

Analysis of *adenomatous polyposis coli* gene expression, *APC* locus-microsatellite instability and *APC* promoter methylation in the progression of melanocytic tumours

Monika Korabiowska¹, Thilo Schlott¹, Nils Siems¹, Anegret Müller², Carlos Cordon-Cardo³, Gösta Fischer⁴ and Ulrich Brinck⁴

¹Department of Cytopathology; ²Department of General Surgery, University of Göttingen, Göttingen, Germany; ³Division of Molecular Pathology, Memorial Sloan-Kettering Cancer Center, New York, NY, USA and ⁴Department of Pathology, Reinhard Nieter Hospital, Wilhelmshaven, Teaching Hospital of the University of Göttingen, Göttingen, Germany

Adenomatous polyposis coli gene (*APC*) defects have been demonstrated for the first time in familial adenomatous polyposis. Recent reports indicate that the *APC* gene is an intermediary between cell adhesion molecules and the cytoskeleton and that it may function as a gatekeeper of colonic epithelial proliferation. The objective of this study was to analyse *APC*'s presence in lentigos, primary melanomas and melanoma metastases. By immunohistochemistry, *APC* was demonstrated in all lentigos, in 75 out of 88 primary melanomas and in 16 out of 28 melanoma lymphatic metastases. The percentage of immunolabelled tumour cells (*APC* index) in lentigos ranged between 5 and 69%, in primary melanomas between 0 and 98% and in melanoma metastases between 0 and 52%. Statistically significant differences between lentigos and primary melanomas and between lentigos and metastases in *APC* expression were found. In a multivariate analysis, *APC* showed an independent prognostic impact. Analysis of microsatellite instability in the *APC* locus was performed on 29 melanomas. Microsatellite instability was found in 5/29 melanomas and loss of heterozygosity in 1/29 melanomas. Promoter methylation of *APC* was found in 6/10 *APC*-negative primary melanomas and in 9/10 *APC*-negative melanoma lymphatic metastases investigated. We conclude about important role of *APC* alterations for melanoma progression.

Modern Pathology (2004) 17, 1539–1544, advance online publication, 16 July 2004; doi:10.1038/modpathol.3800238

Keywords: *APC*; microsatellite instability; melanoma; loss of heterozygosity; promoter methylation

The prediction that the *adenomatous polyposis coli* gene (*APC*) might have tumour suppressor activity has been supported by the fact that carcinomas developed in patients with familial adenomatous polyposis exhibited a high frequency of loss of heterozygosity in the chromosome 5q21–22 region—typical *APC* locus.^{1–4} Loss of heterozygosity with respect to *APC* usually occurs in the normal allele. This fact suggests that both alleles of the *APC* gene could be inactivated by germline mutation and the somatic allele lost in colorectal carcinomas. *APC* protein is mutated in familial adenomatous poly-

posis patients and in sporadic colorectal tumours. Familial adenomatous polyposis coli is an autosomal dominant inherited disease that predisposes carriers to a high probability of colorectal carcinoma.^{5–7} The function of *APC* is still unclear. Recent evidence indicates that the *APC* protein functions as an intermediary between cell adhesion molecules and the cytoskeleton, that it is involved in signal transduction and in controlling apoptosis.^{8–10} Another study suggested that *APC* acts as a gatekeeper of colonic epithelial cell proliferation. Inactivation of this gatekeeper initiates neoplastic growth by creating a permanent imbalance between cell division and cell death.¹¹ *APC* expression in cutaneous tumours has not been investigated thus far. The main goal of this study was to investigate *APC* expression, microsatellite instability in *APC* locus and promoter methylation status of *APC* in the progression of melanocytic tumours.

Correspondence: PD Dr M Korabiowska, MD, PhD, Department of Cytopathology, University of Göttingen, Robert Koch Str. 40, 37075 Göttingen, Germany.

E-mail: UBRINCK@AOL.COM

Received 19 March 2004; revised and accepted 1 June 2004; published online 16 July 2004

Materials and methods

Tumour Samples

The material investigated consisted of specimens from 152 melanocytic lesions including 36 lentigos, 88 malignant melanomas with known follow-up and 28 melanoma lymphatic metastases. Correlating non-neoplastic reference tissue was also investigated. The tumours were located as follows: head and neck 32, trunk 39, upper extremity 42, lower extremity 39. There were 79 male and 73 female patients, whose age ranged between 21 and 83 years, averaging 56 years. All primary tumours were classified according to Breslow,¹² showing the following pT distribution: pT1–24, pT2–18, pT3–20 and pT4–26.

Immunohistochemical Demonstration of APC Expression

APC gene expression was analysed by applying rabbit polyclonal antibody C-20 raised against a peptide mapping at the carboxy terminus of APC of human origin (Santa Cruz Biotechnology Inc.).

The immunohistochemical reactions in the paraffin-embedded tumour tissue were carried out using the Stravigen Multilink kit (Biogenex Laboratories). The histological sections were mounted on uncoated slides. They were deparaffinated by xylol and then transferred to a descending alcohol series and rinsed with distilled water. Before incubation with primary antibodies, the sections were heated for 10 min on a heat plate (85°C) in citrate buffer (pH=6). Afterwards, incubation with the primary antibodies was carried overnight at 4°C at an antibody concentration of 1:50. Then, the histological specimens were rinsed with Tris buffer solution and incubated at room temperature with link (Stravigen Multilink, Biogenex Laboratories) for 45 min. After the detection reaction was performed using a label (Stravigen Multilink, Biogenex Laboratories) in combination with chromogen fast red (Biogenex Laboratories), the nuclei were counterstained with haematoxylin. In control reactions, primary antibodies were omitted. Sections were evaluated using the CAS200 image analysis system and results were expressed as percentages of immunolabelled cells (indices).

Normal control for immunohistochemical staining was positivity within the epidermis.

DNA-Extraction

Paraffin-embedded tumours and corresponding normal tissue were mounted on glass slides and stained with haematoxylin and eosin. Microdissection was performed on paraffin sections stained briefly with methylene blue. Areas of interest were scraped off the slide and subjected to digestion with proteinase

K 20 mg/ml (Qiagen, Valencia CA, USA). DNA was extracted with the QIAmp DNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendations. The eluted DNA was suspended in 30 µl H₂O and stored at –20°C.

Microsatellite Analysis

Microsatellite analysis of locus *D5S346* (*APC*) was performed according to the protocol of Spirio *et al*¹³ with modification. Primers applied had following sequence: forward 5'-ACTCACTCTAGTGATAA ATCG-3' (labelled with fluorescent dye HEX) and reverse 5'-AGCAGATAAGACAGTATTACTAGTT-3'. Oligonucleotides for PCR were obtained from Perkin-Elmer (Weiterstadt, Germany). Optimal PCR results were achieved by an initial 95°C denaturation step (7 min), followed by 34 cycles at 94°C (1 min)//55°C (1 min)//72°C (1.5 min), followed by a final extension step at 72°C for 5 min. The PCR mix consisted of 10 × PCR buffer (Perkin-Elmer, Weiterstadt, Germany), 100 pg primer, 10 µM of each dNTP (Perkin Elmer, Weterstadt, Germany), 2 U of Ampli Taq Gold (Perkin-Elmer, Weiterstadt, Germany). The final volume was 50 µl. Resulting PCR products were separated on 3% agarose gels and stained with ethidium bromide. Negative controls were included in each PCR run. Automatic analysis of PCR products was then performed on ABI 310 DNA-Analyzer (Perkin-Elmer, Germany) with GENESCAN software. Internal standard GENESCAN™-350, TAMRA (Perkin-Elmer, Germany) was added to check the size of the PCR fragments analysed. The peak pattern of tumour tissue was compared to that of normal reference tissue. Additional peaks in tumour tissue when compared to normal tissue indicated microsatellite instability. Loss of peaks in tumour tissue compared to normal tissue indicated loss of heterozygosity.

Analysis of APC Promoter Methylation

An amount of 1 µg of DNA isolated from tumour tissue and non-neoplastic reference tissue was treated with sodium bisulphite using the CpGenome DNA Modification Kit (Intergeren Company, Oxford, UK) according to the manufacturer's instructions. A volume of 2 µl of modified DNA (1/5 volume) was used for PCR amplification. Primers were *APC* sense 5'-GGTATATTTTCGAGGGGTACT-3' and *APC* antisense 5'-TTCCCGACCCGGACTCCGC-3' for methylated sequences, *APC* sense 5'-TGTGAGGGTA TATTTTTGAGGGGTAT-3' and *APC* antisense 5'-CTTCTCTCTCCACTTCCCAACCCA-3' for non-methylated sequences. The PCR conditions for methylated *APC*-primers were as follows: 95°C hot start × 15 min, then 40 repetitive cycles of denaturation (95°C × 45 s), annealing (56°C × 60 s), extension (72°C × 60 s) followed by a final 7 min extension at 72°C. The PCR conditions for nonmethylated

APC-primers were as follows: 95°C hot start × 15 min, then 40 repetitive cycles of denaturation (95°C × 45 s), annealing (56°C × 30 s), extension (72°C × 60 s) followed by a final 7 min extension at 72°C. The PCR fragments were separated on a 3% agarose gel.

Statistics

After being scanned into the spread sheet (Microsoft Excel 97), where they were made available to the statistics program via an ODBC driver, the data were analysed using the statistical analysis system (SAS, Version 7.5). on an IBM-compatible PC under Windows NT 4.0. The median and ranges were calculated for all parameters. Mann-Whitney *U*-tests were used for the group comparisons between pT groups). Survival rates were calculated according to the Kaplan and Meier method.¹⁴ Cox regression was the multivariate method used for predicting the survival rate based on several parameters.¹⁵

Results

APC Expression

APC was present in all lentigos investigated and APC expression ranged between 5 and 69%. Out of 88 primary tumours, 75 were APC-positive. Index of APC-labelled cells oscillated in primary melanomas between 0 and 98%. APC expression was detected in 16 out of 28 melanoma lymphatic metastases. APC index in this group reached values between 0 and 52% (Table 1, Figure 1a–c).

Comparison of Diagnostic Groups Investigated

Highly significant differences with regard to APC expression were found between lentigos and primary melanomas as well as between lentigos and melanoma lymphatic metastases ($P < 0.01$). Comparison of primary melanomas and melanoma lymphatic metastases did not show any significant differences ($P > 0.05$)

Table 1 APC expression in diagnostic groups investigated

Diagnosis	Positive cases	% of APC-positive cells		
		Median	Minimum	Maximum
Lentigo maligna	36/36	49.00	5.00	69.00
Primary melanomas	75/88	18.00	0.00	98.00
Lymphatic metastases	16/28	2.50	0.00	52.00

Relation between pT Groups and APC Expression

APC-positive cells were found in 21/24 pT1, 18/18 pT2, 16/20 pT3 and 20/26 pT4 tumours. APC index reached maximally 98% in pT1 and 70% in pT2 melanomas. In pT3 and pT4 tumours APC index peaked at 52% and 48% of tumour cells, respectively (Table 2). Statistically significant differences were found between pT1 and pT > 1 groups for APC expression ($P < 0.05$).

Microsatellite Instability Analysis in APC Locus (D5S346)

Altogether 29 cases were investigated for the presence of microsatellite instability in APC locus.

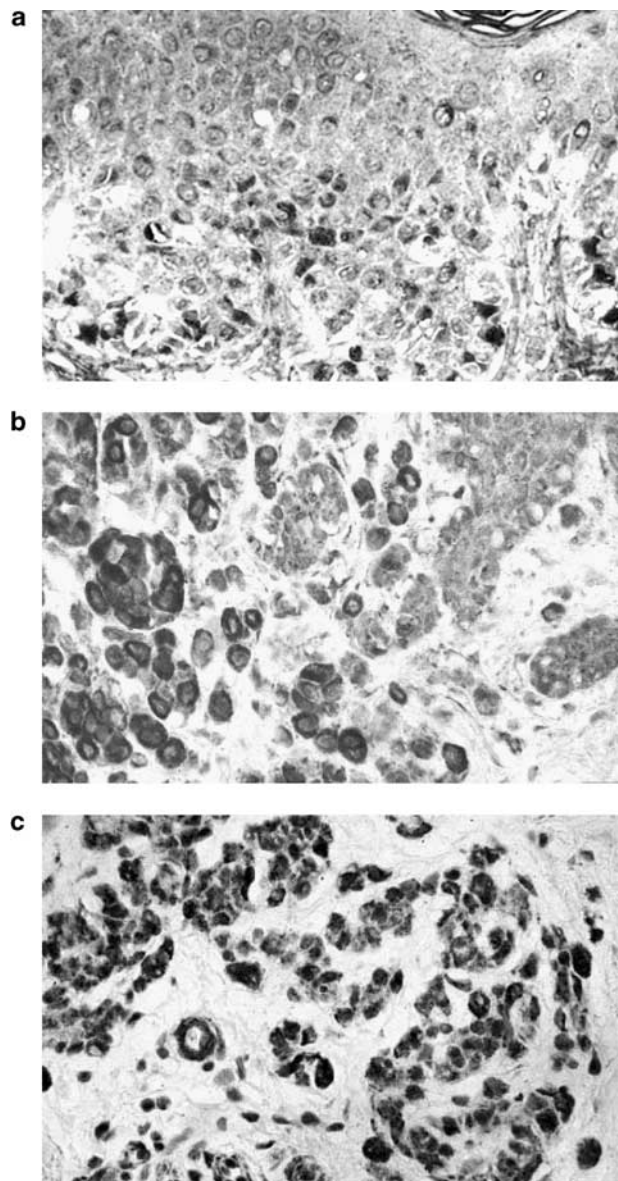


Figure 1 Positive cytoplasmic reaction for APC in primary melanoma and in epidermis (a, b) and in metastatic melanoma cells in lymph node (c), × 400.

Table 2 APC expression in primary melanomas

Group	Positive cases	% of APC-positive cells		
		Median	Minimum	Maximum
pT1	21/24	69.00	0.00	98.00
PT2	18/18	8.00	2.00	70.00
PT3	16/20	12.00	0.00	52.00
PT4	20/26	11.00	0.00	48.00

Microsatellite instability was found in 5/29 cases and loss of heterozygosity only in 1/29 case (Figures 2 and 3).

APC Expression in Cases without Microsatellite Instability in APC Locus

APC-positive cells were found in 17/23 melanomas without microsatellite instability. APC expression ranged between 0 and 63% of tumour cells, reaching median of 15% (Figure 4).

APC Expression in Cases with Microsatellite Instability in APC Locus

Positive reaction for APC was observed only in 1/5 melanomas with unstable APC locus. APC expression ranged in this tumour groups between 0 and 9% (Figure 5).

Comparison of APC Expression in Melanomas with and without Microsatellite Instability

Difference in APC expression between melanomas with and without microsatellite instability in APC locus was significant ($P = 0.0303$).

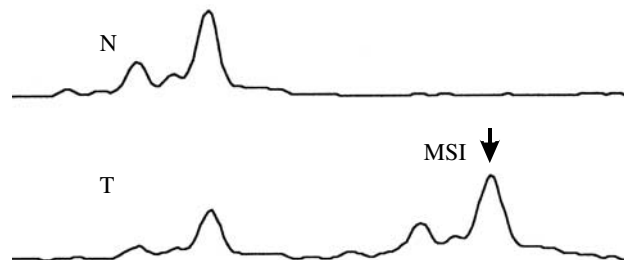
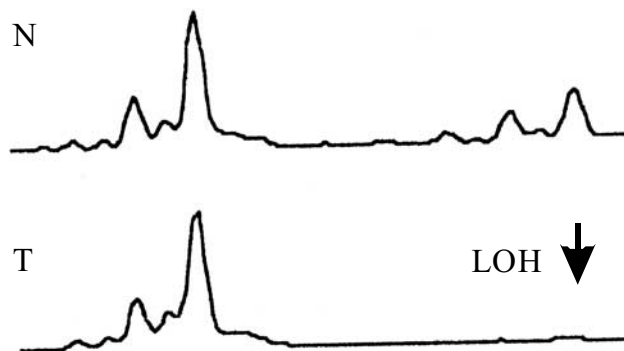
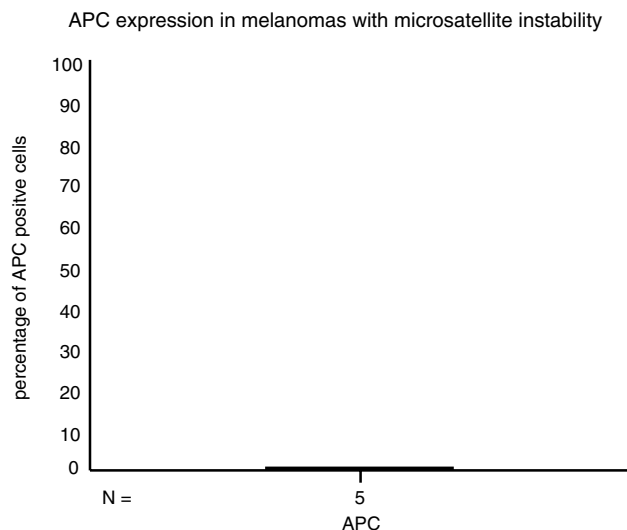
Analysis of Promoter Methylation Status of APC

Methylation of APC promoter was detected in 6/10 APC-negative melanomas without metastatic events associated with them and in 9/10 APC-negative melanoma lymphatic metastases (Table 3, Figure 6).

Prognostic Significance of APC in Malignant Melanomas

Multivariate analysis

In multivariate analysis in both models, forward and backward APC proved to be a significant factor in predicting outcome ($P < 0.05$).

**Figure 2** Example of microsatellite instability in APC locus.**Figure 3** Example of loss of heterozygosity in APC locus.**Figure 4** Boxplot demonstrating APC expression in melanomas without microsatellite instability in APC locus.

Kaplan–Meier Survival Curves

Analysis of Kaplan–Meier survival curves showed that about 45% of APC-positive patients (index $> 0\%$) and none of the APC-negative patients survived 5 years. Difference between both survival curves was significant ($P < 0.05$ —log-rank test) (Figure 7).

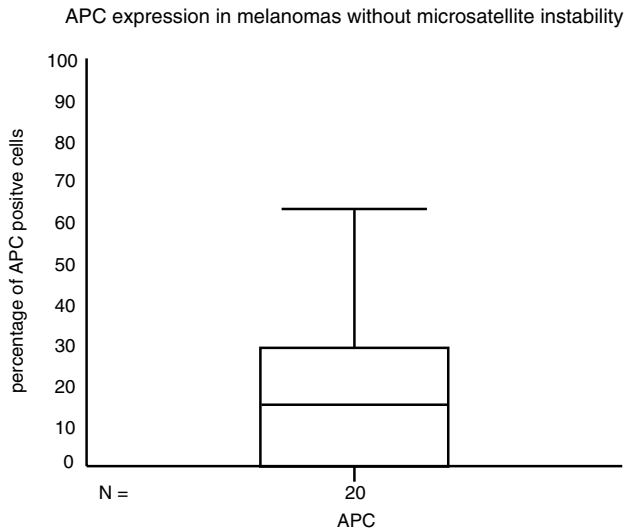


Figure 5 Boxplot demonstrating *APC* expression in melanomas with microsatellite instability in *APC* locus.

Table 3 Frequency of *APC* promoter methylation in *APC*-negative primary and metastatic melanomas

Diagnosis	Number of cases investigated	Number of cases investigated with <i>APC</i> Methylation
Primary melanomas	10	6 (60%)
Lymphatic metastases	10	9 (90%)

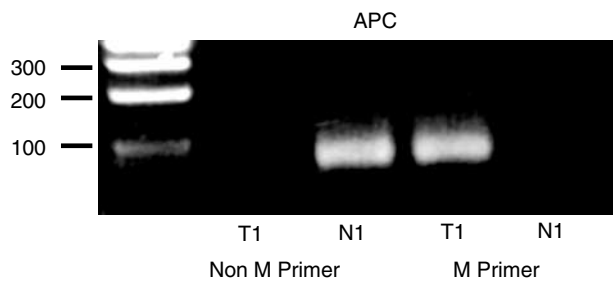


Figure 6 Detection of methylated and nonmethylated *APC* in normal and melanoma tissues in 3% agarose gel. T1—tumour, N1—normal tissue, Non M primer—nonmethylated primer, M primer—methylated primer.

Discussion

Our study demonstrated for the first time that the altered expression of *APC* gene had the prognostic impact in primary melanomas. Thus far, alternations of this gene had been mostly considered to be associated with colon disease. Now that we have shown, altered *APC* expression in tumours outside the gastrointestinal tract allows us to postulate the general role of this gene in tumorigenesis. Similar situations are encountered in genes involved in mismatch repair. While most reports have demonstrated the role of mismatch repair genes in

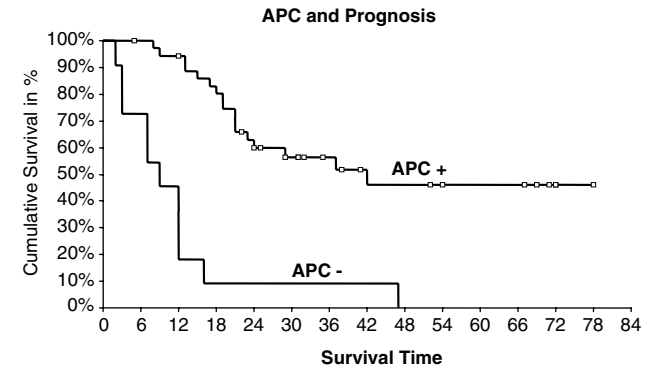


Figure 7 Kaplan–Meier survival curves for *APC*-positive and -negative melanoma patients.

hereditary nonpolyposis colon cancer, it has also been shown to play a crucial role in the progression of several sporadic tumours.^{16–18}

As mentioned in the introduction, the role of *APC* in tumour biology is still unclear, although there has been some speculation concerning the function of this gene.^{8–11} The relation between the *APC* gene and proliferation has been investigated comprehensively. *APC* is known as negative regulator of proliferation (gatekeeper). Loss of expression of this gene reportedly leads to uncontrolled proliferation and dysharmony between proliferation and apoptosis and, ultimately, to accelerated tumour progression.

In this study, we demonstrated a significant difference in *APC* expression between melanomas with and without microsatellite instability in the *APC* locus. Somatic mutations and loss of heterozygosity of *APC* were reported for colorectal, gastric, pancreatic and cholangiocarcinomas.^{19,20} To our knowledge, ours is the first investigation of loss of heterozygosity and microsatellite instability in the *APC* locus in malignant melanomas. In total, 80% of cases with an unstable *APC* locus did not show any *APC* expression on the protein level. Aberrant *APC* promoter methylation has not yet been demonstrated for malignant melanomas. We concentrated our attention on *APC*-negative primary and metastatic melanomas to find possible correlation between loss of *APC* expression and promoter methylation status.

The relation between loss of *APC* expression and promoter methylation has been demonstrated for several sporadic tumours. Aberrant methylation of *APC* was associated with a loss of gene function as was demonstrated for 27% of gallbladder carcinomas.²¹ In prostate cancer, methylation of *cyclin D2* correlated significantly with methylation of *APC* and with clinicopathological features of poor prognosis.²² The methylation frequency of *APC* correlated in prostate cancer with Gleason score.²³ In breast cancer, methylation of *APC* was independently associated with poor outcome and was even more powerful than standard prognostic

parameters.²⁴ In colorectal cancer, the frequency of aberrant promoter methylation of *APC* in malignant tissues was significantly higher than in nonmalignant tissues, but methylation status of *APC* had no clear relationship with clinical parameters.²⁵ Our finding of high frequency of *APC* methylation in malignant melanomas partly explains the mechanism responsible for the loss of *APC* expression and indirectly the influence of *APC* on the disease prognosis.

The other mechanism by which *APC* can influence prognosis in melanoma patients is connected with cell adhesion molecules. Colon cancer cells with mutant *APC* contain abnormally high levels of intracellular β -catenin. When full-length *APC* was added to these cells, cytoplasmic pools of β -catenin were destabilized and eliminated.^{9,26,27} The functional implications for the colocalization of *APC* protein with cytoskeletal microtubules are unknown. One intriguing possibility in light of recent finding that *APC* associates with catenins is that *APC* could relay signals from catenins to the cytoskeleton. This might, in turn, interface with extracellular signals communicated through the cell adhesion molecule *E-cadherin*, which also binds catenins.

The main reason that melanoma therapy is unsuccessful is its metastatic spread. Strong relations between *APC* and cell adhesion molecules suggest that *APC* plays an indirect role in tumour migration. In conclusion, we submit that *APC* should be regarded as an important new factor in progression of malignant melanoma.

References

- Bodmer WF, Bailey CJ, Bodmer J, *et al*. Localization of the gene for familial adenomatous polyposis on chromosome 5. *Nature* 1987;328:614–616.
- Herrera L, Kakati S, Gibas L, *et al*. Brief clinical syndrome: Gardner syndrome in a man with an interstitial deletion of 5q. *Am J Med Genet* 1986; 25:473–476.
- Kang YK, Kim WH, Lee HW, *et al*. Mutation of *p53* and *k-ras* and loss of heterozygosity of *APC* in intrahepatic cholangiocarcinoma. *Lab Invest* 1999;79:477–483.
- Miyaki M, Tanaka K, Kikashi-Yanoshita R, *et al*. Familial polyposis: recent advances. *Crit Rev Oncol Hematol* 1995;19:1–31.
- Groden J, Thliveris A, Samowitz W, *et al*. Identification and characterization of the familial adenomatous polyposis coli gene. *Cell* 1991;66:589–600.
- Miyoshi Y, Nagase H, Ando H, *et al*. Somatic mutations of the *APC* gene in colorectal tumors: mutation cluster region in the *APC* gene. *Hum Mol Genet* 1992;1: 229–233.
- Nishisho I, Nakamura Y, Miyoshi Y, *et al*. Mutations of chromosome 5q21 genes in FAP and colorectal patients. *Science* 1991;253:665–669.
- Mulkens J, Poncin J, Arends JW, *et al*. *APC* mutations in human colorectal adenomas: analysis of the mutation cluster region with temperature gradient gel electrophoresis and clinicopathological features. *J Pathol* 1998;185:360–365.
- Smith KJ, Levy DB, Maupin P, *et al*. Wild-type but not mutant *APC* associates with the microtubule cytoskeleton. *Cancer Res* 1994;54:3672–3675.
- Rubinfeld B, Robbins P, El-Gamil M, *et al*. Stabilization of *beta-catenin* by genetic defects in melanoma cell lines. *Science* 1997;275:1790–1792.
- Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. *Cell* 1996;87:159–170.
- Breslow A. Thickness, cross-sectional areas and depth of invasion in the prognosis of cutaneous melanoma. *Ann Surg* 1979;172:902–908.
- Spirio L, Joslyn G, Nelson L, *et al*. A Ca repeat 30–70 kbs downstream for the adenomatous polyposis coli (*APC*) gene. *Nucleic Acids Res* 1991;19: 6348.
- Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. *J Am Statist Assoc* 1958; 53:457–460.
- Cox DR. Regression models and life tables. *J Statist Soc B* 1972;34:187–222.
- Korabiowska M, Dengler H, Kellner S, *et al*. Decreased expression of *MLH1*, *MSH2*, *PMS1* and *PMS2* in pigmented lesions indicates accumulation of failed DNA repair along with malignant transformation and tumour progression. *Oncol Rep* 1997;4: 653–655.
- Leung SY, Yuen ST, Chung LP, *et al*. *hMLH1* promoter methylation and lack of *hMLH1* expression in sporadic gastric carcinomas with high frequency microsatellite instability. *Cancer Res* 1999;59:159–164.
- Piccinin S, Gasparotto D, Vukosavljevic T, *et al*. Microsatellite instability in squamous cell carcinomas of the head and neck related to field cancerization phenomena. *Br J Cancer* 1998;78:1147–1151.
- Nakamura Y. The role of the *adenomatous polyposis coli (APC)* gene in human cancers. *Adv Cancer Res* 1993;62:65–87.
- Ding SF, Delhanty JDA, Bowles L, *et al*. Loss of constitutional heterozygosity on chromosomes 5 and 17 in cholangiocarcinoma. *Br J Cancer* 1993;67: 1007–1010.
- House MG, Wistuba II, Argani P, *et al*. Progression of gene methylation in gallstone disease leading to gallbladder cancer. *Ann Surg Oncol* 2003;8:882–889.
- Padar A, Sathyanarayana UG, Suzuki M, *et al*. Inactivation of *cyclin D2* gene in prostate cancers by aberrant promoter methylation. *Clin Cancer Res* 2003; 9:4730–4734.
- Kang GH, Lee S, Lee HJ, *et al*. Aberrant CpG island hypermethylation of multiple genes in prostate cancer and prostatic intraepithelial neoplasia. *J Pathol* 2003; 202:233–240.
- Muller HM, Widschwendter A, Fiegl H, *et al*. DNA methylation in serum of breast cancer patients: an independent prognostic factor. *Cancer Res* 2003;63: 7641–7645.
- Lin SY, Yeh KT, Chen WT, *et al*. Promoter CpG methylation of tumor suppressor genes in colorectal cancer and its relation to clinical features. *Oncol Rep* 2004;11:341–348.
- Shtutman M, Zhurinsky J, Simcha I, *et al*. The *cyclin D1* gene is a target of the *beta-catenin/LEF-1* pathway. *Proc Natl Acad Sci USA* 1999;96:5522–5527.
- Gumbiner BM. Carcinogenesis: a balance between *beta-catenin* and *APC*. *Curr Biol* 1997;7:443–446.