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Original Citation:

Mutations of the APC gene in human sporadic colorectal cancers / De Filippo C.; Luceri C.; Caderni G.; Pacini M.; Messerini L.; Biggeri A.; Mini E.; Tonelli F.; Cianchi F.; Dolara P.. - In: SCANDINAVIAN JOURNAL OF GASTROENTEROLOGY. - ISSN 0036-5521. - STAMPA. - 37:(2002), pp. 1048-1053.

Availability:

This version is available at: 2158/766127 since:

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Mutations of the *APC* Gene in Human Sporadic Colorectal Cancers

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De Filippo C, Luceri C, Caderni G, Pacini M, Messerini L, Biggeri A, Mini E, Tonelli F, Cianchi F, Dolara P. Mutations of the *APC* gene in human sporadic colorectal cancers. *Scand J Gastroenterol* 2002;37:1048-1053.

Background: Mutations of the *APC* gene are reported to occur frequently in sporadic colorectal adenomas and adenocarcinomas. We studied *APC* gene mutations in cases of human sporadic colorectal cancer in order to evaluate their correlation with pathologic characteristics and clinical prognosis. **Methods:** Most of the mutations of the *APC* gene (95%) are nonsense or frame shift mutations, encoding for truncated APC proteins. For this reason, mutation detection of the *APC* gene was performed using the in vitro synthesized protein (IVSP) assay, analysing the region between nucleotide 2058 and nucleotide 5079 of the gene, containing the mutation cluster region. **Results:** Out of 58 cases of colorectal cancer, 29 presented a mutated form of *APC* (mutation frequency 50%). We did not find a statistically significant correlation between *APC* gene mutation and age, sex, localization of the primary tumour, grading, Crohn-like lymphoid reaction or presence of residual adenoma. Tumours with low invasivity (Dukes' stages A and B) were less frequently mutated (12/27, 44.5%) than tumours of Dukes' stage C (15 out of 21, 71.4%), which developed macroscopically secondary metastasis with variable latency after surgery. Highly invasive tumours with synchronous metastases (Dukes' stage D) had, instead, a low frequency of *APC* mutations (20%, 2/10) ($P = 0.02$, compared with Dukes' stages A, B and C). **Conclusions:** These data suggest that more aggressive Dukes' stage D tumours develop metastasis by means of an unknown mechanism, independent of *APC* mutation.

Key words: *APC*; colorectal cancer; IVSP

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Of all the malignancies of the gastrointestinal tract, colorectal cancer is the most widely studied at the genetic level. According to a popular model (1, 2), colorectal carcinogenesis proceeds through a series of genetic alterations in which loss or inactivation of the tumour suppressor *APC* (adenomatous polyposis coli) gene occurs very early.

The human *APC* gene is mutated in familial adenomatous polyposis (FAP) and in sporadic colorectal tumours (3-5). This gene contains an open reading frame of over 8500 nucleotides (6, 7) and encodes for a cytoplasmic protein of 300 kDa that associates with the cytoskeleton (8) and binds to β -catenin (9), a protein present in adherent junctions, suggesting that the *APC* product might regulate cell adhesion and cell-to-cell communication (10). In fact, β -catenin mediates the interaction of E-cadherin with alpha-catenin and the actin cytoskeleton. Recent evidence indicates that when the *APC* tumour suppressor gene is inactivated, β -catenin can translocate into the nucleus, acting as a transcriptional regulator and promoting the expression of *c-myc* and cyclin D1 (11, 12). Therefore, the *APC*- β -catenin complex appears to be a binary switch. In the absence of outside stimulation by growth factors, *APC* mediates β -

catenin degradation and promotes cell migration. When *APC* loses its function, the up-regulation of β -catenin induces the down-regulation of cell migration and the interaction of β -catenin with Tcf-lef (T cell factor lymphoid enhancer factor), altering gene expression and promoting cell proliferation (13, 14).

About 50%-60% of the somatic mutations of the *APC* gene are clustered in a region of 700 bp, designated in humans as the mutations cluster region (MCR), corresponding to the β -catenin binding domain II (15). Both germ line and somatic mutations (95%) in this gene are nonsense or frame shift mutations, encoding for truncated *APC* proteins, which lack their carboxy-terminal half (15, 16). It was recently shown that the *APC* protein has a highly conserved nuclear export signals 3' region near the MCR (17), and that the ability of *APC* to exit from the nucleus is critical in tumour suppression.

Variable frequencies of *APC* mutations in sporadic colorectal cancer (ranging from 40% to 80%) have been reported (15, 16, 18, 19), but there are still no data in the literature about the prognostic significance of *APC* gene alterations.

In colorectal cancer, conventional pathological staging has served as the standard measure of prognosis. Prediction of survival is straightforward for patients with tumours confined

to the muscularis propria (Dukes' stage A) or with metastatic disease (Dukes' stage D). However, the outcome of intermediately advanced cancer cases (Dukes' stages B and C) is less predictable (20).

The aim of this study was to identify whether a clinical profile is correlated to a particular APC status. Therefore, we correlated APC gene mutations or its loss of heterozygosity (LOH) in locus D5S346 with a series of features at the time of initial diagnosis, such as age, sex, localization of primary tumour, Dukes' stage, grading, type of invasive margin in the histology (expanding or infiltrating), Crohn-like lymphoid reaction, presence of residual adenomas within cancer, relapse rate, distant metastasis and survival.

Methods

Patients, clinical and pathological characterization

Tissue from 58 adenocarcinomas and the respective normal mucosa were obtained from patients undergoing surgical colectomy for sporadic colon cancer between 1994 and 1997 at the University Hospital of Florence, Italy. Patients with a family history of colorectal cancer, FAP or inflammatory bowel disease were excluded from the study. Patients' ages ranged from 23 to 82 years (63.0 ± 1.4 ; mean \pm standard error (*s*)). Samples of tumour tissues and of normal mucosa were frozen immediately after surgery in liquid nitrogen and stored at -80°C until analysis. All slides were reviewed by a pathologist (LM) unaware of mutational analysis and clinical outcome. Tumour histotype and grading were defined according to WHO directives (21). In each case we evaluated the pattern of cancer growth (expanding or infiltrating), the occurrence of Crohn-like lymphoid reaction at the periphery of the tumour and the presence of a residual adenoma within the tumour.

All cases were staged using a modified Dukes' system (22) as follows: A = cancer limited to bowel wall, B = cancer with extramural spread, C = cancer with lymph node metastasis, D = cancer with distant metastasis.

With regard to cancer localization, three anatomical sites were considered: proximal colon (from caecum to transverse colon), distal colon (from splenic flexure to sigmoid-rectum) and rectum.

Patient characteristics studied included: age, sex, relapse (months), survival (months) and site of metastasis.

The study was approved by the Regional Ethics Committee in accordance with the Helsinki declaration of 1975, and written informed consent was obtained from all subjects.

DNA extraction

Genomic DNA was extracted from frozen tumours using the QiAmp tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol.

IVSP (in vitro synthesized protein) assay

The region between nucleotide 2058 and nucleotide 5079

of the APC gene was analysed for mutations using the in vitro synthesised protein (IVSP) assay (23). In brief, two overlapping segments of the APC gene (segment S2 from nt. 2058 to nt. 3651 and segment S3 from nt. 3297 to nt. 5079) were amplified using polymerase chain reaction (PCR) as follows: 200 ng of genomic DNA; 350 ng each of the appropriate primers: segment S2 5'-GGATCCTAATACGACTCACTA-TAGGG AGACCACCATGGATGCATGTGGAACCTTTGTGG-3' and 5'-CTCTTGGCATTAGAT GAAGGTGTGGA-CG-3'; segment S3 5'-GGATCCTAATACGACTCACTA-TAGGGA GACCACCATGGTTTCTCCATACAGGTCACGG-3' and 5'-GGAGGATCCTGTAGGA ATGGTATCTCG-3'.

Two units of Taq Polymerase (Advanced Biotechnologies, Surrey, UK) were used in a 50 μl PCR reaction (10 mM Tris-HCl, 1.5 mM MgCl_2). Amplifications were performed in a thermal cycler (Perkin Elmer 9700, PE Applied Biosystems, Foster City, Calif., USA) for 36 cycles of 40-s denaturation (95°C), 90-s annealing (59°C), 100-s extension (72°C). All PCR reactions included a 5-min extension period (72°C) after the 36th cycle. PCR reaction mixtures (4 μl), purified with chloroform, were used as templates in 25 μl in a linked transcription-translation system (TNT T7 Quick Coupled transcription/translation system, Promega, Madison, Wis., USA) containing 10 μCi of [^{35}S]-methionine (Amersham Italia, Milano, Italy) for 90 min (transcription/translation) at 30°C . Samples were diluted in sample buffer, denatured for 5 min at 95°C and analysed on 12.5% sodium dodecylsulphate-polyacrylamide gel. Proteins were visualized by autoradiography.

LOH analysis

LOH analysis was performed using a PCR-based approach with primers amplifying a (CA) $_n$ locus (D5S346) located 30–70 Kb downstream of the APC gene, which is highly polymorphic. One of two primers from this marker was first end-labelled with $\gamma^{33}\text{P}$ -ATP (2000 Ci/mmol; NEN, Köln, Germany) and T4 DNA polynucleotide kinase (Pharmacia Biotech Italia, Cologno Monzese, Milano, Italy). PCR reactions were carried out in a 15 μl volume containing about 150 ng of genomic DNA, 1X PCR buffer, 1.5 mM MgCl_2 , 0.25 mM dNTPs, 0.2 mM primers, 0.2 μl of the end-labelled primer (10 ng/ μl) and 1.25 units of Taq polymerase (Advanced Biotechnologies, Surrey, UK). Amplifications were performed in a thermal cycler (Perkin Elmer 9700, PE Applied Biosystems, Foster City, Calif., USA) for 30 cycles of 1-min denaturation (94°C), 1-min annealing (60°C), 1-min extension (72°C) and a final extension at 72°C for 5 min. PCR products were separated on a 7% polyacrylamide-urea-formamide gel and visualized by autoradiography.

Sequence analysis

PCR products of mutants were purified for sequencing analysis from agarose gels (QIAquick gel extraction kit, Qiagen, Hilden, Germany). PCR templates were sequenced

using internal primers and DNA sequencing kit dRhodamine Terminator Cycle Sequencing Ready Reaction (ABI PRISM, Perkin Elmer Applied Biosystems, Foster City, Calif., USA) following the manufacturer's protocol.

Statistical analysis

The Fisher exact test and extension to general $R \times C$ tables were performed when appropriate. The level of significance was set at $P < 0.05$, two-sided (24). The correlation between APC alterations and relapse and survival were carried out using univariate and multivariate models by Cox regression analysis in order to identify independent survival factors. Kaplan-Meier curves, used to estimate survival probability as a function of time and patient relapse or survival differences, were analysed by the log-rank test. The statistical analyses were done using STATA 6 Statistical Software (StataCorp, College Station Tx., USA).

Results

Out of 58 cases of colorectal cancer, 29 presented a mutated form of APC (mutation frequency 50%). Using IVSP assay, we found that all 29 tumours had at least 1 mutation in the S3 region, as shown in Fig. 1. Of the 29 mutated tumours, 6 had 2 different mutations in this gene (5 in the S2 region and 1 in the S3 region) and 3 had LOH in D5AS346 locus. Of the 35 mutations identified, 51.4% were insertions, 20% were deletions and 28.6% were nonsense mutations (Table I). All insertions or deletions produced a frame shift that would truncate the APC protein. Almost all the mutations (26/29, 89%) occurred in the S3 region at the MCR, involving β -catenin binding domain II. We also analysed allelic losses in locus D5AS346, localized on chromosome 5, between APC and MCC genes. Four out of 43 informative tumours showed allelic losses (9.3%) and 3 out of 4 were also mutated in APC; the 3 tumours with LOH had a mucinous histotype.

We did not find any significant correlation between APC status and age, sex, localization of primary tumour, grading, growth pattern at the invasive margin, presence of Crohn-like lymphocyte reaction and presence of residual adenomas within the tumour (Table II).

Tumours with low invasivity (Dukes' stages A and B) were less frequently mutated (12/27, 44.5%) than tumours with higher invasivity (Dukes' stage C, 15/21, 71.4%), but this difference did not reach statistical significance ($P = 0.0824$). However, highly invasive tumours (Dukes' stage D) showed a surprisingly low frequency of APC mutations (2/10, 20%) ($P = 0.020$; Table II). Tumours located in the rectum tended to be more frequently mutated in APC (12/20, 62%) than tumours located in other sites, but this difference was not statistically significant ($P = 0.148$; Table II).

We observed that patients with tumour of Dukes' stage C who developed secondary metastasis (i.e. metastases which probably were present but not macroscopically detectable at the time of surgery) had tumours with a high frequency of

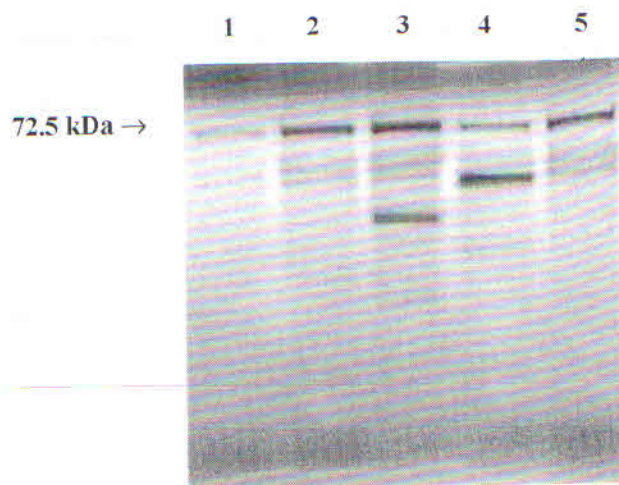


Fig. 1. IVSP analysis of APC fragment S3 on colorectal tumours. Lanes 1, 2 and 5 show bands of APC wild-type proteins (72.5 kDa), while lanes 3 and 4 illustrate bands of truncated APC proteins (40.5 kDa and 47.5 kDa, respectively).

Table I. Sequence analysis of APC mutations

| Tumours | Nucleotide | Nucleotide change | Effect of mutation |
|---------|------------|-------------------|--------------------|
| B1 | 4168 | Ins. G | Frameshift |
| B3 | 4587 | Ins. G | Frameshift |
| B4 | 3898 | CAG → TAG | Stop Codon |
| B5 | 4496 | Ins. A | Frameshift |
| C2 | 3877 | Del. A | Frameshift |
| C4 | 3874 | GAA → TAA | Stop Codon |
| C4 | 3901 | GAA → TAA | Stop Codon |
| C8 | 3752 | Del. A | Frameshift |
| C10 | 4293 | Ins. G | Frameshift |
| C10 | 3134 | Ins. G | Frameshift |
| C12 | 4174 | Ins. C | Frameshift |
| C15 | 4658 | Ins. GGGG | Stop Codon |
| C16 | 4496 | Ins. A | Frameshift |
| C19 | 3843 | Del. GAAAA | Frameshift |
| C20 | 4655 | Ins. G | Frameshift |
| C20 | 2654 | GCA → TGA | Stop Codon |
| C21 | 3882 | Del. A | Frameshift |
| C27 | 4608 | Ins. G | Frameshift |
| C29 | 3898 | CAG → TAG | Stop Codon |
| P16 | 4608 | Ins. G | Frameshift |
| P19 | 4219 | Ins. G | Frameshift |
| P19 | 2607 | TAC → TAG | Stop Codon |
| P20 | 4359 | Ins. T | Frameshift |
| P21 | 4303 | CAA → TAA | Stop Codon |
| P22 | 3946 | AAG → TAG | Stop Codon |
| P28 | 4680 | Del. G | Frameshift |
| P30 | 3755 | Del. CT | Frameshift |
| P38 | 4686 | Ins. C | Frameshift |
| P38 | 2562 | Ins. A | Frameshift |
| P40 | 3910 | Ins. G | Frameshift |
| P43 | 4163 | Ins. G | Frameshift |
| P45 | 4366 | CGA → TGA | Stop Codon |
| P47 | 4281 | Ins. T | Frameshift |
| P48 | 4410 | Ins. G | Frameshift |
| P48 | 3551 | Del. A | Frameshift |

Ins. = Insertion; Del. = Deletion.

Table II. Correlation between APC status and pathological features of tumours

| | Mutated APC | wt APC | P value |
|--------------------------|-------------|------------|---------|
| Dukes' stage | | | |
| A/B (n = 27) | 12 (44.5%) | 15 (55.5%) | |
| C (n = 21) | 15 (71.4%) | 6 (28.6%) | 0.020 |
| D (n = 10) | 2 (20%) | 8 (80%) | |
| Grading* | | | |
| G1 (n = 3) | 2 (66.6%) | 1 (33.3%) | |
| G2 (n = 40) | 19 (47.5%) | 21 (52.5%) | NS |
| G3 (n = 7) | 3 (42.8%) | 4 (57.2%) | |
| Mucinous (n = 7) | 5 (71.4%) | 2 (28.6%) | |
| Type of invasive margin* | | | |
| Expanding (n = 20) | 10 (50%) | 10 (50%) | NS |
| Infiltrating (n = 35) | 17 (48.6%) | 18 (51.4%) | |
| Ex adenoma | | | |
| Yes (n = 13) | 7 (53.8%) | 6 (46.2%) | NS |
| No (n = 45) | 22 (48.8%) | 23 (51.2%) | |
| Crohn-like reaction* | | | |
| Yes (n = 9) | 4 (44.4%) | 5 (55.6%) | NS |
| No (n = 46) | 23 (50%) | 23 (50%) | |
| Tumour localization | | | |
| Proximal colon (n = 17) | 5 (29.4%) | 12 (70.6%) | NS |
| Distal colon (n = 21) | 12 (57.1%) | 9 (42.9%) | |
| Rectum (n = 20) | 12 (60%) | 8 (40%) | |
| LOH in D5S3 locus* | | | |
| Yes (n = 4) | 3 (75%) | 1 (25%) | NS |
| No (n = 52) | 27 (51.9%) | 25 (48.1%) | |

* Number of data missed per category: 1 for grading, 3 for growth, 3 for Crohn and 2 for LOH in D5S3 locus.

APC mutations (8 out of 10, 80%), but this difference was not statistically significant ($P = 0.079$, Table III). Thirteen out of 15 tumours of Dukes' stage C and mutated in APC also presented an infiltrating margin in histological sections (86%). Eight out of 13 of these tumours presented a secondary metastasis and the APC mutations generated a loss of β -catenin-binding domain (6 out of 8 tumours were mutated in MCR and the other 2 tumours had mutations upstream of the MCR). Secondary metastases of 8 tumours with APC mutations were in the liver (5 out of 8, 62.5%), in lymph nodes (2 out of 8, 25%) and local (1 out of 8, 12.5%).

On the contrary, patients with synchronous metastases (macroscopically evident at time of surgery and classified as Dukes' stage D) carried a lower frequency of APC mutations

Table III. Correlation between APC status of tumours and prognosis of the patients

| | Mutated APC | APC wt | P value |
|------------------------|-------------|------------|---------|
| Synchronous metastasis | | | |
| Yes (n = 10)* | 2 (20%) | 8 (80%) | 0.040 |
| No (n = 45) | 26 (57.7%) | 19 (42.3%) | |
| Secondary metastasis | | | |
| Yes (n = 10) | 8 (80%) | 2 (20%) | 0.077 |
| No (n = 45) | 20 (43.7%) | 25 (56.3%) | |
| Relapse | | | |
| Yes (n = 10) | 8 (80%) | 2 (20%) | NS |
| No (n = 35) | 18 (51.4%) | 17 (48.6%) | |

* All tumours with synchronous metastasis are classified as Dukes' stage D. At the follow-up, we missed 3 patients.

Table IV. Correlation between synchronous metastasis and growth pattern

| | Synchronous metastasis | | |
|-----------------------|------------------------|------------|---------|
| | Yes | No | P value |
| Expanding (n = 20) | 0 (0%) | 20 (44.4%) | 0.009 |
| Infiltrating (n = 35) | 10 (100%) | 25 (55.6%) | |
| Total (n = 55)* | | | |

* No information was available about invasive margin for 3 samples out of 58.

compared to patients without synchronous metastases (2/10, 20% and 26/45, 57.7%, respectively, $P = 0.040$; Table III).

We found a significant association between Dukes' stage and growth pattern at the invasive margin. Tumours with high invasivity (Dukes' stages C and D) were more often infiltrating than expanding (stage C: 15 infiltrating out of 19, 78.9% and stage D: 7 infiltrating out of 9, 77.7%; $P < 0.05$).

The growth pattern at the invasive margin significantly influenced relapse. In fact all tumours that developed a secondary metastasis presented an infiltrating margin (10 out of 10, $P < 0.05$; Table IV).

The survival curves of patients according to Dukes' stage is reported in Fig. 2. Univariate analysis performed on 54 patients (no survival data were available for 4 patients) showed, as expected, a significant correlation between survival and Dukes' stage C ($P < 0.001$). On the contrary, there was no significant difference in survival time between patients with APC mutations and those with no mutation.

Multivariate analysis also showed a significant correlation between survival and metastasis ($P < 0.05$). Fourteen out of 15 patients died due to metastatic cancer, with the exception of 1 whose death was unrelated to cancer. The only patient who survived had a tumour located in the recto-sigmoidal junction.

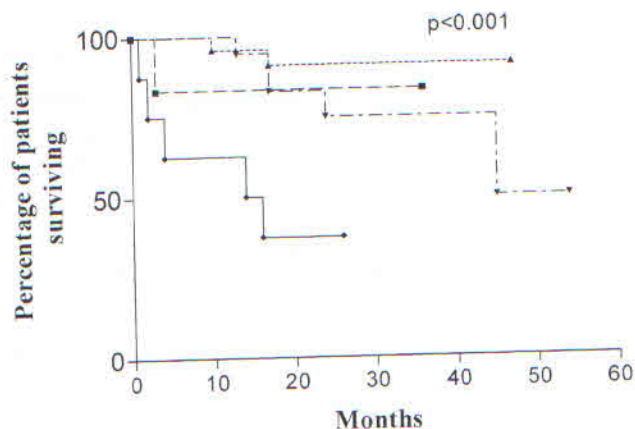


Fig. 2. Kaplan-Meier survival curve of patients according to Dukes' stage ($P < 0.001$). (■ Dukes' A; ▲ Dukes' B; ▼ Dukes' C; ◆ Dukes' D).

Discussion

APC is considered the 'gatekeeper' gene in sporadic colon carcinogenesis and in some hereditary colorectal cancer syndromes (5). Variable frequencies of *APC* mutations have been reported in human colorectal cancer, ranging from 40% to 80%. Powell et al. (16) presented evidence that *APC* mutations occur early during colorectal tumourigenesis. Sequence analysis of 41 colorectal tumours revealed that most carcinomas (60%) and adenomas (63%) contained a mutated *APC* gene. The frequency of such mutations remained constant as tumours progressed to more aggressive stages. This finding provided support for identifying *APC* gene mutation as an initial step in colorectal carcinogenesis (1). However, other authors reported a lower mutation rate of *APC* in sporadic colorectal carcinogenesis (21% in 24 adenocarcinomas tested) (18).

Our analysis indicates that *APC* mutation is indeed a frequent genetic event (50%) in sporadic colorectal cancer. The differences in frequency reported in the literature are probably explained by variations in the methodologies used to detect *APC* mutations. In fact, while Powell analysed all 8500 bp (16), Deuter et al. (18) restricted his analysis to the MCR (700 bp). We analysed the *APC* mutations by IVSP on a 3021 bp region of exon 15 (nt 2058-5079). Therefore, we cannot exclude that analysis of a larger region of the gene could further increase the detection of *APC* mutation. Another possible problem of this analysis could be associated with the contamination by non-transformed cells in tumour samples. To clarify this problem, we performed a dilution experiment, by mixing a PCR product of mutated DNA (S3 fragment) with a wild-type PCR product, in the following proportions: 1:1, 1:2, 1:5, 1:10 and 1:20. Using these mixtures as template for IVSP analysis we found that a truncated protein was still detectable after a 1:10 dilution with wild-type sequences (data not shown). On the basis of these results, we do not think that dilution of the tumour tissue with non-transformed cell should lead to a systematic under-detection of *APC* mutation with IVSP assay.

In our analyses we found that 89% (26 out of 29) of the detected *APC* mutations are clustered in the MCR, which represents only 8% of the entire coding sequence. Such a high frequency of somatic mutations in the central region of this gene had been previously reported (15, 16, 25). Our results confirm that a mutation in this region, which corresponds to the β -catenin binding domain II, is a critical step in colorectal carcinogenesis. Inactivation of the *APC* gene through mutation plus LOH (17%) results in inactivation of both alleles, as described by (25).

Although *APC* mutations were frequently found in these tumours, mutations were not correlated significantly with age, sex, localization of primary tumour, grading, growth pattern at the invasive margin, Crohn-like lymphoid reaction and the presence of residual adenomas within tumour.

Tumours from Dukes' stages A and B patients were less

frequently mutated (44.5%) than those of Dukes' stage C (71.4%). These data suggest that *APC* mutations are not only an early event but may also occur during later stages of carcinogenesis, as proposed by other authors (16, 19, 26, 27).

The overall role of *APC* mutation seemed very complex in our cases. We observed that 86% of the Dukes' stage C tumours with *APC* mutations presented an infiltrating invasive margin and developed secondary metastases after a variable period of time.

APC mutations lead to the loss of the β -catenin-binding domain and to the accumulation of β -catenin, which binds TCF4 and causes transcriptional activation of a number of genes like *c-myc* (11) and up-regulates the expression of the metalloproteinase-7 (MMP-7) matrix (28), explaining the invasivity of the tumour into neighbouring tissues. However, we also observed that highly invasive tumours, located preferentially in the rectum and presenting synchronous metastases at the time of surgery (Dukes' stage D), had a very low frequency of *APC* mutations (20%). The loss of *APC* function might be associated with the delayed metastatic process (secondary metastasis) observed in Dukes' stage C tumours. On the contrary, the aggressive synchronous metastasis of Dukes' stage D tumours seems to be almost independent of the molecular mechanisms regulated by the *APC* gene.

It appears, therefore, that Dukes' stages C and D tumours do not represent progressive stages of the disease, but are distinct in terms of genetic alterations. Our observation needs to be confirmed by analysis of a larger number of patients. Al-Mulla et al. (29) have also suggested in a recent paper that tumours of Dukes' stages C and D differ, since a reduced copy number of chromosome arm 17p (containing p53) is significantly associated with Dukes' stage C, whereas an increase in copy number of 6p and 17q was associated with Dukes' stage D.

In conclusion, our data suggest that alterations in the *APC* gene mutations are involved both in tumour growth and in tumour progression, controlling progression of metastasis in Dukes' stage C; on the contrary, the more aggressive phenotype of the tumours in Dukes' stage D could be *APC*-independent.

Acknowledgements

Supported by grants from the European Community FAIR program (grant no. PL95/0653), the European Community QLRT programs (grants no. 1999/00346 and 1999/00505), the Ministero dell'Università e della Ricerca Scientifica e Tecnologica and the University of Florence (Florence, Italy).

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Received 28 December 2001

Accepted 10 April 2002