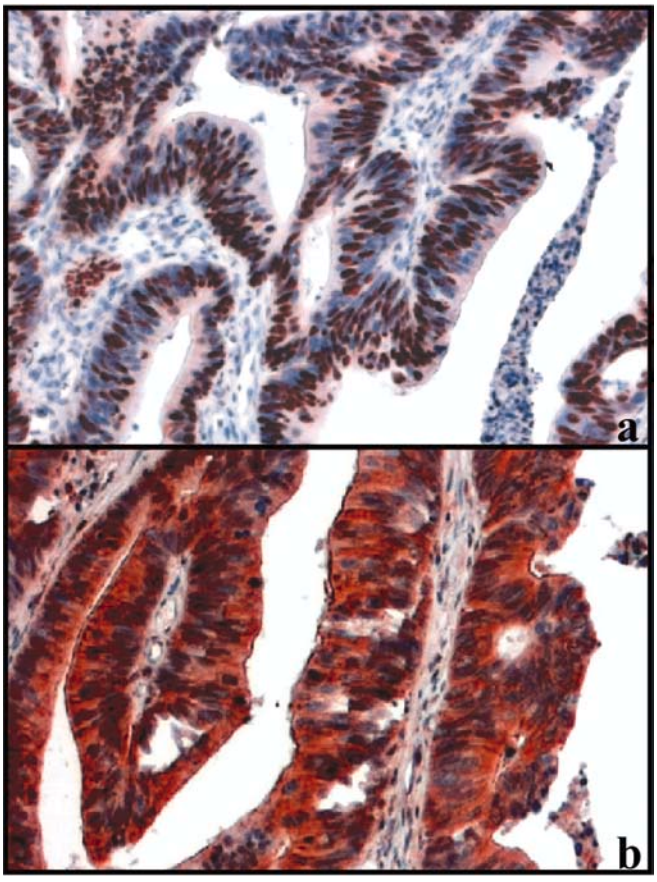

Fig. 1 Immunohistochemical staining for p53 **a** and NM23/NDPK **b** in colorectal carcinoma (CRC). Marked p53 nuclear expression in most of the glandular cells of a moderately differentiated CRC (magnification, ×250, a). Intense nm23-H1 cytoplasmic staining in almost all the glandular cells of the same tumor (magnification, ×400, b)

DFS was measured from the day of primary surgery to the date of the first relapse (locoregional or metastatic). Significant differences among survival curves were checked by the log-rank test and Wilcoxon test, or a trend test when appropriate. Multivariate analysis was carried out by means of Cox's logistic regression model, using a backward procedure (Cox 1972). P-values less than 0.05 were considered significant.

Results

Immunohistochemical analysis of TP53

Forty-eight percent of the cases analyzed (77/160) presented positive staining for TP53. Of these, eighty-seven percent (67/77) presented exclusively positive nuclear staining, 9% (7/77) showed positive nuclear staining together with staining of the cytoplasm, and 4% (3/77) showed staining of the cytoplasm only.

Immunohistochemical analysis of NM23-H1

Positive immunostaining for NM23-H1 was primarily confined to the cytoplasm, while the nuclei stained negatively, and was observed in 106/160 tumors (66%). Of 106 positive tumors, 49 (46%) were strongly positive ($>50\%$) and 57 (54%) were weakly positive (10–50%).

Flow cytometric analysis

Flow cytometry was performed to obtain adequate DNA histograms for all normal and tumoral tissues. The coefficients of variation of the DNA-diploid peak ranged from 2.5% to 4.8% (median 3.4%). DNA-aneuploidy was found in 120/160 cases (75%), while 18% of these (22/120) showed multiclonality. The SPF ranged from 2.1% to 32.6% (median: 18.3% and interquartile range: 14.1–21.7%). The median SPF of DNA-aneuploid tumors was 19.2% while that of the DNA-diploid tumors was 12.4% ($P<0.01$). By using the SPF median value as cut-off point, tumors were accordingly divided into low (≤ 18.3) and high (>18.3) SPF tumors.

Relationship between TP53 and NM23-H1 expression with biological and clinicopathological data

Table 2 shows the significant relationships between TP53 protein expression and tumor site ($P<0.05$) and DNA-ploidy ($P<0.05$). Moreover, no significant association was observed between TP53 protein expression and the other cytometric and clinicopathological variables analyzed (data not shown).

There was no significant difference in tumor site, histologic grade, presence of inflammation, Dukes' stage, lymphohematic invasion, lymph node status, SPF, type of surgery, mucinous pattern of the tumoral cells, and type of tumoral growth between patients with NM23-H1 positive colorectal cancer and those with NM23-H1 negative tumors (data not shown).

The distribution of TP53 positive and negative tumors did not show any significant difference in the NM23-H1 staining patterns ($P=0.943$). DNA-aneuploidy was associated with distal tumors ($P<0.01$), histological grade (G3) ($P<0.05$), advanced Dukes' stage (C and D) ($P<0.01$), lymph node metastases ($P<0.01$), and high SPF ($>18.3\%$) ($P<0.01$) (Table 3).

Table 2 Significant relationships between TP53 expression, site, and DNA ploidy

Site	TP53 expression		
	Negative	Positive	*P*
Proximal tumors	21	10	
Distal tumors	62	67	<0.05
DNA-ploidy			
Diploid	29	11	
Aneuploid monoclonal	44	54	
Aneuploid multiclonal	10	12	<0.05
Total	83	77	

Table 3 Relationships of DNA-ploidy to clinicopathological and biological variables of CRCs

	DNA-ploidy		*P*
	Diploid (%)	Aneuploid (%)	
Total	40 (25)	120 (75)	
Site			
Proximal tumors	19 (61)	12 (39)	
Distal tumors	21 (16)	108 (84)	< .01
Tumor grade			
G1	12 (48)	13 (52)	
G2	21 (21)	81 (79)	< .05
G3	7 (21)	26 (79)	
Dukes' stage			
A + B	30 (33)	61 (67)	
C + D	10 (14)	59 (86)	< .01
Node status			
Negative	32 (32)	67 (68)	
Positive	8 (13)	53 (87)	< .01
SPF			
≤ 18.3%	32 (40)	47 (60)	
> 18.3%	8 (10)	73 (90)	< .01

Uni- and multivariate analysis of prognostic factors

The median follow-up time in our study group was 71 months (range 34–115 months). The median survival of the whole group was 43 months. At univariate analysis, distal cancers ($P < 0.05$), advanced Dukes' stage ($P < 0.01$), positive node status ($P < 0.05$), lymphohematic invasion ($P < 0.01$), DNA-aneuploidy ($P < 0.01$), and high SPF ($P < 0.01$) proved to be significantly related to quicker relapse, whereas these same factors ($P < 0.05$, $P < 0.01$, $P < 0.01$, and $P < 0.01$, respectively) – and, in addition, infiltrative tumor growth ($P < 0.01$), prominent lymphocytic infiltration ($P < 0.05$), and non-curative resection ($P < 0.01$) – were significantly related to shorter overall survival (data not shown). Figures 2 and 3 show: a) the probability of disease-free survival; and overall survival b) according to DNA-ploidy and SPF, respectively. The significant variables at univariate analysis were entered in a multivariate Cox's logistic regression model with backward elimination. The major significant predictors for both disease relapse and death were advanced Dukes' stage, aneuploid tumors, and high SPF, while lymphohematic invasion was the only independent factor for relapse and non-curative resection for death (Table 4).

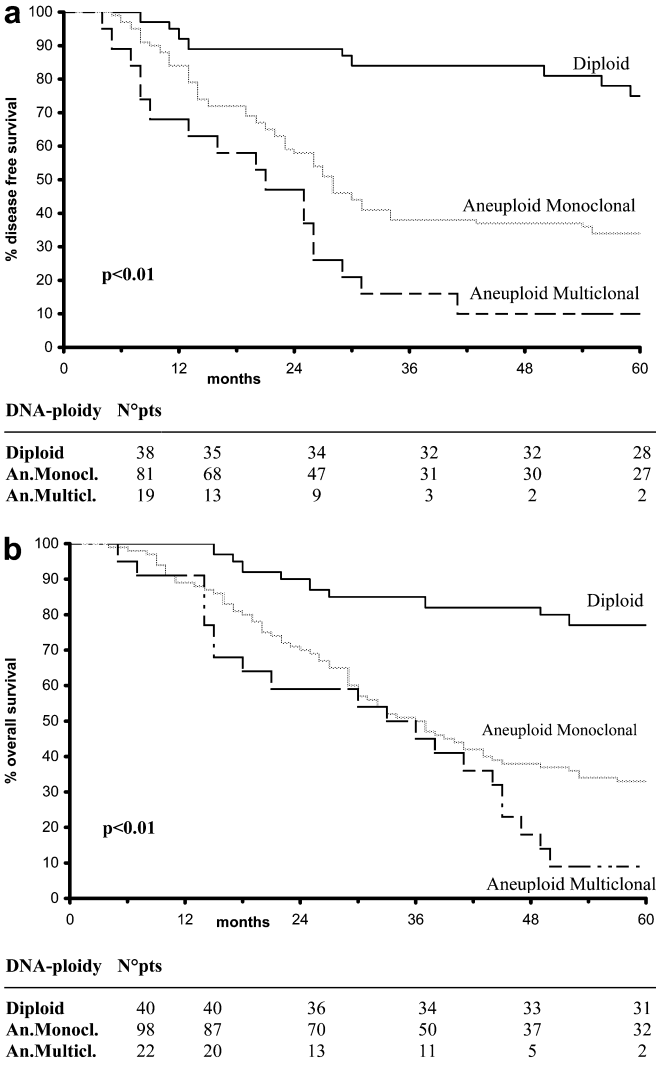

Fig. 2 Disease-free survival **a** and overall survival **b** of 160 patients with colorectal carcinoma (CRC) according to DNA-ploidy

Discussion

Although it has been recognized for some time that p53 mutations play an important role in the development and progression of CRCs (McLeod and Murray 1999), the importance of an increased expression of this protein is still not clear, since this does not always occur as a result of mutations inactivating its functionality. Increased expression, in fact, may also be the physiological consequence of different types of stress and is linked to the action of other factors, such as MDM 2, which regulate its turnover (Lane and Hall 1997).

The expression of NM23 presents an even more complex situation, since the results reported in literature, once again controversial, have not yet been able to explain how NM23 is involved in the metastatization process (Gazzeri et al. 1996; Martinez et al. 1995; Nakamura et al. 1998; Tannapfel et al. 1995).

In our study we focused attention on TP53 and NM23-H1 expression and flow cytometric variables, in the attempt to clarify the potential role of these factors in the development of CRCs and to evaluate their possible prognostic significance. We found a TP53 overexpression frequency of 48% (77/160); other authors report results in CRCs ranging from 42% to 74% (Auvinen et al. 1994; Bouzourene et al. 2000; Jansson et al. 2001; Kaserer et al. 2000; Scott et al. 1991). Moreover, our results showed NM23-H1 expression in 66%

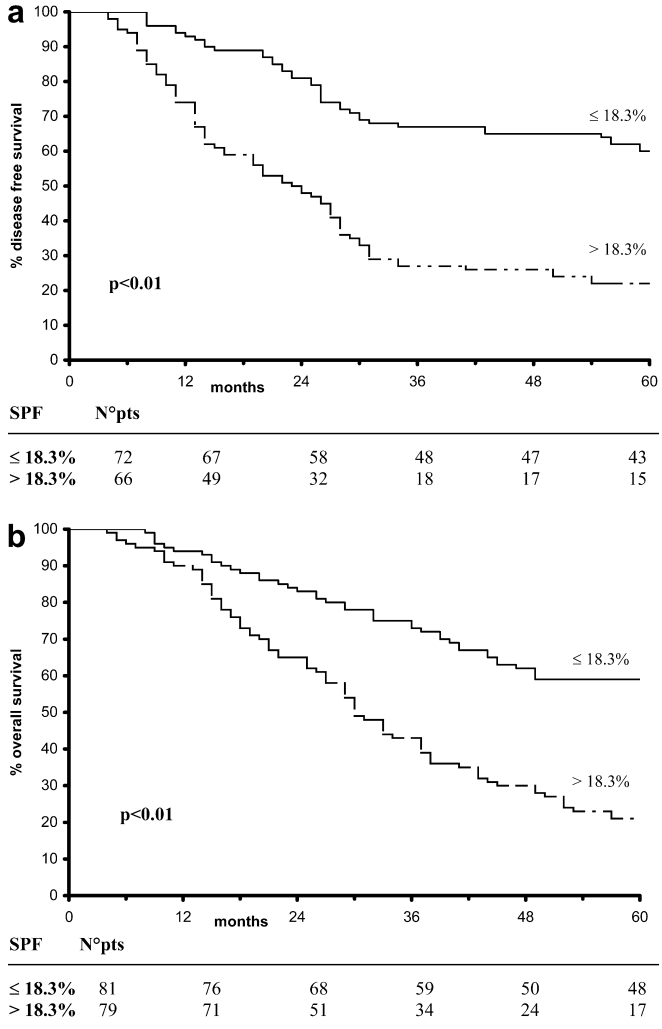

Fig. 3 Disease-free survival **a** and overall survival **b** of 160 patients with colorectal carcinoma (CRC) according to S-phase fraction (SPF)

(106/160) of the tumor cell cytoplasm while the nuclei stained negatively: 54% (57/106) displayed a moderately homogeneous positivity (≤ 50%) while 46% (49/106) showed strong NM23 immunoreactivity (> 50%), in accordance with more recent reports in literature, where cytoplasm staining ranges from 27% to 79% (Indinnimeo et al. 2000; Lee et al. 2001; Nesi et al. 2001; Sarris et al. 2001; Tabuchi et al. 1999).

Variability in TP53 and NM23-H1 immunoreactivity is mainly due to tumoral heterogeneity and the specific features of the patient cohorts included in the study, such as histopathologic staging, grading, and site of the tumor. Furthermore, the TP53 staining pattern may be influenced by the antibody used for the identification of the protein. In fact, the monoclonal antibody DO7 used in our study made it possible to identify a mainly positive nuclear staining, compared to the more positive cytoplasmatic result reported in other studies where the polyclonal antibody CM1 was used (Bosari et al. 1994; Sun et al. 1992). Our study, in fact, showed TP53 cytoplasmatic positivity in only ten of the cases. Moreover, the choice of a median cut-off point of 5% further reduces the risk of obtaining false positive results due to background or artefacts.

The DNA-aneuploidy rate observed in our study was of 75%, which is among the highest so far reported in literature (range from 39% to 89%) (Ross 1996; Silvestrini 2000), probably due to the multiple sampling performed in all of the cases studied, which considerably reduces the probably of missing aneuploid clones at analysis. In fact, CRCs are heterogeneous, both from the histopathologic point of view and also with regard to cellular DNA content (Flyger et al. 1999; Quirke et al. 1985). Furthermore, our high DNA-aneuploidy rate might depend on the choice of freezing to –80 °C rather than paraffin-embedding as the sample storage method. Although the analysis of paraffin-embedded samples permits the retrospective evaluation of a large number of cases with suitable follow-up, this type of storage may compromise the reliability of the results due to the presence of a relatively large quantity of debris, to poor histogram resolution, and to high coefficients of variation.

In the first part of our investigation, we analyzed the relationships between p53 and nm23-H1 gene product expression and the flow cytometric variables and clinicopathological characteristics. TP53 overexpression was significantly associated with DNA aneuploidy

Table 4 Cox proportional hazards analysis to predict the HR of relapse or death in CRC patients (*HR* hazard ratio, *CI* confidence interval)

	Relapse (n = 138)		Death (n = 160)	
Dukes' stage D vs A	HR (95% CI) 3.02 (1.26–7.24)	P value < .05	HR (95% CI) 7.04 (3.05–16.3)	P value < .01
Surgery Non-curative resection vs curative resection			3.80 (1.77–8.19)	< .01
Lymphohematic invasion Present vs none	2.18 (1.22–3.89)	< .01		
DNA-ploidy An Monoclonal vs diploid An Multiclonal vs diploid	2.81 (1.36–5.84) 6.76 (2.92–15.6)	< .01 < .01	2.36 (1.17–4.76) 4.76 (2.13–10.7)	< .05 < .01
SPF > 18.3% vs ≤ 18.3%	2.71 (1.68–4.39)	< .01	2.46 (1.57–3.83)	< .01

($P < 0.05$); this is not surprising since an incorrect functionality of the p53 "guardian of genomic integrity" might be responsible for genomic alterations leading to a greater probability of the development of cell populations containing aneuploid DNA. This latter, in its turn, was associated with high SPF, which might result from higher proliferative activity of the aneuploid clones or else from a prolongation of the S-phase in an altered cell cycle, once again leading to a higher risk of further genetic alterations and higher probability of the development of populations containing aneuploid DNA.

In our series TP53 overexpression was more frequent in distal carcinomas of the colon ($P < 0.05$) in accordance with several other authors, suggesting that the occurrence of particular gene alterations is dependent on the tumor site. Weisburger (Weisburger 1991) suggests that distal and proximal tumors might involve different types of epidemiological behavior, and subsequently Beart et al. (Beart et al. 1983) assumed the existence of two different pathways of tumoral progression for CRCs originating in the two tracts. This idea was enhanced by the additional observation of a different distribution of cell DNA content in tumors of the right and left colon reported by many authors (Delattre et al. 1989; Lanza et al. 1998; Meling et al. 1993). We also found an extremely significant association between aneuploid DNA and distal tumors ($P < 0.01$). The fact that cell DNA content is an important indicator of CRC progression (Silvestrini 2000) also emerges from our study, where tumors with aneuploid DNA are mainly undifferentiated ($P < 0.05$), at advanced stages ($P < 0.01$) and accompanied by lymph node metastases ($P < 0.01$).

Although several authors have recently suggested that abnormalities in the nm23-H1 gene or of its expression are found in particularly aggressive tumors and which give rise to lymph node and distant metastases (Martinez et al. 1995; Tannapfel et al. 1995; Yamagushi et al. 1993; Wang et al. 1993), our own data failed to disclose any significant association between NM23-H1 expression and the clinicopathological and biological variables analyzed. Thus, our study, like many others, indicates that the role and importance of the nm23 gene in the development of tumoral metastases is questionable. Several authors, in fact, have found an association between the presence of mutations in the gene nm23 and the development of metastases in CRCs (Wang et al. 1993). Other researchers have demonstrated that NM23-H1 expression was significantly lower in advanced stages of CRCs and with lymph node and liver metastases (Yamagushi et al. 1993; Martinez et al. 1995; Tannapfel et al. 2000). On the other hand, other authors have not found any apparent differences in the mutational status of nm23-H1 with regard to metastatic potential (Myeroff and Markowitz 1993), either in NM23-H1 expression at various tumoral stages (Lindmark 1996), or where metastases are present (Haut et al. 1991; Lee et al. 2000).

Furthermore, with regard to the different biological variables analyzed, our study did not show any significant difference between the NM23-H1 staining pattern and TP53 positive and negative tumors, nor did we find any association between NM23 immunoreactivity and flow cytometry variables (DNA ploidy and SPF). In our opinion, therefore, a clearer understanding of the role of nm23-H1 in the process of tumoral progression and metastatization of CRCs can only be reached by further research aimed at the evaluation of expression of the other nm23 genes, of the genetic alterations occurring in these genes, and possible associations with other genetic changes.

In our prospective study, based on univariate and multivariate analyses with established prognostic indicators (such as Dukes' stage, tumor grade, and lymphatic invasion), we found that DNA-ploidy and SPF, but not TP53 or NM23-H1 expression, are significant and independent prognostic factors for disease-free survival (DFS) and overall survival (OS) in patients with CRC who have undergone surgical resection.

Whether cell DNA content is a significant prognostic factor in CRC is still not clear from the data reported in literature (Silvestrini 2000). While several authors have suggested that DNA-ploidy is an independent variable (Kimura et al. 1996; Lanza et al. 1998; Salud et al. 1999; Scott et al. 1987; Witzig et al. 1991), others have reported that this biological variable is not associated with clinical outcome in CRC (Purdie et al. 2000; Tonouchi et al. 1998; Zarbo et al. 1997). These conflicting results may be partly due to several factors, such as patient selection, number of cases studied, intratumoral heterogeneity, sampling methods, analytic techniques, lack of standardization, and inadequate control of the techniques from one laboratory to another, and interpretation of results.

From the clinical point of view, CRCs containing multiple abnormal stemlines ("DNA-multiploid tumors") might have a more adverse prognosis than those containing a single abnormal stemline (Cosimelli et al. 1998). In our own study, in fact, multivariate analysis of the patient sub-group with multiclonal tumors showed both a higher risk of disease relapse and of death.

Despite the use of different mathematical models, the prognostic value of SPF in CRCs seems to be clearer. Literature reports almost all agree that this biological variable is a major determinant of biological aggressiveness and that it has a predictive role in clinical outcome (Dandone et al. 2001; Salud et al. 1999). This is in accordance with our own results, where SPF was identified as an independent prognostic factor.

With regard to the prognostic impact of p53 alterations in CRC patients, mutations of this gene seem to be much more important than variations of expression levels (Borresen-Dale et al. 1998; Bosari et al. 1995), a fact confirmed by other authors and by our own results, where TP53 expression did not prove to be significant for prognosis (Allegra et al. 2002; Kandioler et al. 2002).

This study was not able to define any association between NM23-H1 staining pattern and disease-free and overall survival for CRC patients; in fact, although several other authors have reported an association between NM23-H1 expression and the clinical outcome of

patients, (Barney et al. 1998; Qin et al. 2000), others have not observed, as we did, any involvement of nm23-H1 in patient survival time (Cheah et al. 1998; Heys et al. 1998; Lee et al. 2001; Lindmark 1996).

In conclusion, our results indicate that DNA aneuploidy and high SPF are associated with a poor clinical 5-year outcome, while, on the contrary, the prognostic role of NM23-H1 and TP53 expression is still to be clarified.

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