PTEN mutations are common in sporadic microsatellite stable colorectal cancer

Authors: Najah T. Nassif[*,#,1], Glenn P. Lobo[#,1], Xiaojuan Wu[2], Christopher J.A. Henderson[2], Carl D. Morrison[3,4], Charis Eng[3,5,6,7], Bin Jalaludin[8] and Eva Segelov[1]

Affiliations: [1]Cancer Research Laboratories, South West Sydney Clinical School, University of New South Wales, Liverpool Hospital, Liverpool, NSW 2170, Australia. [2]Department of Anatomical Pathology, South Western Area Pathology Service, Liverpool Hospital, Liverpool, NSW 2170 Australia. [3]Clinical Cancer Genetics and Human Cancer Genetics Programs, Comprehensive Cancer Center, [4]Department of Pathology, and [5]Division of Human Genetics, Department of Internal Medicine, [6]Division of Human Cancer Genetics, Department of Molecular Virology, Immunology and Medical Genetics, The Ohio State University, Columbus OH 43210, USA. [7]Cancer Research UK Human Cancer Genetics Research Group, University of Cambridge, Cambridge CB2 2XZ, UK. [8]Epidemiology Unit, School of Public Health and Community Medicine, University of New South Wales, Liverpool Hospital, Liverpool, NSW 2170, Australia.

[*]Correspondence: Dr Najah Nassif, Department of Medicine, South West Sydney Clinical School, University of New South Wales, Level 4, Health Services Building, Cnr Goulburn & Campbell Sts, Liverpool, NSW 2170, Australia. Phone: +61 2 9828 4977; Fax: +61 2 9828 4970; E-mail: n.nassif@unsw.edu.au

[#]These authors contributed equally to this work

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Abstract

The tumour suppressor gene *PTEN*, located at chromosome sub-band 10q23.3, encodes a dual specificity phosphatase that negatively regulates the phosphatidylinositol 3'-kinase (PI3K) / Akt-dependent cellular survival pathway. *PTEN* is frequently inactivated in many tumour types including glioblastoma, prostate and endometrial cancers. While initial studies reported that *PTEN* gene mutations were rare in colorectal cancer, more recent reports have shown an approximate 18% incidence of somatic *PTEN* mutations in colorectal tumours exhibiting microsatellite instability (MSI+). To verify the role of this gene in colorectal tumorigenesis, we analysed paired normal and tumour DNA from 41 unselected primary sporadic colorectal cancers for *PTEN* inactivation by mutation and/or allelic loss. We now report *PTEN* gene mutations in 19.5% (8/41) of tumours and allele loss, including all or part of the *PTEN* gene, in a further 17% (7/41) of cases. Both *PTEN* alleles were affected in over half (9/15) of these cases. Using immunohistochemistry, we have further shown that all tumours harbouring *PTEN* alterations have either reduced or absent PTEN expression and this correlated strongly with later clinical stage of tumour at presentation (p=0.02). In contrast to previous reports, all but one of the tumours with *PTEN* gene mutations were microsatellite stable (MSI-), suggesting that *PTEN* is involved in a distinct pathway of colorectal tumorigenesis that is separate from the pathway of mismatch repair deficiency. This work therefore establishes the importance of *PTEN* in primary sporadic colorectal cancer.

Introduction

Colorectal cancer (CRC) constitutes the second most common cause of cancer deaths in many Western countries. Colorectal carcinogenesis is a multistep process with tumours displaying considerable genetic heterogeneity. A number of genetic and epigenetic alterations resulting in the inactivation of tumour suppressor genes and/or the activation of oncogenes have been described (Chung, 2000; Ilyas et al., 1999). DNA mismatch repair deficiency, observed in most cases of the familial syndrome hereditary nonpolyposis colon cancer, occurs in 10-15% of sporadic colorectal cancers (Aaltonen et al., 1993; Ionov et al., 1993; Thibodeau et al., 1993). Some of these molecular changes, such as mismatch repair deficiency, have been shown to correlate with the pathological features of the tumour and clinical course of the disease (Elsaleh, 2001; Elsaleh et al., 2001a; Elsaleh et al., 2001b; Elsaleh et al., 2000; Watanabe et al., 2001). The range of genetic alterations involved in colorectal carcinogenesis is incomplete and further key genes involved in the pathogenesis of CRC remain to be identified.

The tumour suppressor gene *PTEN* (phosphatase and tensin homologue deleted on chromosome 10), also known as *MMAC1* (mutated in multiple advanced cancers 1) and *TEP1* (TGFβ-regulated and epithelial cell-enriched phosphatase 1), is located on chromosome sub-band 10q23.3. *PTEN* encodes a dual specificity phosphatase displaying homology to the cytoskeletal proteins tensin and auxilin (Li & Sun, 1997; Li et al., 1997; Steck et al., 1997). PTEN is a protein and lipid phosphatase whose major substrate is the second messenger phosphatidylinositol 3,4,5-triphosphate (PIP-3), produced by the activity of phosphoinositide 3-kinase (PI3K). An important downstream target of PI3K is protein kinase B (PKB)/Akt which controls cell proliferation and protects cells from apoptosis. The role of PTEN in this pathway is to keep levels of PIP-3 low. Loss of PTEN function results in increased PIP-3 concentration and consequent PKB/Akt

hyperactivation leading to protection from various apoptotic stimuli (Downward, 1998; Maehama & Dixon, 1998; Myers et al., 1998). PTEN is now known to function as a tumour suppressor through negative regulation of the PI3K/Akt pathway (Li et al., 1998; Stambolic et al., 1998; Wu et al., 1998) among other roles.

PTEN was considered a good candidate for involvement in colorectal tumorigenesis for a number of reasons. First, allele loss close to the *PTEN* locus has been reported in 30% of sporadic CRC (Frayling et al., 1997). Second, somatic mutations or deletions of *PTEN* have been observed in many other sporadic cancers including glioblastoma (Wang et al., 1997), prostate cancer (Cairns et al., 1997), melanoma (Guldberg et al., 1997; Whiteman et al., 2002) and endometrial cancer (Mutter et al., 2000), in which the presence of *PTEN* mutations has been found to be a prognostic indicator (Minaguchi et al., 2001). Third, germline mutations of *PTEN* are associated with the autosomal dominant Cowden and Bannayan-Zonana syndromes (Liaw et al., 1997; Marsh et al., 1997; Marsh et al., 1999), characterised by multiple hamartomas of many tissues including the gastrointestinal tract. Cowden syndrome is also associated with an increased risk of breast, thyroid and endometrial neoplasia (Eng, 2000; Eng & Peacocke, 1998; (Marsh et al., 1998). Germline *PTEN* mutations have also been recently implicated in Proteus and Proteus-like syndromes (Smith et al., 2002; Zhou et al., 2001; Zhou et al., 2000b). Finally, colon cancer has been described in *Pten*$^{+/-}$ chimeric mice (Di Cristofano et al., 1998) but is not a known component of Cowden syndrome (Eng, 2000), although rare Cowden individuals have been found to have colorectal cancers (Eng, unpublished observations).

Initial reports of *PTEN* mutation screening in sporadic CRC found somatic mutations to be uncommon (Chang et al., 1999; Negoro et al., 2000; Okami et al., 1998; Wang et al., 1998). However, more recent analyses have reported a 17-19% incidence of *PTEN* mutations in microsatellite unstable (MSI+) colorectal tumours and concluded that *PTEN*

mutations could be selected for during tumorigenesis, but as a later event (Dicuonzo et al., 2001; Guanti et al., 2000; Shin et al., 2001; Zhou et al., 2002b). To verify the role of *PTEN* in sporadic CRC and to explore its role in MSI- tumours, we screened a series of 41 unselected primary sporadic colorectal tumours for *PTEN* mutations and deletions. In contrast to previous studies, we report the detection of *PTEN* gene mutations in 19.5% of sporadic colorectal tumours, all but one of which were microsatellite stable (MSI-). Furthermore, all tumours with *PTEN* gene alterations (mutation and/or deletion) showed a reduction or absence of PTEN expression, and this correlated clinically with later stage of disease at diagnosis.

Results

Tumour MSI status

Seven of the 41 (17%) tumours exhibited microsatellite instability (MSI+). Two of these were of the MSI-L phenotype (CRC 23 and 42) and five were MSI-H (CRC 7, 17, 18, 46 and 52) (Table 1). None of the tumours showing LOH at 10q23 were MSI+ and only one of the tumours with mutations was MSI+ (CRC 17). This tumour harboured two separate mutations in exon 8, affecting both alleles. The first mutation was a frameshift mutation caused by a deletion of 4 bp (ACTT) corresponding to nucleotides 955-958 of the *PTEN* mRNA sequence. The second was a missense mutation (N323K) caused by a base change (T→A) which effectively lengthened the $(A)_6$ tract within exon 8 to $(A)_7$.

PTEN mutation screening

Paired normal and tumour DNA samples from 41 unselected primary sporadic colorectal tumours were screened for mutations of the *PTEN* coding and flanking intronic sequences (Figure 1), as well as for allelic loss at the *PTEN* locus. Ten somatic *PTEN* mutations and one previously-reported germline polymorphism (Sato et al., 2002) were identified in 8/41 (19.5%) patients (Figure 2). All sequence changes were confirmed by re-amplification and sequence analysis of an independent PCR product. Only one of the mutations (del 955-958) has been previously described (Ali et al., 1999; Bonneau & Longy, 2000; Kurose et al., 1998; Lin et al., 1998; Rhei et al., 1997). Six tumours harboured two or more mutations of *PTEN*, affecting both alleles. One tumour (CRC 1) demonstrated loss of one allele with mutation of the remaining allele. Of the 8 tumours with *PTEN* mutations, two (CRC 2 and 37) harboured homozygous somatic changes with the remainder having heterozygous somatic changes (Table 1).

The ten somatic mutations were located in exons 1, 3, 5, 7 and 8 (Figure 2). Eight were missense, one was nonsense and one was a frameshift mutation caused by the deletion of 4 bp (ACTT) within the *PTEN* coding region in exon 8. This mutation was detected in two tumours (CRC 17 and CRC 48A) from unrelated patients. Five mutations occurred in exon 5 (K125E, K125X, E150Q, D153Y D153N), which encodes the phosphatase domain of the protein. Six missense mutations (K62R, Y65C, K125E, E150Q, D153Y, D153N) and one nonsense mutation (K125X) fell within the N-terminal phosphatase domain of the protein (residues 7-185). The K125E and K125X mutations lie in the core phosphatase motif HCXXGRXXR (residues 123-130). Two further missense mutations (V217A, N323K) occurred within the C-terminal domain (residues 186-351) of the protein. Representative sequences of somatic mutations are presented (Figure 3).

Apart from the primary tumour, patient CRC 37 also had a coincident polyp removed at the time of surgery. The *PTEN* mutation observed in exon 5 (E150Q) in the carcinoma, was not seen in the matched polyp sample from the same patient.

Loss of heterozygosity (LOH) at the PTEN locus

Using an initial five polymorphic markers, allele loss at the *PTEN* locus was observed in 8 of the 41 (19.5%) tumours (Figure 4). Two tumours (CRC 12 and 14) demonstrated apparent homozygous deletion of *PTEN* with decreased amplification intensity of marker alleles but constant reference (β-globin exon 2) amplification intensity. CRC 12 showed homozygous deletion of the two flanking markers (D10S579 and D10S541) with all intragenic markers being non-informative. Retention of both alleles at D10S1735 defined the deletion boundary between D10S541 and D10S1735. CRC 14 showed homozygous deletion at D10S579 and AFMa086wg9 with LOH at IVS8 +32 T/G and retention of both alleles at D10S541 and D10S1735. Retention of heterozygosity at D10S1687, defined the boundary of deletion between this marker and D10S579. CRC 1 demonstrated LOH at

two intragenic marker loci with mutation of the remaining *PTEN* allele. The remaining five tumours demonstrated LOH (hemizygous deletion) without mutation of the remaining *PTEN* allele (Figure 4). Combined *PTEN* mutation and deletion data shows that of the 15 tumours with *PTEN* alterations (7 with mutations only, 5 with hemizygous deletion, 2 with homozygous deletion and 1 with concurrent LOH and mutation), both alleles were affected in over half (9/15) of the cases.

Tumour PTEN expression

Immunohistochemical analysis of normal colonic epithelium showed that PTEN expression was predominantly cytoplasmic (++ immunoreactivity, n=41), with some normal to weak nuclear staining (++, n=15; +, n=26; Figure 5). Analysis of tumour sections containing normal cells, vascular endothelium (internal positive control) and tumour cells showed a reduction of cytoplasmic staining intensity in 19 tumours (46%) and a total absence of staining in 10 tumours (24%) (Table 2). All tumours harbouring alterations of the *PTEN* gene demonstrated a reduction or absence of cytoplasmic PTEN expression (Figure 5). PTEN expression was retained in 12/41 (29%) tumours, none of which harboured any *PTEN* gene alterations (Table 2). Tumours with suspected homozygous deletions including *PTEN* showed a corresponding absence of PTEN expression (-, n=2) while tumours with hemizygous *PTEN* deletion all showed a corresponding decrease in PTEN expression (+, n=5) (Figure 5). Interestingly, the homozygous deletion in CRC 12 (deleted at D10S579 and D10S541) must have included the *PTEN* gene as PTEN expression was absent. CRC 12 showed homozygous deletion of the two flanking markers (D10S579 and D10S541) with all intragenic markers being non-informative

In addition, 12 tumours showed reduced or absent PTEN immunoreactivity in the absence of any *PTEN* genetic alteration suggesting a role for epigenetic factors affecting PTEN expression in these tumours (Figure 5).

Clinicopathological correlations

Clinical characteristics were compared according to MSI status, because of the known correlations of MSI with certain clinicopathological features. MSI+ tumours were associated with being right sided and occurring in patients with younger age (p=0.03). In the 34 MSI- tumours, there was a significant association between reduced or absent PTEN staining in the tumour and later tumour stage (p=0.02). This was consistent with the trend seen between reduced or absent staining and incidence of death from CRC (no deaths in the 10 patients with normal tumour staining but 6 deaths in the 18 patients with reduced or absent staining). This association did not reach statistical significance but follow-up times were relatively short. There was a trend towards MSI- tumours without *PTEN* mutation or deletion occurring on the left side of the colon, but there was no association with sex, age of the patient, or grade of the tumour.

Discussion

This report demonstrates that the *PTEN* tumour suppressor is frequently altered in primary sporadic CRC. Structural *PTEN* abnormalities (mutation and/or deletion) were present in 37% of primary tumours and affected both alleles in over half of the cases. All but one of the tumours with *PTEN* mutations were microsatellite stable (MSI-). All tumours with structural *PTEN* abnormalities showed a reduction or absence of PTEN expression in the neoplastic cells, and, conversely, no tumours that retained PTEN staining harboured *PTEN* alterations. In contrast to MSI+ CRC, known to harbour somatic *PTEN* mutations, none of the mutations detected in MSI- CRC occurred in the coding mononucleotide tracts in exons 7 and 8 (Zhou et al., 2002a). This suggests that the *PTEN* gene is involved in a distinct pathway of colorectal tumorigenesis that is separate from the pathway of mismatch repair deficiency. Interestingly, CRC 37 harboured a homozygous somatic mutation (E150Q) that was not present in the matched polyp sample of the same patient and further suggests that *PTEN* mutation may be involved in CRC progression.

Reduced or absent PTEN expression has previously been reported in 35% of MSI+ CRC, of which 15-20% were shown to have *PTEN* alterations (Zhou et al., 2002b). In contrast, reduced expression has previously been documented in only approximately 17% of MSI- tumours and no structural *PTEN* alterations were documented in these tumours (Taniyama et al., 2001; Zhou et al., 2002b). Complete absence of PTEN expression has not been previously reported in any MSI- (or MSI status unknown) colorectal cancer (Taniyama et al., 2001; Zhou et al., 2002b) and this is the first report to correlate reduced or absent PTEN expression with *PTEN* structural abnormalities in a significant number of MSI- CRC.

PTEN inactivation appears to occur through different mechanisms in different tumour types and more than one mechanism can co-exist within a single tumour type (Taniyama et al., 2001; Zhou et al., 2002b). In our series of sporadic CRC, *PTEN* inactivation, with consequent reduction or absence of PTEN expression, appeared to occur by three distinct mechanisms. First, structural biallelic inactivation, through mutation of both alleles, homozygous deletion or a combination of mutation and deletion, occurred in 22% of tumours, and accounted for over half of the cases in which *PTEN* alterations were detected. While structural biallelic inactivation is common in glioblastoma multiforme, cervical carcinoma and endometrial cancer (Duerr et al., 1998; Kong et al., 1997; Kurose et al., 2000; Mutter et al., 2001; Mutter et al., 2000; Zhou et al., 1999), it has not been previously observed in CRC.

Second, monoallellic inactivation by mutation or hemizygous deletion of *PTEN* was found in a similar proportion (20%) of tumours in our series. Interestingly, hemizygous deletion led to reduced PTEN expression while monallelic mutation of *PTEN* led to either reduced or absent expression. It is possible that the remaining allele harbours a mutation occurring in a region of the gene not analysed in this study, or that epigenetic factors may be acting on the remaining allele. Interestingly, hemizygous *PTEN* knockout mouse models have demonstrated that loss of a single *PTEN* allele is sufficient for tumorigenesis (Di Cristofano et al., 1999; Di Cristofano et al., 1998; Podsypanina et al., 1999; Stambolic et al., 1998), and hemizygous deletion of *PTEN* appears to be common in some tumour types such as breast and prostate cancers (Feilotter et al., 1998; Singh et al., 1998).

We also identified a third group of tumours with reduced or absent PTEN expression but without any detectable *PTEN* structural genetic alterations. It is postulated that *PTEN* may be inactivated by epigenetic mechanisms alone, suggesting that biallelic epigenetic silencing may also be a mechanism of *PTEN* inactivation in sporadic CRC. Biallelic epigenetic silencing is the predominant mechanism of *PTEN* inactivation in metastatic

melanoma (Zhou et al., 2000a) and occurs in a small subset of epithelial ovarian carcinomas (Salvesen et al., 2001; Zhou et al., 2000a).

Mutations of the *PTEN* gene were detected in 19.5% of primary sporadic colorectal tumours (MSI-, n=7; MSI+, n=1). Nine novel, and one previously reported, mutations were detected in this study (Ali et al., 1999; Bonneau & Longy, 2000; Kurose et al., 1998; Lin et al., 1998; Rhei et al., 1997; Sato et al., 2002). While *PTEN* mutations have been reported in 17-19% of MSI+ CRC (Dicuonzo et al., 2001; Guanti et al., 2000; Shin et al., 2001), they have not been previously documented in a significant proportion of MSI- tumours (Chang et al., 1999; Negoro et al., 2000; Okami et al., 1998; Wang et al., 1998). Our detection of a higher frequency of *PTEN* mutations in sporadic MSI- CRC, in contrast to previous reports, may be attributed to several factors. First, the technique of dideoxy fingerprinting (ddF) for mutation screening is known to be more sensitive and less dependent on the nature of the mutation, than single strand conformation polymorphism (SSCP) (Sarkar et al., 1992) used in all but one of the previous studies (Liu & Sommer, 1994).

Furthermore, SSCP has been shown to be highly insensitive in detecting germline *PTEN* mutations in Proteus syndrome (0% detection rate) (Eng et al., 2001). Second, our study involved microdissection of tumour specimens to minimise contamination of normal tissue DNA. Third, there may be population differences in the incidence of *PTEN* mutations in CRC, similar to that observed in endometrial cancer (Maxwell et al., 2000).

Exon 5, encoding the phosphatase domain of the protein, a region crucial for tumour suppressor function, appeared to be targeted with 50% of mutations occurring in this exon. Overall, the N-terminal phosphatase domain (residues 7-185) was the site of 6 missense and one nonsense mutation, including the K125E and K125X mutations that lie within the core phosphatase motif of PTEN and are predicted to alter at least the lipid phosphatase activity essential for PTEN tumour suppressor function (Han et al., 2000;

Lee et al., 1999). Interestingly, codon 125, an invariant lysine residue, appears to be a target with two distinct mutations occurring at this site. Two missense (V217A, N323K) and one frameshift mutation were located within the C-terminal domain of the protein. This region is also essential for tumour suppressor function and partial or total absence of phosphatase activity has been demonstrated to result from amino acid changes outside the phosphatase domain (Georgescu et al., 1999; Han et al., 2000).

The frameshift mutation (del ACTT 955-958) was observed as a heterozygous change in two tumours (CRC 17 and 48A) from unrelated patients. The deletion of 4 bp changes codon 319 to a termination codon leading to a truncated PTEN protein lacking predicted phosphorylation sites (specific tyrosine and/or serine residues) and the C-terminal C2 domain that is important for phospholipid binding (Lee et al., 1999; Miller et al., 2002). Interestingly, one of the tumours harbouring this mutation was MSI+ (CRC 17), whilst the other was MSI- (CRC 48A). This mutation has been previously reported in endometrial cancer (Kurose et al., 1998; Lin et al., 1998). Neither this nor the second mutation present in the MSI+ (N323K) tumour was typical of deficient mismatch repair, suggesting that the tumorigenic mechanism involving *PTEN* is independent from that of mismatch repair.

Similar to the clinicopathological correlations now well established for MSI+ tumours (Elsaleh, 2001; Elsaleh et al., 2001a; Elsaleh et al., 2001b; Watanabe et al., 2001), there may be certain characteristics associated with tumours harbouring *PTEN* alterations. The association between reduced or absent PTEN staining and later clinical stage at presentation is significant, and the trend towards abnormal PTEN staining and death from CRC, if verified in a large cohort, may translate into clinical use of PTEN expression to stratify prognostic groups, and hence, help to tailor adjuvant chemotherapy. The demonstration of *PTEN* mutations in 19.5% of sporadic colorectal tumours, and LOH in a similar proportion, with consequent reduction or abolition of PTEN expression in all

cases, redefines the role of this important tumour suppressor gene in sporadic CRC, particularly MSI- tumours. If subsequent *in vitro* functional studies confirm cellular dysfunction, *PTEN* may become a potential therapeutic target in CRC. *In vitro* and *in vivo* demonstration of enhanced sensitivity of *PTEN*-deficient tumours to inhibition of the FRAP/RAFT/TOR component of the P13K intracellular pathway by the rapamycin analogue CCI-779, has led to the postulate that such drugs may have clinical efficacy in human *PTEN*-deficient cancers (Neshat et al., 2001; Yu et al., 2001). Data presented here provides a molecular basis for the inclusion of patients with CRC in clinical trials of such novel anticancer agents.

Materials and methods

Patients and tissue samples

Samples of tumour, coincident polyps, matching blood and normal colon were collected prospectively with the informed consent of patients who underwent surgery in the South Western Sydney Area Health Service during the period 2000-2002. All samples are archived in the South Western Sydney Colorectal Tumour Bank. Consecutive unselected cases were examined except where insufficient material was available. Clinical details were obtained from the database of the Tumour Bank. The lack of a family history of colorectal cancer or other familial cancer syndrome was ascertained by detailed questionnaire.

Forty-two primary colorectal cancers from 39 patients were studied. Two patients (CRC 30 and 48) had synchronous, distinct tumours (CRC 30A, CRC 30B and CRC 48A, CRC 48B) at the time of surgical resection and these were treated as separate entities. Of the 39 patients, 17 were females and 22 were males; mean age at diagnosis was 67 years (SD=10.9 years); and the mean duration of follow-up from date of diagnosis was 1.6 years (SD=0.8 years). Nine patients (23.1%) had died, all from colorectal cancer, with a mean age at death of 72.0 years (SD=12.2 years).

Of the 41 tumours, 23 (56.1 %) occurred on the right side of the colon (caecum, ascending colon or transverse colon) and 18 were left sided (descending colon, sigmoid colon or rectum). This distribution reflects the clinical practice of pre-operative chemoradiation for locally advanced rectal tumours, hence the lower availability of fresh tissue from left-sided tumours. Histologically, one tumour was purely mucinous. The remainder were adenocarcinomas, with three tumours showing areas of mucinous carcinoma and one displaying focal signet ring morphology. Clinical stage and histological grade were classified by standard criteria. Two tumours were grade 1, 31

tumours were grade 2 and 8 tumours were grade 3. Clinical staging at time of diagnosis was classified as Stage I-IV with 18 tumours being Stage I and II (early stage) and 23 tumours being Stage III and IV (late stage). Stage I corresponds to the TNM staging of T1, N0, M0 or T2, N0, M0; Stage II corresponds to T3, N0, M0 or T4, N0, M0; Stage III corresponds to any T, N1, M0 or any T, N2, M0 and Stage IV corresponds to any T, any N, M1 (Beahrs, 1997).

Fresh tissue samples of tumour and normal colonic epithelium (distant to the tumour site) were snap frozen in liquid nitrogen and stored at -80^{o}C. Histological sections of a region immediately adjacent to the tumour sample taken for storage were processed for diagnostic purposes and reviewed by a dedicated histopathologist (CJH). For mutation screening, frozen sections (15 μm) were prepared from stored tumour specimens. The first, middle and last slides (5μm) were stained as reference slides. Manual microdissection was carried out on the unstained slides under low power light microscopy (20-40X) by scraping of individual cell populations with a 28-gauge needle. Normal cells adjacent to the tumour were collected separately.

DNA extraction

DNA was isolated from microdissected tumour cells and specimens of normal colonic mucosa using the RapidGene genomic DNA purification kit (Amresco) or the Qiagen DNA Mini system (QIAGEN), according to the manufacturer's instructions. DNA was extracted from peripheral blood leukocytes using the DNA isolation kit for mammalian blood (Roche Molecular Biochemicals).

Analysis of MSI status

Paired tumour and constitutional DNA samples were analysed using a panel of 10 microsatellite markers as previously described (Kahnoski et al., 2003). Samples were classified as MSI-L (low level microsatellite instability) if instability was observed at 20-40% of loci assayed or MSI-H (high level microsatellite instability) if instability was observed at over 40% of loci assayed (Boland et al., 1998).

Amplification of the PTEN coding region

DNA from paired normal and tumour tissue samples was screened for *PTEN* mutations by dideoxy fingerprinting (ddF). The entire *PTEN* coding and flanking intronic sequences were amplified by PCR. Primers used were as described (Guldberg et al., 1997), with the exception of exon 5 which was amplified into two overlapping segments (A and B) using the primers PTENEx5a-Fwd (5'-TATGCAACATTTCTAAAGTTACC-3') with PTENEx5a-Rev (5'-GCACATATCATTACAGTTC-3') and PTENEx5b-Fwd (5'-AATG GCTAAGTGAAGATGACAA-3') with PTENEx5b-Rev (5'-AAGAAACCCAAAATCT GTTTTCC-3') respectively. PCR was carried out in a final volume of 50 μL containing 25 ng DNA, 20 pmol each primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0) and 0.5 U *Taq* polymerase (Amersham-Pharmacia Biotech). An initial denaturation step (95 ^{o}C, 5 min) was followed by 34 cycles of denaturation (95 ^{o}C, 45 s), primer annealing (50-60 ^{o}C, 45 s) and extension (72 ^{o}C, 45 s). PCR was ended with a final extension (72 ^{o}C, 10 min) step. An aliquot of each PCR product was analysed on 2% agarose gels to check the efficiency of PCR.

Dideoxy fingerprinting analysis

Following amplification, PCR products were purified using the JetQuick PCR product cleanup system (Genomed). Amplicons were eluted in 35 μL of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) and 5 μL was used to estimate DNA concentration by electrophoresis against known standards. Typically, 4 μL of purified PCR product was subjected to a cycle sequencing reaction using the Sequitherm Excel II system (Epicentre Technologies) with the appropriate forward PCR primer and a single ddNTP. The sequencing reactions were carried out in a final volume of 6 μL containing 20 pmol forward primer essentially as described by the manufacturer. Sequencing products were mixed with an equal volume of loading buffer (10 mM NaOH, 95% deionised formamide, 0.05% bromophenol blue and 0.05% xylene cyanol), denatured at 95 ^{o}C for 5 min and quick chilled on ice. An aliquot (4 μL) was electrophoresed (12 W, 22 ^{o}C, 4.5 h) on 0.5 X MDE (Mutation Detection Enhancement: FMC BioProducts) gels. Products were visualised by autoradiography. Samples showing migration shifts were selected for sequencing analysis using the amplification primers. Samples were subjected to both manual (SequiTherm Excel II: Epicentre Technologies) and automated sequencing (Applied Biosystems ABI 377 at the Westmead Sequencing Facility or ABI 3700 at the Sydney University and Prince Alfred Macromolecular Analysis Centre (SUPAMAC), Australia). Products were sequenced in both directions and mutations verified on both DNA strands. Samples with sequence variations were re-amplified and re-sequenced from a second independent PCR product to ensure reproducibility of detected sequence changes. For those samples in which the mutation(s) could not be unambiguously identified by sequence analysis, the primary PCR product was cloned (TOPO TA Cloning, Invitrogen) and 10 clones were sequenced.

Analysis of LOH at the PTEN locus

Allelic loss at the *PTEN* locus was determined using the markers D10S1687, D10S579, D10S541 and D10S1735, that flank the *PTEN* gene, as well as three intragenic markers: AFMa086wg9 (intron 2), IVS4 +109 ins/del TCTTA and IVS8 +32 T/G. Marker positions and primer sequences for D10S579, D10S541, D10S1735 and AFMa086wg9 were obtained from the Whitehaed Institute for Biomedical Research/MIT Centre for Genome Research maps and contigs at http://www-genome.wi.mit.edu and from the Genome Database. Primers for the amplification of IVS4 +109 ins/del TCTTA and IVS8 +32 T/G were as described by Zhou et al (Zhou et al., 2000a) and Hansen et al (Hansen et al., 2001) respectively. After amplification, the IVS4 +109 ins/del and IVS8 +32 T/G genotypes were determined by differential digestion of the respective PCR products with the restriction endonucleases *Afl*II and *Hin*dII (Roche Molecular Biochemicals) respectively (Dahia et al., 1997; Zhou et al., 2000a). Digestion products were separated on 3% agarose gels.

Each of the markers D10S1687, D10S579, D10S541, D10S1735 and AFMa086wg9 were co-amplified with exon 2 of the β-globin gene that was used as an internal standard to monitor amplification efficiency. A 280 bp region containing exon 2, and part of the flanking intron 3, of the β-globin gene was amplified using the primers BGE2-Fwd (5'-CTCTGCCTATTGGTCTATTTTCCC-3') and BGE2-Rev (5'-GAAAACATCAAGGGT CCCATAGAC-3'). Amplification was performed in a final volume of 25 μl containing 25 ng genomic DNA, 20 pmol each respective primer (forward primers were 5'-HEX labelled with the exception of BGE2-Fwd which had a 5'-FAM label), 0.2 mM dNTPs, 50 mM KCl, 10 mM Tris-HCl (pH8.3), 1.5 mM $MgCl_2$ and 0.5 U *Taq* polymerase (Amersham-Pharmacia Biotech). PCR was initiated by a 5 min denaturation (94 ^{o}C) followed by 28 cycles of denaturation (94 ^{o}C, 45 s), primer annealing (50-60 ^{o}C, 45 s) and extension (72 ^{o}C, 45 s). PCR cycling was ended with a 10 min extension (72 ^{o}C) step. The

products were checked by electrophoresis on 2% agarose gels. Fluorescently-labelled products were then electrophoresed on an Applied Biosystems ABI 3700 system (SUPAMAC, Australia). Analysis of peak signal areas, corresponding to fluorescence intensity, was carried out using the GeneScan 3.1 analysis software (Applied Biosystems). Ratios of signal intensities were calculated for the two alleles at each marker locus. Allele loss was defined according to the following formula: LOH index = (T2/T1)/(N2/N1), where T was the tumour sample, N was the matched normal sample, 1 and 2 were the intensities of the smaller and larger alleles, respectively (Giraud et al., 1997). Loss of an allele was established if the ratio was < 0.67 or > 1.3.

Initially, five markers were analysed (D10S579, AFMs086wg9, IVS4, IVS8, D10S541) in all samples. Those samples showing allele loss at the extreme centromeric or telomeric markers were analysed with a further two markers (D10S1687 and D10S1735) to define the deletion boundaries. Overall, samples were scored as showing LOH if allele loss was observed at two or more of the five initial marker loci analysed.

Determination of the allelic distribution of individual mutations

Where more than one mutation occurred in an individual exon, the particular exon was re-amplified and cloned using the TOPO TA cloning system (Invitrogen). Where mutations occurred in more than one exon of the gene in the same tumour, RT-PCR amplification of a region encompassing all mutations was carried out and the RT-PCR product cloned using the same system. Ten clones were sequenced to determine the allelic distribution of detected mutations.

RNA extraction and RT-PCR analysis

Where necessary, total RNA was isolated from microdissected tumour cells using the High Pure RNA Tissue kit (Roche Molecular Biochemicals) according to the

manufacturer's instructions. Total RNA was reverse transcribed and amplified using the Superscript One Step RT-PCR system (Gibco BRL) as described by the manufacturer. RT-PCR was used to amplify the complete *PTEN* open reading frame using the exonic primers PTENEx1-Fwd (5'-ATGACAGCCATCATCAAAGAG-3') and PTENEx9-Rev (5'-GACTTTTGTAATTTGTGTATGC-3'). The RT-PCR products were subsequently purified, cloned and sequenced.

PTEN Immunohistochemical analysis and scoring

Histological stage and tumour grade was determined by a dedicated pathologist (CJH) and a paraffin block representative of each tumour was selected. Immunohistochemical staining was carried out on histological sections using the PTEN monoclonal antibody 6H2.1 (Cascade Bioscience, USA). This antibody, raised against the last 100 C-terminal amino acids of human PTEN, has been used by various investigators (Gimm et al., 2000; Perren et al., 1999; Zhou et al., 2000a; Zhou et al., 2002b). Essentially, tissue sections (3 μm) were deparaffinised by soaking in xylene and hydrated by passing through a graded series of ethanol. Antigen retrieval was carried out by incubation of slides for 40 min at 99°C in target retrieval solution (DAKO). Endogenous peroxidase was quenched by soaking the slides in 3% hydrogen peroxide for 5 min.

Automated immunohistochemical staining was carried out using a DAKO immunoautostainer (Universal Staining System). Slides were sequentially incubated with a dilution (1:300) of the PTEN primary antibody (Ab) clone 6H2.1, biotinylated secondary antibody (DAKO) and peroxidase-labelled streptavidin reagent (DAKO). All incubations were performed at room temperature. Finally, 3'-3' diaminobenzidine (DAKO) was used as the coloured chromogen which gives a brown product. Slides were counterstained with Harris haematoxylin, mounted and viewed under a light microscope.

Vascular endothelium, present in all sections, served as an internal positive control to monitor variation and act as a reference for determining intensity of staining in each slide as previously described (Gimm et al., 2000; Perren et al., 1999; Zhou et al., 2000a; Zhou et al., 2002b). In addition to positive controls, negative controls were slides treated identically to the tumour sections with the exception that they were incubated with a negative control reagent (DAKO) instead of the primary PTEN (6H2.1) antibody.

Four independent observers (CE, CDM, XW and CJH) reviewed the slides and determined the immunostaining patterns and staining intensities of all slides blindly (with no prior knowledge of *PTEN* gene alteration status). Scoring was concordant in over 95% of cases, and where opinions differed, slides were re-reviewed and a consensus score applied. Immunostaining intensity of the vascular endothelium was scored as ++. Immunostaining intensities (cytoplasmic and nuclear) in tumour and normal bowel were compared to the vascular endothelium present within each section. Staining intensities equal to that of vascular endothelium were scored as ++ (normal or retained); weak or decreased staining intensity as + (reduced); and no immunostaining as - (absent). Any immunostaining intensity stronger than that of vascular endothelium was operationally graded as +++ (increased).

Statistical analyses

Analyses were conducted for all tumours and for the subgroup of MSI- tumours. Continuous variables were compared using the t-test. Associations between categorical variables were determined by the chi square test or the Fisher exact test when subgroup numbers were low. All analyses were conducted using SAS version 8 (SAS Institute Inc. Cary, NC, USA). A p-value of ≤ 0.05 was taken as denoting statistical significance.

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References

Aaltonen, L.A., Peltomaki, P., Leach, F.S., Sistonen, P., Pylkkanen, L., Mecklin, J.P., Jarvinen, H., Powell, S.M., Jen, J., Hamilton, S.R. & et al. (1993). *Science*, **260,** 812-6.

Ali, I.U., Schriml, L.M. & Dean, M. (1999). *J Natl Cancer Inst*, **91,** 1922-32.

Beahrs, O.H. (1997). *American Joint Cmmittee on Cancer: AJCC Cancer Staging Manual*. Lippincott-Raven: Philadelphia, pp 83-90.

Boland, C.R., Thibodeau, S.N., Hamilton, S.R., Sidransky, D., Eshleman, J.R., Burt, R.W., Meltzer, S.J., Rodriguez-Bigas, M.A., Fodde, R., Ranzani, G.N. & Srivastava, S. (1998). *Cancer Res*, **58,** 5248-57.

Bonneau, D. & Longy, M. (2000). *Hum Mutat*, **16,** 109-22.

Cairns, P., Okami, K., Halachmi, S., Halachmi, N., Esteller, M., Herman, J.G., Jen, J., Isaacs, W.B., Bova, G.S. & Sidransky, D. (1997). *Cancer Res*, **57,** 4997-5000.

Chang, J.G., Chen, Y.J., Perng, L.I., Wang, N.M., Kao, M.C., Yang, T.Y., Chang, C.P. & Tsai, C.H. (1999). *Eur J Cancer*, **35,** 647-51.

Chung, D.C. (2000). *Gastroenterology*, **119,** 854-65.

Dahia, P.L., Marsh, D.J., Zheng, Z., Zedenius, J., Komminoth, P., Frisk, T., Wallin, G., Parsons, R., Longy, M., Larsson, C. & Eng, C. (1997). *Cancer Res*, **57,** 4710-3.

Di Cristofano, A., Kotsi, P., Peng, Y.F., Cordon-Cardo, C., Elkon, K.B. & Pandolfi, P.P. (1999). *Science*, **285,** 2122-5.

Di Cristofano, A., Pesce, B., Cordon-Cardo, C. & Pandolfi, P.P. (1998). *Nat Genet*, **19,** 348-55.

Dicuonzo, G., Angeletti, S., Garcia-Foncillas, J., Brugarolas, A., Okrouzhnov, Y., Santini, D., Tonini, G., Lorino, G., De Cesaris, M. & Baldi, A. (2001). *Clin Cancer Res*, **7,** 4049-53.

Downward, J. (1998). *Curr Opin Cell Biol*, **10,** 262-7.

Duerr, E.M., Rollbrocker, B., Hayashi, Y., Peters, N., Meyer-Puttlitz, B., Louis, D.N., Schramm, J., Wiestler, O.D., Parsons, R., Eng, C. & von Deimling, A. (1998). *Oncogene*, **16,** 2259-64.

Elsaleh, H. (2001). *Gastroenterology*, **121,** 230-1.

Elsaleh, H., Powell, B., McCaul, K., Grieu, F., Grant, R., Joseph, D. & Iacopetta, B. (2001a). *Clin Cancer Res*, **7,** 1343-9.

Elsaleh, H., Shannon, B. & Iacopetta, B. (2001b). *Gastroenterology*, **120,** 1309-10.

Elsaleh, H., Powell, B., Soontrapornchai, P., Joseph, D., Goria, F., Spry, N. & Iacopetta, B. (2000). *Oncology*, **58,** 52-9.

Eng, C. (2000). *J Med Genet*, **37,** 828-30.

Eng, C. & Peacocke, M. (1998). *Nat Genet*, **19,** 223.

Eng, C., Thiele, H., Zhou, X.P., Gorlin, R.J., Hennekam, R.C. & Winter, R.M. (2001). *Lancet*, **358,** 2079-80.

Feilotter, H.E., Nagai, M.A., Boag, A.H., Eng, C. & Mulligan, L.M. (1998). *Oncogene*, **16,** 1743-8.

Frayling, I.M., Bodmer, W.F. & Tomlinson, I.P. (1997). *Cancer Genet Cytogenet*, **97,** 64-9.

Georgescu, M.M., Kirsch, K.H., Akagi, T., Shishido, T. & Hanafusa, H. (1999). *Proc Natl Acad Sci U S A*, **96,** 10182-7.

Gimm, O., Perren, A., Weng, L.P., Marsh, D.J., Yeh, J.J., Ziebold, U., Gil, E., Hinze, R., Delbridge, L., Lees, J.A., Mutter, G.L., Robinson, B.G., Komminoth, P., Dralle, H. & Eng, C. (2000). *Am J Pathol*, **156,** 1693-700.

Giraud, S., Choplin, H., Teh, B.T., Lespinasse, J., Jouvet, A., Labat-Moleur, F., Lenoir, G., Hamon, B., Hamon, P. & Calender, A. (1997). *J Clin Endocrinol Metab*, **82,** 3487-92.

Guanti, G., Resta, N., Simone, C., Cariola, F., Demma, I., Fiorente, P. & Gentile, M. (2000). *Hum Mol Genet*, **9,** 283-7.

Guldberg, P., thor Straten, P., Birck, A., Ahrenkiel, V., Kirkin, A.F. & Zeuthen, J. (1997). *Cancer Res*, **57,** 3660-3.

Han, S.Y., Kato, H., Kato, S., Suzuki, T., Shibata, H., Ishii, S., Shiiba, K., Matsuno, S., Kanamaru, R. & Ishioka, C. (2000). *Cancer Res*, **60,** 3147-51.

Hansen, L., Jensen, J.N., Ekstrom, C.T., Vestergaard, H., Hansen, T. & Pedersen, O. (2001). *Diabetologia*, **44,** 237-40.

Ilyas, M., Straub, J., Tomlinson, I.P. & Bodmer, W.F. (1999). *Eur J Cancer*, **35,** 335-51.

Ionov, Y., Peinado, M.A., Malkhosyan, S., Shibata, D. & Perucho, M. (1993). *Nature*, **363,** 558-61.

Kahnoski, K., Khoo, S.K., Nassif, N.T., Chen, J., Lobo, G.P., Segelov, E. & Teh, B.T. (2003). *J Med Genet* (in press).

Kong, D., Suzuki, A., Zou, T.T., Sakurada, A., Kemp, L.W., Wakatsuki, S., Yokoyama, T., Yamakawa, H., Furukawa, T., Sato, M., Ohuchi, N., Sato, S., Yin, J., Wang, S., Abraham, J.M., Souza, R.F., Smolinski, K.N., Meltzer, S.J. & Horii, A. (1997). *Nat Genet*, **17,** 143-4.

Kurose, K., Bando, K., Fukino, K., Sugisaki, Y., Araki, T. & Emi, M. (1998). *Jpn J Cancer Res*, **89,** 842-8.

Kurose, K., Zhou, X.P., Araki, T. & Eng, C. (2000). *Genes Chromosomes Cancer*, **29,** 166-72.

Lee, J.O., Yang, H., Georgescu, M.M., Di Cristofano, A., Maehama, T., Shi, Y., Dixon, J.E., Pandolfi, P. & Pavletich, N.P. (1999). *Cell*, **99,** 323-34.

Li, D.M. & Sun, H. (1997). *Cancer Res*, **57,** 2124-9.

Li, J., Simpson, L., Takahashi, M., Miliaresis, C., Myers, M.P., Tonks, N. & Parsons, R. (1998). *Cancer Res*, **58,** 5667-72.

Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S.I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S.H., Giovanella, B.C., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M.H. & Parsons, R. (1997). *Science*, **275,** 1943-7.

Liaw, D., Marsh, D.J., Li, J., Dahia, P.L., Wang, S.I., Zheng, Z., Bose, S., Call, K.M., Tsou, H.C., Peacocke, M., Eng, C. & Parsons, R. (1997). *Nat Genet*, **16,** 64-7.

Lin, W.M., Forgacs, E., Warshal, D.P., Yeh, I.T., Martin, J.S., Ashfaq, R. & Muller, C.Y. (1998). *Clin Cancer Res*, **4,** 2577-83.

Liu, Q. & Sommer, S.S. (1994). *PCR Methods Appl*, **4,** 97-108.

Maehama, T. & Dixon, J.E. (1998). *J Biol Chem*, **273,** 13375-8.

Marsh, D.J., Dahia, P.L., Zheng, Z., Liaw, D., Parsons, R., Gorlin, R.J. & Eng, C. (1997). *Nat Genet*, **16,** 333-4.

Marsh, D.J., Kum, J.B., Lunetta, K.L., Bennett, M.J., Gorlin, R.J., Ahmed, S.F., Bodurtha, J., Crowe, C., Curtis, M.A., Dasouki, M., Dunn, T., Feit, H., Geraghty, M.T., Graham, J.M., Jr., Hodgson, S.V., Hunter, A., Korf, B.R., Manchester, D., Miesfeldt, S., Murday, V.A., Nathanson, K.L., Parisi, M., Pober, B., Romano, C., Eng, C. & et al. (1999). *Hum Mol Genet*, **8,** 1461-72.

Marsh, D.J., Coulon, V., Lunetta, K.L., Rocca-Serra, P., Dahia, P.L., Zheng, Z., Liaw, D., Caron, S., Duboue, B., Lin, A.Y., Richardson, A.L., Bonnetblanc, J.M., Bressieux, J.M., Cabarrot-Moreau, A., Chompret, A., Demange, L., Eeles, R.A., Yahanda, A.M., Fearon, E.R., Fricker, J.P., Gorlin, R.J., Hodgson, S.V., Huson, S., Lacombe, D., Eng, C. & et al. (1998). *Hum Mol Genet*, **7,** 507-15.

Maxwell, G.L., Risinger, J.I., Hayes, K.A., Alvarez, A.A., Dodge, R.K., Barrett, J.C. & Berchuck, A. (2000). *Clin Cancer Res*, **6,** 2999-3005.

Miller, S.J., Lou, D.Y., Seldin, D.C., Lane, W.S. & Neel, B.G. (2002). *FEBS Lett*, **528,** 145-53.

Minaguchi, T., Yoshikawa, H., Oda, K., Ishino, T., Yasugi, T., Onda, T., Nakagawa, S., Matsumoto, K., Kawana, K. & Taketani, Y. (2001). *Clin Cancer Res*, **7,** 2636-42.

Mutter, G.L., Ince, T.A., Baak, J.P., Kust, G.A., Zhou, X.P. & Eng, C. (2001). *Cancer Res*, **61,** 4311-4.

Mutter, G.L., Lin, M.C., Fitzgerald, J.T., Kum, J.B., Baak, J.P., Lees, J.A., Weng, L.P. & Eng, C. (2000). *J Natl Cancer Inst*, **92,** 924-30.

Myers, M.P., Pass, I., Batty, I.H., Van der Kaay, J., Stolarov, J.P., Hemmings, B.A., Wigler, M.H., Downes, C.P. & Tonks, N.K. (1998). *Proc Natl Acad Sci U S A*, **95,** 13513-8.

Negoro, K., Takahashi, S., Kinouchi, Y., Takagi, S., Hiwatashi, N., Ichinohasama, R., Shimosegawa, T. & Toyota, T. (2000). *Dis Colon Rectum*, **43,** S29-33.

Neshat, M.S., Mellinghoff, I.K., Tran, C., Stiles, B., Thomas, G., Petersen, R., Frost, P., Gibbons, J.J., Wu, H. & Sawyers, C.L. (2001). *Proc Natl Acad Sci U S A*, **98,** 10314-9.

Okami, K., Wu, L., Riggins, G., Cairns, P., Goggins, M., Evron, E., Halachmi, N., Ahrendt, S.A., Reed, A.L., Hilgers, W., Kern, S.E., Koch, W.M., Sidransky, D. & Jen, J. (1998). *Cancer Res*, **58,** 509-11.

Perren, A., Weng, L.P., Boag, A.H., Ziebold, U., Thakore, K., Dahia, P.L., Komminoth, P., Lees, J.A., Mulligan, L.M., Mutter, G.L. & Eng, C. (1999). *Am J Pathol*, **155,** 1253-60.

Podsypanina, K., Ellenson, L.H., Nemes, A., Gu, J., Tamura, M., Yamada, K.M., Cordon-Cardo, C., Catoretti, G., Fisher, P.E. & Parsons, R. (1999). *Proc Natl Acad Sci U S A*, **96,** 1563-8.

Rhei, E., Kang, L., Bogomolniy, F., Federici, M.G., Borgen, P.I. & Boyd, J. (1997). *Cancer Res*, **57,** 3657-9.

Salvesen, H.B., MacDonald, N., Ryan, A., Jacobs, I.J., Lynch, E.D., Akslen, L.A. & Das, S. (2001). *Int J Cancer*, **91,** 22-6.

Sarkar, G., Yoon, H.S. & Sommer, S.S. (1992). *Genomics*, **13,** 441-3.

Sato, K., Tamura, G., Tsuchiya, T., Endoh, Y., Sakata, K., Motoyama, T., Usuba, O., Kimura, W., Terashima, M., Nishizuka, S., Zou, T. & Meltzer, S.J. (2002). *Virchows Arch*, **440,** 160-5.

Shin, K.H., Park, Y.J. & Park, J.G. (2001). *Cancer Lett*, **174,** 189-94.

Singh, B., Ittmann, M.M. & Krolewski, J.J. (1998). *Genes Chromosomes Cancer*, **21,** 166-71.

Smith, J.M., Kirk, E.P., Theodosopoulos, G., Marshall, G.M., Walker, J., Rogers, M., Field, M., Brereton, J.J. & Marsh, D.J. (2002). *J Med Genet*, **39,** 937-40.

Stambolic, V., Suzuki, A., de la Pompa, J.L., Brothers, G.M., Mirtsos, C., Sasaki, T., Ruland, J., Penninger, J.M., Siderovski, D.P. & Mak, T.W. (1998). *Cell*, **95,** 29-39.

Steck, P.A., Pershouse, M.A., Jasser, S.A., Yung, W.K., Lin, H., Ligon, A.H., Langford, L.A., Baumgard, M.L., Hattier, T., Davis, T., Frye, C., Hu, R., Swedlund, B., Teng, D.H. & Tavtigian, S.V. (1997). *Nat Genet*, **15,** 356-62.

Taniyama, K., Goodison, S., Ito, R., Bookstein, R., Miyoshi, N., Tahara, E., Tarin, D. & Urquidi, V. (2001). *J Pathol*, **194,** 341-8.

Thibodeau, S.N., Bren, G. & Schaid, D. (1993). *Science*, **260,** 816-9.

Wang, S.I., Puc, J., Li, J., Bruce, J.N., Cairns, P., Sidransky, D. & Parsons, R. (1997). *Cancer Res*, **57,** 4183-6.

Wang, Z.J., Taylor, F., Churchman, M., Norbury, G. & Tomlinson, I. (1998). *Am J Pathol*, **153,** 363-6.

Watanabe, T., Wu, T.T., Catalano, P.J., Ueki, T., Satriano, R., Haller, D.G., Benson, A.B., 3rd & Hamilton, S.R. (2001). *N Engl J Med*, **344,** 1196-206.

Whiteman, D.C., Zhou, X.P., Cummings, M.C., Pavey, S., Hayward, N.K. & Eng, C. (2002). *Int J Cancer*, **99,** 63-7.

Wu, X., Senechal, K., Neshat, M.S., Whang, Y.E. & Sawyers, C.L. (1998). *Proc Natl Acad Sci U S A*, **95,** 15587-91.

Yu, K., Toral-Barza, L., Discafani, C., Zhang, W.G., Skotnicki, J., Frost, P. & Gibbons, J.J. (2001). *Endocr Relat Cancer*, **8,** 249-58.

Zhou, X., Hampel, H., Thiele, H., Gorlin, R.J., Hennekam, R.C., Parisi, M., Winter, R.M. & Eng, C. (2001). *Lancet*, **358,** 210-1.

Zhou, X.P., Gimm, O., Hampel, H., Niemann, T., Walker, M.J. & Eng, C. (2000a). *Am J Pathol*, **157,** 1123-8.

Zhou, X.P., Kuismanen, S., Nystrom-Lahti, M., Peltomaki, P. & Eng, C. (2002a). *Hum Mol Genet*, **11,** 445-450.

Zhou, X.P., Li, Y.J., Hoang-Xuan, K., Laurent-Puig, P., Mokhtari, K., Longy, M., Sanson, M., Delattre, J.Y., Thomas, G. & Hamelin, R. (1999). *Int J Cancer*, **84,** 150-4.

Zhou, X.P., Loukola, A., Salovaara, R., Nystrom-Lahti, M., Peltomaki, P., de la Chapelle, A., Aaltonen, L.A. & Eng, C. (2002b). *Am J Pathol*, **161,** 439-47.

Zhou, X.P., Marsh, D.J., Hampel, H., Mulliken, J.B., Gimm, O. & Eng, C. (2000b). *Hum Mol Genet*, **9,** 765-8.

Table 1 Alterations of the *PTEN* gene and effect on PTEN expression in sporadic microsatellite stable and unstable colorectal cancers

Tumour ID	MSI Status	Nucleotide Change	Predicted Protein or RNA Change	*PTEN* Allele(s) Affected[a]	LOH at 10q[b]	PTEN Ab Staining
1	-ve	AAA→GAA	K125E	A1 + A2[c]	LOH[d]	Reduced
2	-ve	C→G[e] GAT→AAT[g]	- D153N	A1 + A2 A1 + A2	ROH[f]	Absent
3	-ve	GAT→TAT GTC→GCC	D153Y V217A	A1 A2	ROH	Reduced
5	-ve	-	-	A1 or A2	LOH	Reduced
6	-ve	-	-	A1 or A2	LOH	Reduced
12	-ve	-	-	A1 + A2	HD[h]	Absent
14	-ve	-	-	A1 + A2	HD	Absent
17	+ve	Del ACTT (955-8)[i] AAT→AAA	Stop at 319 N323K	A1 A2	ROH	Absent
19	-ve	AAA→AGA TAC→TGC	K62R Y65C	A1 A2	ROH	Reduced
20	-ve	C→G AAA→TAA	- K125X	A1 + A2 A1 or A2	ROH	Absent
27	-ve	-	-	A1 or A2	LOH	Reduced
34	-ve	-	-	A1 or A2	LOH	Reduced
37	-ve	GAG→CAG[g]	E150Q	A1 + A2	ROH	Reduced
45	-ve	-	-	A1 or A2	LOH	Reduced
48A	-ve	Del ACTT (955-8)[i]	Stop at 319	A1 or A2	ROH	Absent

[a] Affected *PTEN* allele(s) inferred from cloning, sequencing and LOH analysis.
[b] LOH was determined from analysis of the flanking markers D10S579 and D10S541 as well as the 3 intragenic markers AFMa086wg9, IVS4 +109 ins/del ACTT and IVS8 +32 G/T.
[c] Individual *PTEN* alleles have been arbitrarily named A1 and A2.
[d] Loss of heterozygosity at two or more of the 5 marker loci.
[e] Homozygous germline polymorphism. Numbering is relative to the translational start site (+1).
[f] Retention of heterozygosity.
[g] Homozygous somatic mutation.
[h] Suspected homozygous deletion at two or more marker loci.
[i] Base numbering is from the *PTEN* mRNA sequence with the translational start site as +1

Table 2 PTEN immunohistochemical analysis of primary sporadic colorectal tumours

PTEN ABNORMALITY	**TUMOUR PTEN EXPRESSION[a]**								
	All Tumours (n=41)			**MSI- Tumours (n=34)**			**MSI+ Tumours (n=7)**		
	Retained (++)	**Reduced (+)**	**Absent (-)**	**Retained (++)**	**Reduced (+)**	**Absent (-)**	**Retained (++)**	**Reduced (+)**	**Absent (-)**
PTEN MUTATION									
1 Allele	0	0	1	0	0	1	0	0	0
2 Alleles	0	3	3	0	3	2	0	0	1
LOH AT 10q									
1 Allele	0	5	0	0	5	0	0	0	0
2 Allele	0	0	2	0	0	2	0	0	0
PTEN MUTATION + LOH	0	1	0	0	1	0	0	0	0
NO *PTEN* ALTERATION	12	10	4	10	8	2	2	2	2
TOTAL TUMOURS	**12**	**19**	**10**	**10**	**17**	**7**	**2**	**2**	**3**

[a]Change in cytoplasmic PTEN antibody staining intensity

Legends to figures

Figure 1 Dideoxy fingerprinting (ddF) analysis of *PTEN*. Autoradiographs of gels showing germline and somatic migration shifts (**a-f**). Those samples showing band shifts were chosen for sequence analysis. **(a)** germline shift observed in CRC 20 exon 1; Somatic shifts observed in**: (b)** CRC 3 exon 7; **(c)** CRC 48A exon 8; **(d)** CRC 17 exon 8; **(e)** CRC 2 exon 5 and **(f)** CRC 37 exon 5. The symbols used are: G, germline or constitutional DNA; T, tumour DNA; N, normal bowel DNA; A, DNA extracted from normal cells adjacent to the tumour.

Figure 2 Mutations of the *PTEN* gene in primary sporadic colorectal cancer. Schematic representation of the *PTEN* gene showing the type and position of each detected sequence variation. The germline sequence changes (polymorphism) is presented below the bar and the somatic mutations above the bar. Heterozygous mutations are shown in plain type, homozygous mutations have been underlined and mutations detected more than once are indicated.

Figure 3 Sequence analysis of novel *PTEN* somatic mutations. Samples showing migration shifts **(a-e)** were sequenced to determine the nature of the suspected mutation. **(a)** Biallelic mutation of *PTEN* in CRC 19. Two mutations (K62R and Y65C) were detected in exon 3 of CRC 19. The mutant sequence was cloned and each mutation was found to be located in a different clone suggesting that each mutation occurred on a different *PTEN* allele. **(b)** Heterozygous somatic mutation (D153Y) in CRC 3, exon 5; **(c)** heterozygous somatic mutation (V217A) in CRC 3 exon 7; **(d)** homozygous somatic mutation (E150Q) in CRC 37 exon 5; **(e)** homozygous somatic mutation (D153N) in CRC

2 exon 5. The symbols used are: G, germline or constitutional DNA; T, tumour DNA. The arrows indicate the position(s) of the sequence variation(s).

Figure 4 Analysis of LOH at 10q23. **(a)** Schematic representation of the *PTEN* locus showing the microsatellite markers within and flanking the PTEN gene. The relative positions of markers are indicated. **(b)** Hemizygous deletion at the *PTEN* locus in CRC 1 at D10S541 is shown in the two top panels. Amplification of the larger allele (T2) is reduced in the tumour compared to the germline (N2) DNA. Apparent homozygous deletion at the *PTEN* locus in CRC 12 at D10S579 is shown in the two lower panels. Amplification of both alleles (T1 and T2) is reduced in the tumour DNA compared to the germline or constitutional (N1 and N2) DNA with retention of comparable amplification intensity of the PCR amplification standard (BG) in the germline and tumour DNA. **(c)** The deletion status of each polymorphic marker and the respective patient are shown. The open circles represent the presence of two intact alleles, the half-shaded circles indicate LOH at the particular marker and the black circles indicate homozygous deletion of a marker. No symbol has been shown in the cases where a particular marker(s) was non-informative. ND indicates that the analysis was not done.

Figure 5 PTEN expression in primary sporadic colorectal tumours. Immunostaining of tumours was carried out using a monoclonal PTEN antibody (6H2.1) as described. PTEN expression is indicated by the brown chromogenic reaction. Tumours shown in B-I are MSI- and tumour J is MSI+. Arrows indicate the positively-staining vascular endothelial cells present in all sections. **(a)** adjacent normal colonic epithelium showing strong (++) cytoplasmic and nuclear PTEN expression; **(b)** Retention (++) of cytoplasmic expression with absence of structural *PTEN* alterations (CRC 38); **(c)** Reduced (+) PTEN expression with monoallelic *PTEN* mutation (CRC 1); **(d)** Reduced (+) expression with biallelic

PTEN mutation (CRC 19); **(e)** Reduced (+) expression with hemizygous *PTEN* deletion (CRC 45); **(f)** Absence (-) of PTEN expression and biallelic *PTEN* mutation (CRC 20); **(g)** and **(h)** Absent (-) PTEN expression and suspected homozygous deletion including *PTEN* (CRC 12 and 14); **(i)** Absent (-) PTEN expression without detectable *PTEN* structural alteration (CRC 13); **(j)** Absence (-) of PTEN expression with biallelic *PTEN* mutation in the MSI+ tumour CRC 17.