



# Loss of p27 expression and microsatellite instability in sporadic colorectal cancer

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Accepted 30 September 2006

## KEYWORDS

p27;  
Fhit;  
MSI;  
Colorectal cancer;  
hMlh1;  
hMsh2

## Summary

**Background:** The role of the loss of p27 protein expression in the oncogenesis of colorectal cancer is still in debate. In this study, we prospectively examined the immunohistochemical expression of p27 in 108 consecutive colorectal cancers, and we analysed the relationship with the results, the clinicopathological data, microsatellite instability (MSI) and other genetic alterations of tumours.

**Methods:** Unselected patients (108) who underwent curative colorectal resection for sporadic colorectal cancer in a three-year period were evaluated for MSI using 6 microsatellite markers, and for the presence of p27, p53, Fhit, Mlh1 and Msh2 proteins by means of immunostaining. The relationships between these markers were analysed. p27 protein expression was examined for association with disease recurrences and survival.

**Results:** Lack of p27 expression was noted in 33 out of 108 (30.5%) colorectal cancer cases ( $P < 0.05$ ). This altered expression was significantly higher in proximal cancers ( $P < 0.05$ ), mucinous tumours ( $P < 0.001$ ), poorly differentiated histology ( $P < 0.01$ ), cancers with MSI ( $P < 0.05$ ), tumours with altered expression of Mlh1 ( $P < 0.01$ ), of Msh2 ( $P < 0.05$ ), and of Fhit ( $P < 0.01$ ). Overall survival was better in the patient group with altered level of phenotypic p27 expression, although the difference does not reach statistical significance ( $P = 0.069$ ). The analysis performed only for patients with tumour at stage II showed significantly better survival when the tumour exhibited altered p27 expression ( $P < 0.02$ ).

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*Conclusions:* The results of the present study support the hypothesis that altered expression of p27 may be part of the genetic pathway involving MSI, which is responsible for the development of some colorectal cancers.

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## Introduction

The p27<sup>kip1</sup> gene (p27) is located on chromosome 12p13, and it is a member of the universal cyclin-dependent kinase inhibitor family [1]. In addition to its role as a cyclin-dependent kinase inhibitor, p27 is a putative tumour suppressor gene: loss of p27 protein expression may result in tumour development and/or progression; however, this loss of expression does not appear to result from gene mutations [2,3]. A study reported that p27 has a role in regulating drug resistance in solid tumours [4], some studies have implicated p27 as a promoter of apoptosis [5], and many studies have shown that p27 has a role in regulating differentiation in some tissues [6,7].

A large number of studies have characterized p27 as a prognostic factor in various human cancers, including colorectal adenocarcinoma. Results of studies on patients with colorectal cancer have revealed discrepancies. Several studies have reported the lack of p27 expression as being associated with short patient survival [8–11], an association not found, however, in some others [12,13]. Some studies have suggested that p27 is an independent prognostic factor for patients specifically with early stage colorectal cancers [8,10], whereas others [9,14] have observed that p27 expression is an independent prognostic marker for patients with stage III colorectal cancer (CRC). The reasons for these contradictory results are not known; the admixture of patient population for different types of colorectal cancer may be affecting these findings. It is well known that two different genetic pathways are involved in the carcinogenesis of colorectal cancer, one involving chromosomal instability and the other involving microsatellite instability (MSI) [15]. The first pathway includes mutation in p53 and K-ras and loss of heterozygosity (LOH) of chromosome regions such as 4p, 5q, 8p, 17p and 18q, 22q [16,17]. The second pathway is characterized by disruption of the DNA mismatch repair system (MMR), which normally maintains sequence fidelity during DNA replication [18].

In this study, we prospectively examined the immunohistochemical expression of p27 in 108 consecutive colorectal cancers, and we analysed the relationship with the results, the clinicopathological data, MSI, and other genetic alterations of tumours.

## Patients and methods

### Study population

The study population consisted of 120 unselected consecutive patients who had undergone curative colorectal

resection for sporadic CRC between January 1997 and April 1999 at our surgical unit. All cases were deemed sporadic, based on the absence of relevant family history as recorded prospectively at initial patient interview. A curative operation was defined as one in which no macroscopic tumour remained at the end of surgery and in which histopathologic examination of the operative specimen showed no tumour at the margins of resection. Distant metastases at the time of resection were excluded by preoperative liver ultrasonography, chest X-ray and intraoperative exploration. Seven patients were excluded on the basis of insufficient tissue for analysis, 5 patients were excluded because they were lost at follow-up, leaving 108 patients for study (55 males and 53 females).

During the study period, a uniform surgical management protocol was adopted.

Proximal colon was defined as the large bowel proximal to the splenic flexure, and distal colon was defined as the large bowel distal to the splenic flexure.

All specimens underwent histopathological analysis by the same gastrointestinal pathologist (B.C.), who was unaware of the interim results of molecular genetic and immunohistochemical analysis. In accordance with the classification of tumours by the World Health Organisation [19], tumours were defined as mucinous when 50% or more of the tumour mass consisted of accumulated mucin, mostly extracellular; the other tumours were classified as “adenocarcinoma, not otherwise specified”. According to Rosai [20] tumours were stratified into two categories: low grade (including grades I and II) and high grade (grade III). Due to their overall worse prognosis [26] mucinous carcinomas were included in the high grade category.

A mean of 12 lymph nodes were examined per case. Tumours were staged in accordance with the TNM system.

Patients with stage III colon cancer under 75 years received adjuvant chemotherapy [21]. The drug regimen for chemotherapy was 375 mg/m<sup>2</sup>/d 5-FU and 20 mg/m<sup>2</sup>/d levamisole, 5 days/week every 4 weeks for 6 months. Patients with rectal cancer received irradiation therapy administered in a dosage of 40 Gy, divided into 16 daily doses of 2.5 Gy each (4 doses/week for 4 weeks) before surgery [22].

Patients were observed at 3-month intervals for 24 months after the completion of therapy, then every 6 months for 3 years, and then yearly. History and physical examination, complete blood cell and platelet count, liver chemistries, ultrasound and carcinoembryonic antigen measurement were performed at each visit, and chest X-ray, colonoscopy and CT were performed once a year.

Local recurrence was defined as the re-growth of the tumour in and around the tumour bed—including the

pericolonic fat, the adjoining mesentery and lymph nodes—or in the suture or staple line of the bowel anastomosis, occurring either alone or in conjunction with generalised recurrence. We adopted the methodology to be followed in the reporting of studies of recurrences after resection of colorectal tumours recently suggested by Dent et al. [23].

Ethical approval for the study was obtained from the Human Ethics Committee of the University of Parma.

### DNA preparation, LOH and MSI testing

Specimens of freshly resected colorectal carcinomas were snap-frozen in liquid nitrogen and subsequently stored at  $-80^{\circ}\text{C}$ . In all cases, fresh specimens of normal colon mucosa were also collected and used as matching controls.

DNA was extracted using the QIAGEN DNeasy tissue kit (QIAGEN, Hilden, Germany) from 15 to 25 cryostat sections (20  $\mu\text{m}$ -thick) of the tumours and matching normal samples. Only tumour samples containing at least 80% of neoplastic cells were included in the study.

### Polymerase chain reaction (PCR)

The following panel of six polymorphic microsatellite markers located on chromosomal regions potentially involved in CRC development and progression was used: D18S58 (18q22-23), D18S61 (18q22) [24], BAT26 (2p16), BAT40 (1p13) [25], D8S254 (8p22) [17], and D4S2397 (4p14-16) [16]. The markers were selected from the Genome database ([www.gdb.org](http://www.gdb.org)) on the basis of chromosomal location and heterozygosity. The PCR conditions and fragment analysis have been described in more detail previously [26].

### Definition of allelic loss (LOH)

An imbalance factor was calculated as the ratio of relative allelic peak area in the tumour DNA to relative allelic peak area in the corresponding normal DNA on the basis of the following formula:

$$\frac{(\text{lower allele/higher allele})_{\text{tum}}}{(\text{lower allele/higher allele})_{\text{norm}}}$$

For informative markers LOH was scored when signal reduction for one allele was 40% or more [27].

### Microsatellite instability (MSI)

The novel appearance in the tumour DNA of one or more alleles, i.e. new peaks in the electropherogram, not present in its paired normal DNA, was the indicator of MSI [28].

Tumours were classified as high frequency MSI (MSI-H) when instability was detected in at least 30% of the interpretable microsatellite markers investigated, or as low-frequency MSI (MSI-L) when instability was found in less than 30% of the markers, in accordance with international criteria [29]. Tumours without MSI were defined as microsatellite-stable (MSS) [29]. For the purposes of this study, MSS and MSI-L cases were considered together [30].

### Immunohistochemical staining

For immunohistochemical analysis the specimens containing tumour and normal glands of the same snap frozen tumours were routinely fixed in buffered 10% formalin and embedded in paraffin. Sections of 5  $\mu\text{m}$  were stained with haematoxylin and eosin for histological diagnosis and with the following primary antibodies: anti-hMsh2 (Clone FE11, Oncogene Research Products, Cambridge, MA, USA; working dilution: 1/20); anti-hMlh1 (clone G168-728, Pharmingen, San Diego, CA, USA working dilution: 1/75), anti-Fhit clone (Polyclonal-ZR44, Zymed Laboratories, San Francisco, CA, USA, working dilution: 1/50), with anti-p27 (clone SX53G8, Dako, Glostrup, Denmark; working dilution: 1/50), and anti-p53 (clone DO7, Dako, Glostrup, Denmark; working dilution: 1/50). The antibodies (Ab), clones, pretreatments, working dilutions, incubation time and localisation of the immunostaining are listed in Table 1.

For antigen retrieval, sections were treated with 10 mM citrate at pH 6.0, in a 750-W microwave oven for three 5-minute cycles. The sections were immunostained with the streptavidin-biotin kit (LSAB2, Dako) in accordance with the manufacturer's specifications and counterstained with haematoxylin. Positive controls were the normal glands of the intestinal crypts for anti-hMsh2, hMlh1, Fhit; peritumoral lymphocytes for anti-p27; colorectal carcinomas strongly positive for anti-p53. Negative controls consisted of substituting the primary antibodies with the normal serum.

### Semiquantitative analysis

p27 was evaluated in a semiquantitative manner by rough estimation of the percentage of immunoreactive nuclei and tumor subdivision into two classes, low and high expressors, using the cut-off level of 50% [31]. For this study, we

**Table 1** Type and characteristics of antibodies used in the study.

Antibodies	Clone	Treatment	Dilution	Incubation $^{\circ}\text{C}$	Type of positivity
hMlh1	G168-728, Pharmingen	Citrate, MW	1:75	o/n, 4	Nuclear
hMsh2	FE11, Oncogene	Citrate, MW	1:20	o/n, 4	Nuclear
p27	SX53G8, Dako	Citrate, MW	1:50	o/n, 4	Nuclear
p53	DO7, Dako	Citrate, MW	1:50	o/n, 4	Nuclear
Fhit	Pab- ZR744, Zymed	Citrate, MW	1:50	o/n, 4	Cytoplasmic

MW, microwave oven; o/n, overnight.

considered only distinct nuclear expression of p27. The normal colonic epithelia served as internal controls for p27 staining.

For the antibodies anti hMlh1, hMsh2 and Fhit the semiquantitative analysis was performed using the following score according to the number of positive tumour cells as follows: 0% (0), <10%(1), 10–50% (2), 51–80% (3), or >80% (4) and the intensity of staining evaluated as weak (1+), moderate (2+) or strong (3+). For each tumour case, the values of the two parameters were multiplied, resulting in scores ranging from 0 to 12 [32,33]. For the purposes of the study, staining of tumour nuclei for hMlh1 and hMsh2 and cytoplasm for Fhit was evaluated as absent (no protein) or present (any evidence). The 0–6 scores were considered as altered expression, 7–12 as preserved expression [32,33].

Finally, the expression of p53 was analysed on the basis of the frequency of positive cells using a cut-off level of 10% [34].

### Statistical analysis

Clinico-pathological and molecular data, immunohistochemical results, recurrence frequency and patient survival were analysed statistically in relation to the p27 protein expression of the tumours.

Contingency tables of the  $\chi^2$  test and/or Fisher exact test were used to evaluate differences between percentages. Factors found to be associated with p27 protein expression were included in a multiple logistic regression analysis which was performed by the SPSS System for Windows release 13.0. Stepwise selection of predictive factors was used.

The statistical analyses for the recurrences and for survival were performed excluding patients with palliative surgical treatment.

Disease-free interval in patients who had recurrence was measured as the interval between the date of resection and the date of diagnosis of recurrence.

Duration of survival was measured from the date of resection until the date of death from any cause or until the censoring date of April 30, 2004. In the survival analysis, deaths due to postoperative complications within 30 days were excluded. Survival curves were drawn according to the method of Kaplan and Meier, and differences in survival and disease-free interval were evaluated by means of the log-rank test.

All reported *p* values are two tailed. Statistical significance level was set at 0.05.

## Results

### Clinical, pathological, and biological features

Mean age of the 108 patients at the time of surgery was 70.1 years (range 41–94 years). The clinical, pathological, and biological features are reported in Table 2.

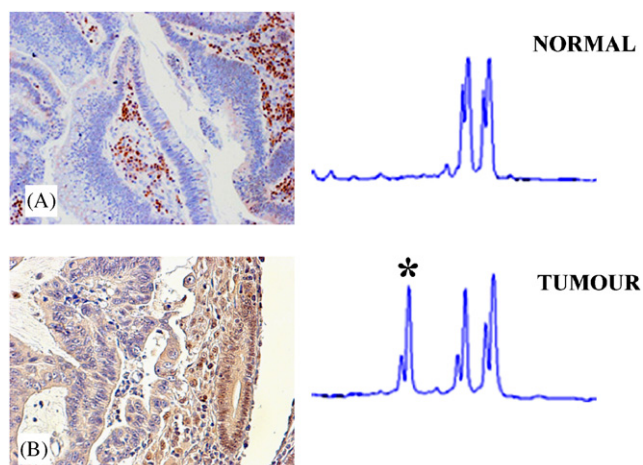
### Genetic alterations

#### p27 expression

Altered (loss) p27 expression was noted in 33 out of 108 (30.5%) colorectal cancer cases (Fig. 1).

**Table 2** Clinicopathological characteristics and expression of p27<sup>kip1</sup> in 108 colorectal adenocarcinomas.

	No.	(%)
Sex		
Male	55	50.9
Female	53	49.1
Tumour location		
Proximal	46	42.6
Distal	62	57.4
Tumour differentiation		
Well-Moderate	67	62.0
Poor	41	38.0
Tumour stage (TNM)		
I	8	7.4
II	54	50.0
III	28	25.9
IV	18	16.7
Tumour type		
Nonmucinous	64	59.3
Mucinous	44	40.7
Expression of p27 <sup>kip1</sup>		
Altered	33	30.6
Normal	75	69.4



**Figure 1** Immunohistochemical expression of p27 and hMlh1 proteins in a patient affected by colorectal carcinoma with microsatellite instability phenotype. (A) Lack of immunohistochemical expression of p27 protein in tumoural tissue with concomitant intense staining of the intratumoural lymphocytes, (B) Lack of immunohistochemical expression of hMlh1 protein in tumoural tissue with concomitant intense staining of normal mucosa nuclei, and (C) Electropherogram of the same tumour showing the appearance of a new peak in tumoural tissue indicating the presence of MSI for D18S58 marker.

When the relationship between these results and the clinicopathological data (age and sex of patients, tumour location, histological type, grading, stage) was analysed, patients with tumours with altered or absent staining for p27 resulted as being older than patients with tumours with normal staining:  $73.48 \pm 10.60$  years versus  $68.65 \pm 12.24$

years ( $P < 0.05$ ); altered staining for p27 was detected in 43.5% of proximal lesions versus 21% of distal lesions ( $P < 0.05$ ), in 56.8% of mucinous tumours versus 12.5% of not otherwise specified adenocarcinomas ( $P < 0.001$ ), and in 46.3% of grade III cancers vs. 20.1% of grade I–II cancers ( $P < 0.01$ ) (Table 3). No significant associations were found between the p27 expression and other clinicopathological parameters (Table 3).

#### Correlations of p27 expression with MSI, LOH of 4p, 8p, and 18q, and expression of Mlh1, Msh2, Fhit, and p53.

Table 4 reports results regarding correlations between MSI status and immunohistochemical expression of Mlh1 and Msh2.

When the relationship between p27 altered expression and other molecular patterns (MSI, LOH of 4p, 8p, and 18q, and expression of Mlh1, Msh2, Fhit, and p53) was analysed, altered staining for p27 was detected in 59% of colorectal

cancers with MSI versus 24% of MSS lesions ( $P < 0.05$ ), in 53.6% of tumours with loss of the expression of Mlh1 versus 23.1% of tumours with normal expression of Mlh1 ( $P < 0.01$ ), in 63.6% of tumours with loss of the expression of Msh2 versus 27.4% of tumours with normal expression of Msh2 ( $P < 0.05$ ), and in 58.8% of lesions with altered expression of Fhit versus 25.9% of lesions with normal expression of Fhit ( $P < 0.01$ ) (Table 5 and Fig. 1). No significant associations were found between the p27 expression and other examined molecular parameters (Table 5).

#### Multiple logistic regression analysis

When factors found to be significantly associated to p27 protein expression at univariate analysis (Tables 2, 3 and 5) were included in a multiple logistic regression analysis, only the histological type of tumours was significantly related to altered p27 protein expression (OR = 9.008, 95% CI: 3.339–24.304,  $p < 0.001$ ).

**Table 3** Relationship between p27 protein expression and clinicopathological findings in 108 patients with colorectal adenocarcinoma (ADK).

	p27 <sup>kip1</sup> protein expression		
	Preserved*	Altered or absent*	$p^\ddagger$
Sex			
Male	41	14	
Female	34	19	n.s.
Tumour localisation			
Proximal-sided	26	20	
Distal-sided	49	13	<0.05
Histological type			
Mucinous	19	25	
ADK NAS <sup>†</sup>	56	8	<0.001
Grading			
Grade I–II	53	14	
Grade III	22	19	<0.01
TNM stage			
I	5	3	
II	37	17	
III	20	8	
IV	13	5	n.s.

\*Preserved: score 12–7; altered or absent: score 6–0.

<sup>†</sup>ADK NAS: adenocarcinoma not otherwise specified.

<sup>‡</sup>n.s.: not significant.

#### Recurrence rate and survival analysis

##### Recurrence rate

The analyses relating to frequency of recurrence of the disease and to survival were carried out using data relating to 89 patients (82.4%) who had undergone curative surgery. No deaths due to postoperative complications within 30 days were observed among this cohort of patients. The minimum, maximum and median follow-up times for all 89 patients were 36, 66 and 54 months.

Overall recurrence rate was 27% (24 patients); 12 patients (13.4%) had local recurrence without metastases, 5 patients (5.6%) had local recurrence with metastases, 7 patients (7.9%) had metastases without local recurrences.

Overall recurrence rates resulted as being lower in patients who had undergone curative surgery for tumours with altered or absent staining for p27 than in patients who had undergone curative surgery for tumours with normal staining; differences, however, do not reach statistical significance ( $P = 0.072$ ) (Table 6). This analysis, performed separately for patients with tumours at stage II and tumours at stage III ( $P = 0.143$  and  $P = 0.328$ , respectively, data not shown) and for patients with proximal tumours and distal tumours ( $P = 0.093$  and  $P = 1$ , respectively, data not shown) did not show any significant difference.

Disease-free interval in the 22 patients who had recurrence was  $17.2 \pm 4.2$  months. No association was found between disease-free interval and altered or absent staining for p27.

**Table 4** Correlations between microsatellite status and immunohistochemical expression of Mlh1 and Msh2.

Mlh1 expression	Msh2 expression	MSS (83 cases)	MSI-H (22 cases)	MSI-L (3 cases)
Normal	Normal	76	2	3
Altered	Normal	6	13	0
Normal	Altered	0	1	0
Altered	Altered	1	6	0

MSS, microsatellite stable; MSI-H, microsatellite high instability; MSI-L: microsatellite low instability.

**Table 5** Relationship between p27 protein expression, MSI, LOH of 4p, 8p, and 18q, and protein expression of Mlh1, Msh2, Fhit, and p53, in 108 patients with colorectal carcinoma.

	p27 <sup>kip1</sup> protein expression		p <sup>†</sup>
	Preserved*	Altered or absent*	
MSI type (97 patients)			
MSI-L and MSS	57	18	
MSI-H	9	13	<0.05
18q LOH (102 patients)			
–	35	20	
+	36	11	n.s.
8p LOH (62 patients)			
–	28	19	
+	11	4	n.s.
4p LOH (87 patients)			
–	47	24	
+	10	6	n.s.
Mlh1 protein expression (106 patients)	18		
Present	13	15	<0.01
Absent			
Msh2 protein expression (106 patients)			
Present	69	26	
Absent	4	7	<0.05
Fhit protein expression (108 patients)			
Present	68	23	
Absent	7	10	<0.01
p53 protein expression (105 patients)			
Present	36	14	
Absent	37	18	n.s.

\*Preserved: score 12–7; altered or absent: score 6–0.

†n.s.: not significant.

**Table 6** Relationship between p27 protein expression and recurrence rates in 89 patients who underwent curative surgery for colorectal carcinoma.

	p27 <sup>kip1</sup> protein expression		P*
	Preserved (61)	Altered or absent (28)	
Overall recurrence rate (%)	32.8	14.3	n.s.
Local recurrences rate (%) (without metastases)	16.4	7.1	n.s.
Distant recurrence rate (%) (without local recurrence)	8.2	7.1	n.s.
Local recurrence+metastasis rate (%)	8.2	0	n.s.

\*n.s.: not significant.

## Survival analysis

Kaplan–Meier univariate survival analysis on the 88 patients who had undergone curative surgery showed better overall survival in the patient group with altered level of phenotypic p27 expression, although the difference does not reach statistical significance ( $P = 0.069$ ).

Univariate survival analysis based on p27 expression and on tumour location also showed no significant differences for

both anatomical sites ( $P = 0.105$  and  $P = 0.475$  for proximal and distal tumours, respectively, data not shown)

Despite the small size of the sample, we also analysed the significance of p27 expression in the complete study population based on the tumour stage, and from the results of this analysis it would seem that patients with tumours exhibiting altered p27 expression have significantly better overall survival only at stage II ( $P < 0.02$ ). Similar analyses at stages I, III and IV did not show statistically significant

differences between patients with altered or normal levels of p27 expression.

## Discussion

The results of our study showed altered expression of p27 in 30.5% of colorectal cancers, a frequency similar to that observed by others [14]. Similar proportions of colorectal cancers with the altered levels of p27 expression were noted at all stages (28–37%). Similarly to studies by Ciaparrone et al. [35], and by Manne et al. [14] we found a significant correlation between p27 expression and tumour grade with grade III colorectal cancers expressing lower p27 expression, and this result seems to support the hypothesis that p27 has a role in regulating differentiation in some tissues [6,7].

We also found that proximal tumours exhibited altered levels of p27 more frequently than did distal tumours. This result is in agreement with the observation of Palmqvist et al. [41], whereas others [10,37] did not find a relationship between p27 and tumour location, and in the experience of Manne et al. [14] distal tumours exhibited altered levels of p27 expression. In the present study, p27 expression was significantly correlated with tumour type, i.e. mucinous tumours exhibited altered levels of p27, and multiple logistic regression showed that tumour histological type is the only clinico-pathological feature independently related to p27 protein expression. The findings of others [14,36,37] did not show this correlation. Although the exact reasons for these contradictory results are not known, the well known marked geographical variations in the epidemiological characteristics of colorectal cancer [38] probably account for these differences. Mucinous histological subtype accounts for 40% of all colorectal cases in the present study, for 4–5% in studies from Japan and Singapore, [39], for 12–14% in studies from Sweden, Taiwan, Argentina [40–42], and for 30% in a study from Greece [43]. Even the localisation of mucinous histological subtypes varies in the different experiences: in our experience it resulted as being greater in the proximal colon, whereas other authors report a higher frequency of mucinous carcinomas in the rectum and/or distal localisations [44], or do not find significant differences [45].

Our study found altered staining for p27 to be correlated also to the MSI+ phenotype. Differences have been revealed in molecular genetic features of cancer cells of the large bowel, leading to the hypothesis that colorectal cancer arises through at least two distinct genetic pathways, one involving MSI and the other involving chromosomal instability [46]. Microsatellites are distributed throughout the genome, predominantly in noncoding regions. When MSI affects the coding region of critical genes for cell physiology, it may lead to loss of gene function, thus contributing to malignant transformation [47]. This genetic pathway has been described in hereditary tumours, where MSI appears to be a good marker for defects of the MMR genes, but it has also been suggested in sporadic cancer [48]. High-frequency MSI occurs in approximately 15% of sporadic cases of colorectal cancers. [48,49]. In the present study, high MSI was detected in 20% of the colorectal cancers studied.

In accordance with previous studies [48,49], colorectal cancers with MSI resulted as being more frequent in right-sided, mucinous adenocarcinomas and in poorly differentiated tumours. The correlation between high MSI tumour and altered expression of p27 protein may result from the analogies in clinicopathological features of tumours with MSI and those with altered expression of p27.

The relationship between p27 immunohistochemical expression and MSI phenotype was examined in some other studies [50,51]. Edmoston et al. [50] had not found any correlation between p27 expression and MSI analysing a series of CRC mostly derived from patients with hereditary nonpolyposis CRC. In another study [51], no correlation was seen when both cytoplasmic and nuclear staining were considered, although predominant nuclear localisation of p27 was associated with mutations in TGF-beta receptor II, a potent inhibitor of epithelial cell growth. Mutation in TGF-beta receptor II occurs predominantly in MSI tumours, in fact, human colon cancer cell lines with high rates of microsatellite instability were found to harbour mutations in the type II TGF-beta receptor (RII) gene [52].

Among the known MMR genes, mutations of hMLH1 and hMSH2 have been predominantly associated with MSI in the Lynch II hereditary non-polyposis colon cancer syndrome, but also in sporadic colorectal cancer [53]. Our data also showed MMR deficiency based on the status of the Mlh1 and Msh2 protein expression as being significantly associated with altered p27 protein expression. The number of cases with loss of Mlh1 and Msh2 expression resulted as being higher than the number of MSI cases. This result, reiterated in other studies [54], could appear to be a discrepancy, since cases with loss of Mlh1 or Msh2 nearly always have MSI. The lack of microdissection for MSI analysis might have played a role in the discrepancy, even though only tumour samples containing at least 80% of neoplastic cells were included in the study, and this value is universally recognised as being valid in analyses of this type. Recent studies had found that MSI-L is a mild microsatellite mutator phenotype characterized by genetic differences from MSS and MSI-H cancers [55]. To take into account this intermediate phenotype the revised Bethesda Guidelines [56] suggest to test a secondary panel including microsatellite markers (e.g. BAT40 and/or MYCL) sensitive for low levels of instability in both sporadic and familial CRC. Therefore, some cases with MSI-L phenotype not recognised using the classical Bethesda panel could account, at least in part, for the discrepancy in our study.

The results of a recent study of ours [33] support the hypothesis that the FHIT gene alteration may be part of the genetic pathway involving MSI, and the results of this study support this hypothesis, since altered staining for p27 which was correlated to MSI also resulted as being correlated to altered expression of Fhit.

A surprising datum arising from our study regards the outcome of patients affected by colorectal cancer with altered expression of p27: recurrence rate seems on the whole to be lower and overall survival to be better in these patients compared to those with cancer with normal p27 expression. Some of these differences do not reach statistical significance, most likely because of the small number of samples observed, although the data certainly contrast with most of the observations in the literature.

Although there exist prognostically significant discrepancies of p27 expression in colorectal cancer, none of the previous studies had detected a significantly favourable prognosis: several studies reported the lack of p27 expression as being associated with short patient survival [8–11,35,57], whereas other studies do not show any association [12,13,58]. In some studies, it was suggested that altered expression of p27 was an independent predictor of poor prognosis specifically for patients with early stage colorectal cancer [8,10], whereas studies by Tenjo et al. [9] observed that altered p27 expression was a predictor of poor prognosis for patients with stage III colorectal cancers. However, no study has hypothesised what the results of the present study seem to suggest: that patients with tumours exhibiting altered p27 expression have a significantly better overall survival, especially stage II patients.

In our opinion the explanation for these apparently surprising data is to be found in the particular composition of the population under study. Forty percent of the population of this study present colonic cancers with mucinous histological subtype. The literature has shown that mucinous carcinoma in the colorectum represents distinct clinicopathologic and genetic features as compared to non-mucinous tumours with a different biological behaviour [40], and that two subtypes of colorectal mucinous carcinoma must be distinguished in relation to MSI [59]. Mucinous cancers with MSI have better survival [59,60]. Altered expression of p27 in this study resulted as being correlated with mucinous subtype of colon cancer and with genomic instability, and 50% of the mucinous cancers with altered expression of p27 had MSI, belonging to the subtype with better survival. In the other studies on this subject few patients had mucinous carcinoma, and histological type did not correlate with altered p27 expression [14,36,37], perhaps because those mucinous cancers did not belong to the subtype with better survival.

We are aware of the explorative nature of this paper, and it is obvious that the hypotheses proposed need scientific verification; however, we feel able to conclude with the observation that the results obtained in a population of patients affected by colorectal cancer with the characteristics studied by us allow for the hypothesis that altered expression of p27 may be part of the genetic pathway involving MSI, which is responsible for the development of some colorectal cancers.

## Acknowledgement

This work was supported in part by a grant from “Lega Italiana per la lotta contro i tumori”.

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