Institute of Pathology
Faculty of Medicine
University of Rostock
Director: Prof. Dr. med. A. Erbersdobler

Gene mutations and losses of heterozygosity

of TP53 and PTEN in colorectal cancer

Inaugural Dissertation

to

obtain the academic degree

Doctor of Medicine

Faculty of Medicine

University of Rostock

Submitted by

Tareq Salem

from Aden/Yemen

Rostock 2014

urn:nbn:de:gbv:28-diss2014-0174-3

Dekan: Prof. Dr. med. E. Reisinger

1. Gutachter: Prof. Dr. Med Friedrich Prall
Institut für Pathologie
Universität Rostock

2. Gutachter: PD Dr. rer. Nat. Michael Linnebacher
Klinik und Poliklinik für Chirurgie
Universität Rostock

3. Gutachter: Prof. Dr. med. Mathias Evert
Institut für Pathologie
Universität Greifswald

Datum der Einreichung; 18. März 2014
Datum der Verteidigung; 15. Oktober 2014

For the soul of my parents

and for my family...

Table of contents

List of tables

- **Tables of the results**

- **Tables of the appendix**

List of figures

Abbreviations

AI	Allelic imbalance
BRAF	Proto-oncogene B-RAF belong to the Raf kinase family (signal transduction protein kinase)
CIN	Chromosomal instability
CRC	Colorectal cancer
LOH	Loss of heterozygosity
MSI	Microsatellite instability
PCR	Polymerase chain reaction
PTEN	Phosphatase and tensin homolog deleted in chromosome ten
ROH	Retention of heterozygosity
SMAD	Homologs to both Drosophilia protein, mother against decapentaplegic (MAD) and Caenorhabditis elegans protein SMA from gene sma for small body size
TP53	Tumour Protein 53
TSG	Tumour suppressor gene

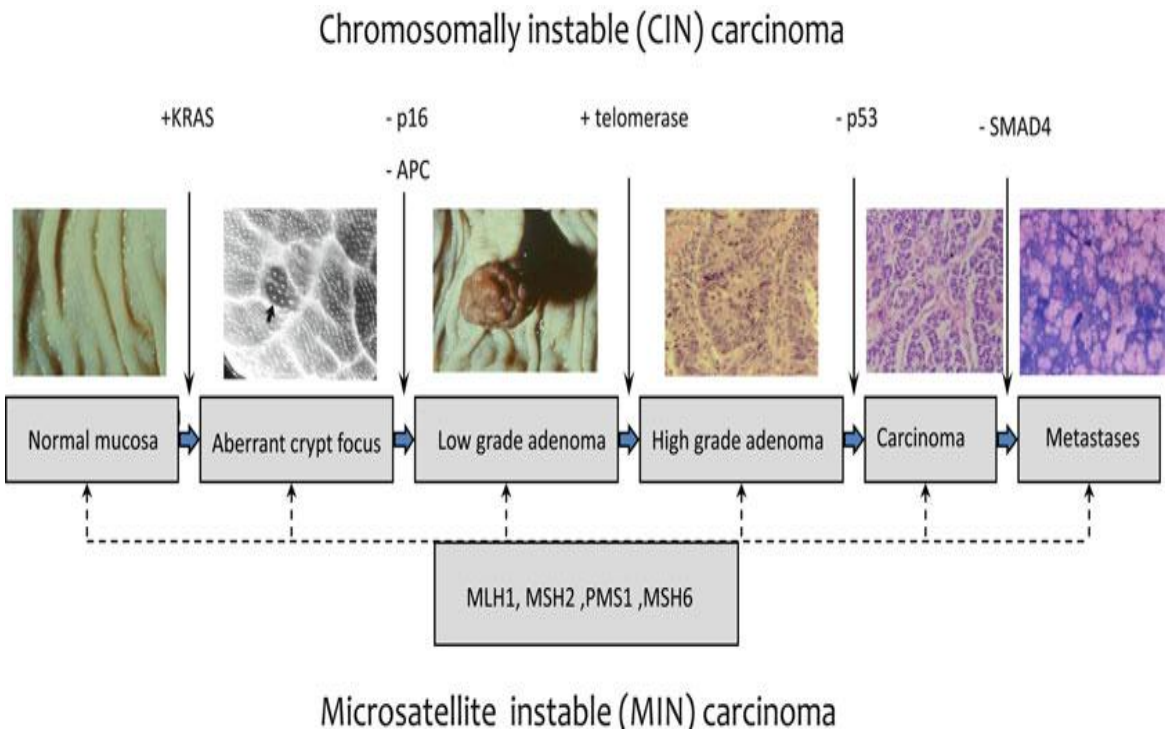
1. Introduction

The proposal of a genetic basis of cancer together with the advances in molecular genetics has provided a deep view into cancer pathogenesis. Nowadays this has made the vision become more obvious about the multistep evolution of cancer by somatic cellular genes mutations and clonal selection of variant progeny with increasing aggressive growth properties. In colon cancer, the essential genetic facts in the understanding of its biology and genetics have emerged and are slowly making their way even into the clinic to contribute in the early diagnosis, therapy and follow-up of colon cancer.

Colorectal cancer (CRC) may develop in several different contexts. The variation in their clinical behaviour and response to therapy exists through various genetic backgrounds and molecular profiles. Based on these varieties, CRC can present as I. pure sporadic, sporadic in the context of inflammatory bowel disease (75-80%) or in II. familial clusters (15-20%) mostly within hereditary familial CRC syndromes (Cardoso J et al, 2007; Calvert PM et al, 2002). Several patients with sporadic CRC carry a familial risk genotype, but have no clear characterized hereditary disease (Goldgar DE et al, 1994; Easton DF, 2008).

Hereditary nonplyposis colon cancer (HNPCC) and familiar adenomatous polyposis (FAP) are the most common and well defined genetic diseases with typical early onset of cancer (Cheah PY, 2009). The genetic screening of families has provided early detection of familial forms of CRC and hence has improved the survival of patients with hereditary variants compared to patients with sporadic types (Lynch HT & de la Chapelle A, 2003).

The first multistep genetic model of colorectal carcinogenesis was proposed by Fearon and Vogelstein, based on the observation of specific genetic changes in benign and malignant lesions (Fearon &Vogelstein, 1990). The main features of this model are mutational activation of oncogenes and inactivating mutation of tumour suppressor genes. The total accumulation of genetic mutations in at least four or five genes is of more importance than the order in which these mutations occur. This model is still considered valid, although a number of genetic events need to be added in order to get the whole picture.



The figure demonstrates the main genetic events in the adenoma carcinoma sequence. Two distinct pathways; namely chromosomal instability (CIN) and microsatellite instability (MIN) are linked throughout colon cancer development. (Liang Cheng, John N. Eble: Molecular surgical pathology, 2013. © Springer Science + Business Media). Note: the abbreviation MSI used instead of MIN for microsatellite instable cases throughout the study.

1.1. Molecular pathways in colorectal carcinogenesis

A simple model of two genetic molecular pathways either microsatellite instability (MSI) or chromosomal instability (CIN) has been initially suggested (Lengauer C et al, 1998). More recently, a third non genetic but rather epigenetic instability pathway was also recognized. Evidence of overlapping between pathways' features has been observed in some tumours (Goel A et al, 2003).

1.1.1. Chromosomal instability pathway

The observation of high-degree CIN in colon cancer has initiated the argument around the role of CIN in cancer onset and/or progression. CIN refers to an accelerated rate of losing or gaining whole chromosomes or large parts of chromosomes during cell division resulting in karyotypic variability from cell to cell. The consequence is an imbalance in chromosome number (aneuploidy), subchromosomal genomic amplifications, and an increased rate of loss of heterozygosity (LOH) (Michor F et al, 2005).

The molecular basis for CIN and the underlying genetic alteration in most CIN cancers have not been yet identified. The DNA genetic alterations are not randomly distributed throughout the genome but target specific genes and CIN causative mutations are selected for tumour cells that enhance the rate of LOH at tumour suppressor genes and up regulate oncogenes by allelic gains (Grady WM, 2004).

Chromosome loss is a common molecular defect in colon cancer similar to other solid tumours. Many investigators explored the relationship between CRC and allele losses at different chromosome loci, where one or more tumour suppressor genes are located (Nowak MA et al, 2002). Chromosomal instability was found in 70% of sporadic CRC, where some of them exhibit recurrence that represents basic chromosomal changes for cancer initiation and progression (Cho KR & Vogelstein B, 1992). The prognostic value of LOH in colorectal cancer was investigated but its significance still remains uncertain (Weber JC et al, 2001).

Advances in scientific research have improved the methodological approaches for CIN measurement such as DNA flow cytometry, allelic imbalance assays (tumour allelotyping/LOH analysis), fluorescent in situ hybridization, and recently, comparative genomic hybridization (CGH). Currently, CGH microarrays (array CGH) have contributed innovative advances in the field due to their ability to identify chromosomal and segmental amplifications and deletions with higher resolution (Geigl JB et al, 2008).

1.1.2. Microsatellite instability pathway

Microsatellite instability is considered a definitive subtype of CRC with a well defined histopathological and therapeutic profile. This pathway is characterized by the loss of mutation mismatch repair (MMR) gene function resulting in tumour-specific frame-shift mutations in stretches of short repetitive DNA sequences (i.e. microsatellite repeats) distributed along the genome (Ionov Y et al, 1993; Thibodeau SN et al, 1993). Notably, MSI tumours increase nucleotide mutation rates, which remain throughout DNA replication resulting in alleles of different size but share near-diploid chromosomal contents (Parsons R et al, 1993; Bhattacharyya NP et al, 1994; Eshleman JR et al, 1998; Jacob S et al, 2002).

Germline defects in MMR genes occur in 5% of all CRC patients with a positive family history, which is the hallmark of the well-known hereditary non-polyposis colorectal cancer syndrome (Lynch HT & Lynch J, 2000). The MSI phenotype found in about 15% of all sporadic colorectal cancer is usually not associated with gross chromosomal changes (Hoang JM et al, 1997; Peltomaki P, 2001).

The most frequently mutated genes in the MSI pathway are transforming growth factor beta receptor II (TGFBR2), Myc-associated factor X (MAX) and Insulin-like growth factor 2 receptor (IGF2R). Inactivating mutations of TGFBR2 is found in approximately 90% of all CRC with MSI phenotype where mostly both alleles are affected (Parsons R, 1995), Hence it is suggested to play an important role in the development of MMR deficient colorectal tumour (Grady WM et al, 1998).

In sporadic CRC, promoter hypermethylation of MutL homolog 1 gene (MLH1) is the key (Issa JP, 2008). The MMR state can be assayed immunohistochemically and/or by PCR microsatellite analysis with the Bethesda panel of microsatellite markers used routinely in clinical practise with a high prognostic and predictive value (Vilar E & Gruber SB, 2010; Geiersbach KB & Samowitz WS, 2011).

Currently due to the frequent association of a specific histological lesion known as sessile serrated adenomas or polyps (SSA/P) with increased risk for CRC development; a new distinct independently alternative pathway known as **serrated pathway** was proposed. This is molecularly characterized by BRAF mutation and promoter methylation of different genes, especially MLH1 gene (Jass JR et al, 2000; Huang CS, 2004).

1.1.3. Epigenetic instability pathway

Pathological epigenetic changes, non-sequence based alterations inherited through cell division, are considered to be involved in the regulation of transcription. An intense DNA hypermethylation has been observed in the gene promoter associated CpG sites. The CpG island methylator phenotype (CIMP) was described as a distinct pathway by epigenetic silencing and inactivating mutations (Egger G et al, 2004).

Epigenetic changes include global DNA hypomethylation, hypermethylation, gene-specific hypo- and hyper-methylation, chromatin alterations, and loss of imprinting. As a net result, these changes may lead to abnormal activation of growth promoting

genes or abnormal silencing of tumour suppressor genes (Feinberg AP & Tycko B, 2004). Gene specific hypermethylation of normally unmethylated promoters applies to many tumour suppressor genes including Wnt signalling genes, like APC in colorectal cancer (Feinberg AP et al, 2006).

Germline epigenetic changes were recently reported. HNPCC cases for which germline mutations in none of the MMR genes could be demonstrated the hypermethylation of the MLH1 gene or MutS homolog 2 (MSH2) gene was found functionally equivalent to inactivating mutation that producing a clinical phenotype similar to HNPCC (Chan TL et al, 2006; Hitchins M et al, 2005; Suter CM et al, 2004).

Loss of imprinting (LOI) of IGF2 is a common epigenetic variant in adults which is associated with increased risk for CRC (Cui H et al, 2003). By testing gene promoter methylation, quantitative DNA analyses of methylations provide more useful information than methylation-specific PCR for the determination of CIMP (Ogino S et al, 2006).

1.2. Tumour suppressor pathway

Tumour suppressor genes (TSGs) are considered the safeguards of the cell. The importance of TSGs is related to the protective function of the protein products against the cell step on the path to cancer. These proteins are implicated in several cellular processes like cell cycle arrest and programmed cell death, indicating its fundamental roles in maintenance of cellular homeostasis.

It is well recognized that germline mutations of TSGs manifest as heritable cancer predisposition syndromes (Malkin D et al, 1992). Generally, one copy of a TSG suffices to control cell proliferation. The demand for two genetic events (Two “hits”) that inactivate both gene alleles was defined and proposed for giving a selective growth advantage to the cell (Knudson AG, 1993; Vogelstein B & Kinzler KW, 2004). TSGs may be partially altered or completely depleted by many genetic events such as missense mutation at gene residues essential for its activity; or by nonsense mutations that result in a truncated protein as well as through deletions or insertion of various chromosomal sizes (Vogelstein B & Kinzler KW, 2004).

However, an epigenetic mechanism such as DNA methylation has also been recorded for TSGs inactivation (Esteller M, 2002). Recently and away from the two hit

theory, a new class of TSGs with haploid insufficiency have been hypothesized. This includes only one genetic event affecting one allele required to cause functional inactivation whereas the single remaining functional allele is insufficient to bring about a wild- type condition and giving the cell selective growth advantage, when challenged by carcinogens (Macleod K, 2000).

Tumour protein 53 (TP53) gene is an ideal model for TSGs, it has been widely recognized and was entitled as the 'Molecule of the Year' (Lane DP, 1992). Studies have implicated the p53 protein as important transcription factor and a multifunctional protein. In its normal condition it represents an important factor in tumour surveillance. Based on these facts p53 was denoted guardian of genome (Vousden KH & Prives C, 2005). Protein 53 is involved in multiple pathways, namely differentiation, senescence, antiangiogenesis, cell cycle arrest and programmed cell death (Lane DP, 1992; Vousden KH & Lu X, 2002; Levine AJ et al, 2006).

Normally, p53 expression is induced by cellular stress such as genetic damage, radiation or imbalance of mitogenic signals which in turn promotes p53 mediated cell growth inhibition via transcriptional activation of cyclin inhibitors like p21. It then ultimately promotes cell death through apoptosis via transcriptional activation of genes such as Bcl-2 associated X (BAX) when the DNA repair machinery cannot cope with the DNA damage load. In absence of cellular stress, the p53 interacts with its main regulator Mouse douple minute homolog 2 (Mdm2) and other proteins keeping p53 expression at a lower level to avoid otherwise harmful effects on normal growth and development (Kubbutat MH et al, 1997).

TP53 is considered a typical TSG, fulfilling the cardinal criteria of the 'two-hit' hypothesis. This was verified by Baker et al. who reported a mutation in one copy of the allele in combination with deletion of the other allele in human colon cancer (Baker SJ et al, 1989). TP53 is found mutated in more than 50% of human cancers. Since TP53 is a tumour suppressor gene, protein function is repressed or lost by mutations, most frequently missense, or deletion of the entire or part of the gene. This mostly occurs in certain gene regions encoding the DNA binding domain. Such mutations impair protein and target DNA binding by disrupting the structure of the domain (Harris CC, 1993).

Since TP53 proved to be halpoinsufficient in studies of mice with a heterozygous p53 status it turned out not to be an ordinary TSG (Venkatachalam S et al, 1998). Only one genetic event can be sufficient to silence the protein. Thus, TP53 does not follow in all aspects the usual mechanism of TSGs. Furthermore, a mutated p53 protein may inhibit normal activity by interfering with wild-type p53 proteins during the formation of homotetramers, the functional p53 unit. The tetramer is thereby deactivated in a dominant-negative fashion, despite only one single p53 mutant protein participating in oligomerization (McLure KG & Lee PW, 1998).

1.3. The genomic landscape of colorectal cancer

The earliest and rate limiting genetic event in colorectal tumour initiation very often is a mutation of the adenomatous polyposis coli (APC) tumour suppressor gene on chromosome 5q21. This is frequently observed at the aberrant crypt foci (ACF) stage in relation to the degree of dysplastic changes that occurs in over than 80% of colon adenocarcinoma (Powell SM et al, 1992; Leslie A et al, 2002). APC acts as a tumour suppressor gene by regulation of the intracellular level of ß-catenin, a key member of the Wnt signal transduction pathway (Korinek V et al, 1997; Morin PJ et al, 1997; Smits R et al, 1999). The second mutational hits is distributed according to the resulting level of residual ß-catenin down-regulating activity (Powell SM et al, 1992; Miyoshi Y et al, 1992; Lamlum H et al, 1999; Albuquerque C et al, 2002). APC gene germline mutation is either inherited or spontaneously as occurs in FAP or one of its variants. This in turn leads to an increase in the rate of initiation of colon adenomas and inevitable progression to carcinoma unless the intestine is not resected surgically (Grady WM & Markowitz SD, 2003; Kinzler K.W. & Vogelstein B, 1996).

Mutation activation of the rat sarcoma (RAS) oncogene is a major part in the molecular genetic analysis of colorectal cancer. It is found in at least 50% of colorectal adenomas larger than 1 cm in size and in carcinomas, but is infrequent in adenomas smaller than 1 cm in size (Forrester K et al, 1987; Vogelstein B et al, 1988). Alternatively, mutations in other oncogenes like BRAF that encodes other members of the RAS pathway are often found among adenomas (Beach R et al, 2005). Activation of this signalling pathway results in an increase of the transcriptional activation of target genes involved in cell proliferation and apoptosis (Kim EC & Lance P, 1997).

Loss of all or part of the long arm of chromosome 18 (18q) was found in at least 50% of large adenomas and 75% of carcinomas (Vogelstein B, Fearon ER, 1988; Vogelstein B, Fearon ER et al 1989; Jen J, 1994). The Deleted in colorectal carcinoma (DCC) tumour suppressor gene was suggested first; later SMAD2 and SMAD4 tumour suppressor genes were indentified in this region. These genes are involved in the Transforming Growth Factor ß (TGF-ß) signalling pathway, an important pathway in the control of cell proliferation, differentiation, migration and apoptosis. TGFBR2 mutations are also frequently found to affect TGF-ß signalling in CRC, mainly among MSI tumours but also in microsatellite stable tumours (Keino-Masu K et al, 1996; Cho KR et al, 1994; Heldin CH et al, 1997; Duff EK & Clarke AR, 1998; Grady WM et al, 1999).

Loss of the short arm of chromosome 17 (17p) is characteristic for colon cancers. TP53 tumour suppressor gene is mapped to this chromosomal region and is considered to be a major colon cancer candidate gene. Inactivation of both TP53 gene alleles can be detected in most intestinal tumours. Mutations in this gene are found in association with the transition from late adenoma to carcinoma and then supposed to be a late event as observed in advanced colon malignancies rather than in the early stages (Lane DP, 1992; Harris CC, 1993; Vogelstein B et al, 1988; Baker SJ, 1990).

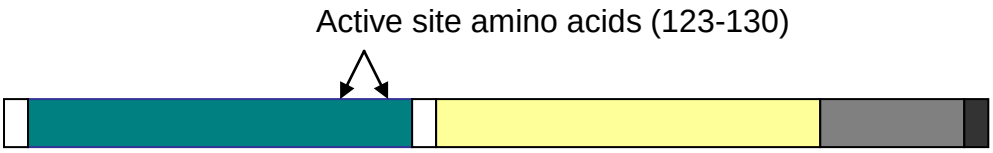
Scientists have detected more than 70% LOH at chromosome 17p and point mutation of the remaining allele in greater than 50% of sporadic CRC (Baker SJ et al, 1989; Harris CC, 1993), TP53 genetic alterations can be measured as aberrant overexpression in immunohistochemical assay, either by direct DNA sequencing or by 17p LOH analysis (Slebos RJC et al, 1996; Baast IO et al, 1994). A p53 expression was used as a marker for TP53 gene mutation, since up to 75% showed agreement with missense mutation (Erhan Y et al, 2002; Guttmejer A N et al, 2000).

Currently new genes have been proposed to be implicated in colon cancer development and metastasis. Examples include gain of chromosome 20q observed in adenoma and Aurora Kinase (AURKA) that represents a candidate oncogenes located at 20q13.2 (Carvalho B et al, 2009). Cycline-dependent kinase 8 (CDK8) located within the frequently amplified chromosome 13 (13q12) was suggested to display oncogenic characteristics (Firestein R et al, 2008). Moreover, SMAD7 is

maps to the 18q locus and has gained a lot of attention (Broderick P et al, 2007). Some of the late chromosomal aberrations suggested are loss of the 8q arm, but not so far tumour suppressor gene has been identified here. Protein tyrosine phosphatase type IVA 3 (PTP4A3 or PRL-3) is the other gene mapping to the region 8q24.3 amplified in late CRC stage and related to metastasis (Saha S et al, 2001).

1.4. PTEN tumour suppressor gene

The novel candidate gene PTEN (phosphatase and tensin homolog deleted in chromosome ten), is also known as mutated in multiple advanced cancer (MMAC) or as transforming growth factor-ß regulated and epithelial-cell-enriched phosphatase (TEP1). It has been identified as a tumour suppressor gene for its lipid phosphatase activity in dephosphorylating the 3-phosphoinositide products (PIP3) of the phosphoinositid-3-kinase (PI3K), thereby resulting in a concomitant decrease in AKT activity, which initiates signals for cellular growth, proliferation and survival (Maehama T & Dixon JE, 1999; Lee JO et al, 1999; Georgescu MM et al, 1999).



Schematic figure for PTEN protein

- Phosphatase domain (amino acids 14-185)
- C2 (libid membrane-binding) domain (amino acids 190-350)
- Carboxy- terminal region (amino acids 351-400)
- PDZ-binding domain (amino acids 401-403)

The PTEN gene spans 105 kb that map to chromosome sub-band 10q23.3 and is divided into nine exons (Steck PA et al, 1997; Li J et al, 1997). The PTEN protein contains two key domains: the phosphatase domain and the lipid membrane binding domain. Both are required for its tumour suppressor functions. In addition to the important role of the lipid phosphatase activity for tumour suppression, other

functions connected to these domains were recently described. Specially, PTEN was detected in the nucleus of both normal and neoplastic cells, which gave PTEN gene more roles in cellular regulatory mechanisms (Chow LM & Baker SJ, 2006).

Independently of its lipid phosphatase activity, PTEN has a tumour suppressor function in the anaphase-promoting complex (ANAPC) and its regulator E-cadherin (CDH1) in the nucleus. Altered ANAPC–CDH1 activity has been implicated in multiple tumour types (Song MS et al, 2011). Furthermore, PTEN was found to participate in regulation of TP53 protein level and activity, through both phosphatase dependent and independent mechanisms (Li AG, 2006).

The frequent loss of PTEN function, through deletion, mutations, and/or decreased expression, is observed in hereditary cancers as well as in many sporadic primary cancers. Germline and somatic mutations found mainly in the protein coding region involve both the phosphatase domain and the poly $(A)_6$ tracts (Dahia PL, 2000; Bonneau D & Longy M, 2000). Most of the germline and somatic mutations were nonsense, frameshift and splice mutations resulting in truncated protein (Ali IU et al, 1999; Bonneau D & Longy M, 2000).

Germline mutations of PTEN are found in hereditary autosomal dominant cancer syndromes, collectively known as PTEN hamartoma tumour syndromes (PHTS). PHTS is characterized by multiple hamartomas in many tissues including the gastrointestinal tract. An increased risk of cancer in the breast, thyroid, and endometrium is characteristic of many of these syndromes (Waite KA & Eng C, 2002).

Hemizygous allelic deletion was almost always responsible for PTEN inactivation in various sporadic tumours, with a frequency approximating 60-80% (Ali IU et al, 1999; Hollander MC et al, 2011). At the same time, PTEN inactivation by somatic mutations is found with different frequency rates through different mechanisms. Additionally more than one mechanism was found in a single tumour type, in which bi-allelic PTEN inactivation was not the predominant mechanism (Hollander MC et al, 2011; Ali IU et al, 1999).

In many sporadic cancers, PTEN is mutated in one allele. But in other tumours PTEN was found to be progressively lost without any evidence of even monoallelic mutation

(Monte NM et al, 2010). Thus, the mechanism of PTEN inactivation with loss of its expression in many sporadic cancers was explained with different combinations of genetic and epigenetic alterations while in others it was attributed to monoallelic deletion (Kurose K et al, 2001; Perren A et al, 1999; Zhou XP et al, 2002; Taniyama K et al, 2001).

Epigenetic mechanisms can explain many cases in which PTEN expression is down-regulated or even totally ablated in the absence of detectable mutation in many cancer types (Zhou XP et al, 2002; Nassif NT et al, 2004; Whang YE et al, 1998; Cairns P et al, 1997). This, for example, was observed in malignant melanomas (Zhou XP et al, 2000).

Moreover, the loss or decrease of either PTEN protein level or function in human cancers has been attributed to a number of other proposed mechanisms such as post-translation modification of PTEN, direct and indirect PTEN inactivation by microRNAs, and to inappropriate subcellular compartmentalization (Salmena L, et al, 2008; Fata JE et al, 2012; Perren A et al, 2000; Hollander MC et al, 2011). Recently, studies suggested that PTEN has a dosage effect and haploinsufficiency may further contribute to tumorigenesis with specific tissue dependent sensitivity to the reduced level (Dahia PL, 2000; Carracedo A et al, 2011).

1.4.1. PTEN as colorectal cancer candidate gene

Regarding colon cancer, PTEN is considered a mutated candidate gene in colon cancer although in low frequency according to mutation frequencies recorded by Wood LD et al (2007). As observed commonly in other sporadic tumours, PTEN somatic alterations, are often associated with other genetic alterations, making a functional role uncertain (Wood LD et al, 2007; Hollander MC et al, 2011).

PTEN inactivation was mentioned in various selected and unselected CRC studies with a heavy predominance for the microsatellite instable type (Shin KH et al, 2001; Negoro K et al, 2000). Allelic deletions at 10q23.3 gene locus have been observed in approximately 30-35% of sporadic CRC (Frayling IM et al, 1997). On the other hand, PTEN somatic mutations were detected in approximately 19% of microsatellite instable (MSI+) CRCs in the coding region, almost exclusively in one of the two poly $(A)_6$ tracts in exon 7 and 8 (Guanti G et al, 2000; Zhou XP et al, 2002). By contrast,

only 5-10% of PTEN somatic mutations have been detected in MSI unknown or microsatellite stable (MSI-) sporadic colorectal tumours (Shin KH et al, 2001; Guanti G et al, 2000).

Gene silencing by promoter hypermythylation was considered as a major mechanism through which the PTEN gene may be inactivated in colon cancer. This is more important than the other mechanisms, particularly in MSI+ colon tumours, where hypermethylation was taken in account as the “second hit” in bi-allelic PTEN inactivation (Goel A et al, 2004). In addition to the mixed genetic/epigenetic alterations reported in colon cancer; PTEN inactivation by dual mutations affecting both alleles was within the registered structural alterations (Nassif NT et al, 2004; Danielsen SA et al, 2008; Dicuonzo G et al, 2001).

PTEN has been studied by immunohistochemistry in various human tissue and was found to be expressed in the majority (Taniyama K et al, 2001). A granular and punctuate manner of PTEN expression is reported in the colon throughout the mucosal and epithelial layers with exclusively cytoplasm predominance in normal and tumour cells (Taniyama K et al, 2001).

Compared to normal mucosa, loss or at least reduction of PTEN expression was recorded for colorectal carcinoma (Hsu CP et al, 2011; Li XH et al, 2009). It has also been designated biologically relevant and predictively reliable whether in early detection of endometrial precancerious lesions or even in colon polyps and advanced metastatic colorectal cancer (Mutter GL et al, 2000; Waniczek D et al, 2013; Sawai H et al, 2008; Lin MS et al, 2011).

1.5. LOH detection by microsatellite analysis

Loss of genomic material is a defining feature of CIN in colorectal cancer whereby, very often, a gene is reduced to a state of homozygosity (Lasko D et al, 1991). Traditionally, LOH was studied by restriction length polyomorphisms (RFLP) (Lothe RA et al, 1988; Solomon E et al, 1987), but with the advent of PCR, polymorphic markers are used widely (Geigl JB et al, 2008; Gruis NA et al, 1993). If informative, PCR amplifying polyomorphic DNA loci with variable repeat sequences of 2-7 nucleotides result in PCR products of different length that represent parental and maternal alleles of a gene, respectively (Cacev T et al, 2006). Thus, the availability of

a broad rang of informative markers for the human genome have made microsatellite assay very popular and used widely (Ellegren H, 2004; Medintz IL et al, 2000; Berti L et al, 2001).

1.5.1. The distinction between ROH, AI and LOH

The accurate scoring of allelic imbalances (AIs) by fluorescence based polymorphic microsatellite PCR method has made possible the detection of even subtle allelic loss in tumour cells by quantification of both size and extent of amplified products more precisely than to the non-quantitative methods. In contrast to MSI, where new alleles are generated, there is some degree of imbalances scored by calculation of the recorded semi-quantitative values (Skotheima RI et al, 2001).

LOH, AI, and retention of heterozygosity (ROH) are different categories which reflect the genetic status of specific gene allele and are calculated according to the ratio of the peak heights of normal and tumour alleles (see section on materials and methods). ROH is scored when the ratio is maintained within the normal range from 0.5 – 2, AI is scored if the ratio exceeds this range. The AI pattern can be obtained either by partial allelic loss or by gain/increased DNA copy number of a particular gene (Geigl JB et al, 2008; Skotheima RI et al, 2001). LOH is assigned in case of a complete loss of a wild type allele (Devilee et al 2001; Tomlinson IP et al, 2002).

1.5.2. The role of tumour xenografts and LCM in the detection of LOH

The application of the laser capture microdissection (LCM) through the successful separation of tumour tissue from the adjacent normal and the human tumour tissue implants into immunodeficient mice eliminate contamination problems and optimize the molecular analysis by providing pure tumour samples (Ellsworth RE et al, 2003; Lo KW et al, 2000; Heinmöller E et al, 2000; Harkes IC et al, 2003; Bartsch D et al, 1999).

The tumour xenografts are neoplastic human cells, supported by nonneoplastic murine stromal cells, which fail to amplify with primers designed from human DNA sequences. Tumour xenograft DNA has been shown to remain stable in relation to the primary tumour DNA regardless of the number of implant passage (Uronis JM et al, 2012; McQueen HA et al, 1991). The possibility of gaining more genetic instabilities during cell propagation either in cell culture or as xenografts has been

often suggested by researchers in many studies related to gene allelotyping. Evidence for such genetic instability has not yet been significantly provided (Reyes G et al, 1996; Hahn SA et al, 1995).

1.6. Aim of the study

This study was undertaken to test if the patterns of molecular aberration of the PTEN gene are consistent with a tumour suppressor gene mechanism. To this end, tumour DNA from colorectal carcinoma xenografts or primary cell lines was used for gene sequencing and allelotyping analyses; gene expression was studied by immunohistochemstry. P53, a paradigm tumour suppressor gene was studied for comparison.

Specifically, the following points were addressed:

1. Frequencies and types of PTEN gene mutations?
2. Frequencies of LOH compared to AI?
3. “Two-hit” mechanism to be recorded?
4. Patterns of gene expression, in relation to molecular aberrations

2. Material and methods

2.1. Tumour specimens

Tumour tissue and normal tissue, for comparison, were obtained from 18 patients who underwent colorectal cancer surgery in the Department of Surgery, University of Rostock, Germany. A small part of each tumour (cubes ca. 3 x 3 x 3 mm) was taken and snap-frozen in liquid nitrogen for storage at -80ºC by the pathologist who later made the dissection and final reports after overnight fixation in buffered formalin. Subcutaneous xenografting into immunodeficient mice and generation of primary tumour cell lines was performed as previously published (Linnebacher M et al, 2010).

DNA was extracted from the tumour xenografts (N = 15) or the primary tumour cell lines (N = 2). For one case (case no. 15) as neither xenograft nor cell line were available tumour DNA was extracted from neoplastic glands isolated from paraffin sections of the primary tumour. Only tumours classified as advanced primary microsatellite stable sporadic standard type colorectal carcinomas (spStd CRC) were included in this study.

2.2. DNA extraction

2.2.1. DNA extraction from paraffin material

Three to five 10 µm thick paraffin tissue sections were collected in a 1.5 ml micro-tube. Sections were deparaffinized by adding 1ml xylene. After vortexting and centrifuging at 10,000 rpm for 5 min, xylene was removed carefully and 1 ml ethanol (95%) was added. Again the mixture was vortexed, followed by centrifugation at 10,000 rpm for 5 min and careful removal of ethanol. This washing step was repeated once. The pellet was dried at 60°C for 10 minutes until the ethanol had completely evaporated. After addition of 100-200 µl digestion puffer (10mM Tris-HCl, 0.1mM EDTA, 0.5% Tween 20) and 10-20 µl Proteinase K (20mg ml^{-1}) (Roche, Germany), tissue was incubated at 56ºC overnight, followed by inactivation of the proteinase K at 95ºC for 15 min. The DNA sample was obtained after cleaning the reaction mixture using the Wizard DNA Clean-Up System (Promega, WI, USA).

2.2.2. DNA extraction from fresh tissue

DNA from frozen fresh tissue and tumour xenografts was extracted using the Nucleo®Spin Tissue Kit (MACHERY-NAGEL, Düren, Germany). Twenty to thirty

cryostat sections (10µm) were placed in a 1.5 ml microcentrifuge tube containing 180 µl cell lysis buffer and 25µl proteinase K and incubated for 24 hours at 56ºC in a shaking incubator until the tissue was completely digested. The following steps were applied according to the manufacturer's instructions. The DNA was eluted with 80µl elution buffer.

2.2.3. Quantitation of DNA concentration and assessment of quality

The quantity of extracted DNA was determined by using the NanoDrop Spectrophotometer ND-1000 (Peqlab). In order to assess the DNA quality we amplified fragments of four housekeeping genes. PCR primers were selected from the human thromboxane synthase gene (TBXAS1, exon 9; GenBank accession No. D34621), human recombination activating gene (RAG1, exon 2; GenBank accession No. M29474), human promyelocytic leukemia zinc-finger gene (PLZF, exon 1; GenBank accession No. AF060568), and human AF4 gene (exon 3; GenBank accession No. Z83679), respectively, to amplify products of 100, 200, 300, and 400 bp (Dongen JJ et al, 2003). The PCR reactions were carried out as multiplex reactions. PCR products were electrophoresed on 2% agarose gels containing 0.5 mg ml^{-1} of ethidiumbromide.

2.3. Mutation analysis

Mutation analysis of the TP53 gene exons 5-8 was carried out by a mutation screening which is well established in our laboratory. Exon 1 of TP53 gene is a non-coding exon. Mutations at TP53 exons 2, 3, 4, 9, 10, and 11 as well as the 9 exons of the PTEN gene were investigated by direct sequencing.

2.3.1 Mutation screening of p53 gene by temperature gradient gel electrophoresis

For mutation screening of the TP53 gene, PCR and subsequent Temperature-Gradient Gel Electrophoresis (PCR-TGGE) were applied as published by Scholz et al. 1993. TGGE separates DNA fragments according to their melting properties allowing even separation of two double-stranded DNA fragments differing in a single base in a polyacrylamid gel with a linear ascending temperature gradient. To detect base changes in high-melting domains and to prevent melting open of this region which results in a loss of sequence-dependent mobility, one of the primers used in the PCR was provided with a GC-clamp at the 5' end. In the presence of a mutation

in the TP53 gene fragment and the wild type (resulting from normal stromal tissue surrounding the tumour), during PCR two homodimers and two heterodimers are formed by denaturation and re-naturation that are separated by TGGE. After silver staining these four products are visible in the gel as 4 bands. In cases with no mutation only one band which corresponds to the homodimer of the wild type is visible (see figure 1).

PCR for TP53 mutation screening was performed in a final volume of 50 µl 1x reaction buffer, containing 50 mM KCl, 1.5 mM $MgCl_2$ (for exon 5, 6, 7), 3 mM for (exon 8), 200 µM of each dinucleotide triphosphate, 0,25 µM of each primer and 1.5 U Taq DNA polymerase (Qiagen, Hilden, Germany). Sequences of primers and the length of amplicons are shown in appendix 1. PCR was performed by using the Sensoquest thermocycler (biolabproducts GmbH) with the following temperature profile: an initial denaturation at 95°C for 5 min, forty cycles with 30 seconds at 94°C, an annealing step of 30 seconds at 58°C for exons 6 and 7, and at 53°C for exons 5 and 8, a polymerization step with 60 seconds at 72°C and a final extension at 72°C for 3 min.

Ten µl from each PCR product mixed with loading dye (0.4% bromphenol blue, 0.4% xylencyanol) were electrophoresed in a 2% agrose gel containing 2% ethidium bromide. Water was used as a negative control, and a case with known mutation served as positive control.

TGGE was performed on an 8% denaturing polyacrolamide gel with 1xMOPS as a running buffer. For easier handling the gel is covalently polymerized on a plastic film using the system from Diagen (Hilden, Germany). After a pre-run of 30 min at 250 V and 20°C to equilibrate the system, 5 µl of the PCR products mixed with 0.5 µl loading buffer (appendix 5) were loaded on the gel. After a run-in time variable for each exons, the current was switched off and the temperature gradient was adjusted (the T-gradient for the different exons was determined before in a perpendicular gel). When the system has reached the T-gradient the run was started again with a different running time for each exon as stated in appendix 2. To detect the DNA bands gels were silver-stained (appendix 5).

2.3.2. DNA sequencing

Mutation analysis was done by Sanger DNA sequencing exons 2-4 and 9-11 of the TP53 gene for the negative cases by TGGE screening, and of the PTEN gene for all the cases. The PCR reactions were carried out with 50 ng DNA in 1x Phire Reaction Buffer (contains 1.5 mM $MgCl_2$), 100 µM of each dNTPs, 0.15 µM of each primer and 0.2 U of Phire Hot Start II DNA polymerase (Finnzymes, Finland), in a total reaction volume of 25 µl. Primer sequences for PCR amplification were taken from ncbi.nlm.nih.gov /tools/primer-blast and are shown in appendix 3.

PCR reactions were performed in Sensoquest thermocycler (biolabproducts GmbH) using a two step protocol. After an initial denaturation step at 98°C for 30 seconds, 38 cycles followed with 20 seconds at 98°C and 40 seconds at 72°C. A final extension step was done at 72°C for 3 min. Amplified products were then analyzed by electrophoresis in a 2% agrose gel. To remove salts, enzymes, nucleotides and non-incorporated primers, PCR products were treated with a combination of Exonuclease I and Shrimp Alkaline Phosphatase (Fermentas). Ten µl PCR product was mixed with 1 µl Exonuclease I and 2 µl Shrimp Alkaline Phosphatase incubated for 15 min at 37°C. Incubation at 80°C for 15 min was followed to inactivate the enzymes.

Sequencing of each DNA strand in both directions (5'→3' and 3'→5') was done using the Big Dye Sequencing Kit v 1.1 (Applied Biosystems): 30-50 ng purified DNA (PCR product) together with 2µl Ready Reaction Mix, 2µl Sequencing Buffer and 10 pmol primer in a total volume of 10 µl was thermocycled as follows: initial denaturation step at 96°C for 5 min, 25 cycles of denaturation at 96°C for 10 seconds and annealing at 55°C for 4 min.

The cycle sequencing product was purified in order to eliminate reaction residuals i.e. oligonucleotides and ddNTPs using Big Dye® XTerminator Purification Kit™ (Applied Biosystems). As specified in the manufacturer's protocol 10 µl of the sample were mixed with 45µl SAM-Solution plus 10µl XTerminator- solution for 30 min followed by vortexing and centrifuging for 2 min. The samples were loaded on an 8 capillary sequencer 3500 Genetic Analyzer (Applied Biosystems). The SeqScape software, version 2.7 was used in sequence analysis.

2.4. Assays for allelic imbalance/loss of heterozygosity

To test for allelic imbalance or loss of heterozygosity, paired tumour and normal tissue DNA were analyzed. To interrogate the TP53 locus we used the three microsatellite markers D17S250, D17S1583 and 17p13.1. The PTEN locus was tested using the three markers D10S541, D10S579 and D10S1765. Primer sequences for PCR amplification were taken from ncbi.nlm.nih UNISTS. Sequences of primers, annealing temperatures, $MgCl_2$ concentrations and the length of amplicons are shown in appendix 4.

The PCR reactions were performed in a volume of 12.5 µl containing 25 ng genomic DNA, 200 µM of each dinucleotide triphosphate, 1x PCR Buffer, 0.5-1.0 µM of each primer and 0.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems). For amplifications we used the Sensoquest thermocycler (biolabproducts GmbH) with the following temperature profile: 1. initial denaturation step at 95°C for 10 min; 2. 35 cycles at 94°C for 30 sec., annealing for 30 sec. and at 72°C for 45 sec.; 3. final extension for 30 min. at 72°C.

The PCR products were diluted (1:10), mixed with 0.3 µl GeneScan-500 LIZ Size Standard (Applied Biosystems) in 24 µl HiDi (Applied Biosystems) and denatured for 3 min. at 95°C. The samples were run on a Genetic Analyzer 3500 (Applied Biosystems). Data were processed by ABI software GeneMapper version 4.0.

Informative cases showed heterozygosity in the microsatellite analysis. Results were analyzed by comparison of the heights of the mean peaks of the tumour alleles and of normal alleles, calculation was performed by the following formula: AI = $(t_2/t_1)/(n_2/n_1)$ where t_1 and t_2 are the peaks' heights of the tumour allele 1 and tumour allele 2, respectively, and n_1 and n_2 are the peaks' heights of the normal alleles. AI was scored when the ratio was < 0.5 or > 2. LOH was assigned to cases showing complete loss of one allele.

2.5. Immunohistochemistry

Consecutive 3-4 µm sections were obtained from the formalin fixed paraffin embedded primary tumours. Blocks selected to contain viable tumour without hemorrhage or pronounced granulocytic inflammation. Epitope retrieval was carried out by incubation of slides in high buffer retrieval solution (DAKO) at 97˚C for 20 min.

The immunohistochemical reactions were performed in an autostainer (Link,DAKO) according to the supplier's protocol. Primary antibodies were mouse monoclonals antibodies against p53 protein (clone Do-7, DAKO) at 1:100 dilutions, and against PTEN protein (clone mAb6H2.1, DAKO) at 1:800 dilutions. Slides were counterstained with hematoxylin and mounted.

P53 immunoreactions were evaluated semi-quantitatively based on the percentage of positive tumour nuclei. A score was applied as follows: none (0%), weak (<10%), moderate (10-50%), or intense (>50%). All tumours showing at least moderate (>10 % positive tumour cells) p53 immunoreactivity were considered p53 positive. A histoscore (H-score) was used for evaluation of PTEN expression. Intensities of cytoplasmic staining of the tumour cells was graded semi-quantitatively on a 3-tiered scale as strong (score 2), weak (score 1) or absent (score 0), taking the immunostaining of vascular endothelium as an internal reference (strong). The proportion of cells without any immunostaining was classified as 0% (score 0), 1 - 50% (score 1), and > 50% (score 2). The final numerical H-score for each case was obtained by multiplication of scores obtained in above categories.

2.6. Statistical analysis

All date were entered into a computerized data bank (Statistical Package for the Social Sciences, SPSS version 15.0). Testing for significance association between cross-tabulated data was done by two-sided Fisher's exact test. $P < 0.05$ was taken as criterion for significance.

3. Results

3.1. Patients' and tumour characteristics

In this study, the mean age of the patients was 71 years, (range 59 to 84 years), 13 patients were males and 5 were females. Most of the tumours (13; 72%) were located in the right colon. Histologically, all the cases were colorectal adenocarcinomas (tubular adenocarcinomas except for one, which was a mucinous adenocarcinoma). Overall, 12 (67%) of the tumours were moderately differentiated while 6 cases (33%) were poorly differentiated. Eight patients (44%) had stage III tumours according to the International Union Against Cancer Classification (UICC). Three patients in stage IV had synchronous metastases in the liver (2 cases) or in the lung (1 case). Molecularly, all the cases were primary microsatellite stable sporadic standard type colorectal carcinomas. The clinical-pathological characteristics are in table 1.

3.2. TP53 gene

3.2.1. Gene mutation screening analysis

TP53 gene mutation analysis was done both with DNA extracted from paraffin sections of the primary tumours as well as with DNA from the xenografts or the primary tumour cell lines. Mutation screening of TP53 exons 5-8 by TGGE revealed mutations in 13 of the 18 tumours (72%). An example of a TGGE analysis is given in figure 1. Most of the mutations were found in exon 8 (44%) (table 2). For the 5 cases in which no mutations were detected by TGGE, direct DNA sequencing of the other TP53 exons (exon 2-4 and 9-11) was done, but this did not reveal any further mutations. Results are demonstrated in table 3.

Table 1

Clinical-pathological characteristics of study cases

Character	Number of cases	%
Age (Years)		
60 - 70	9	50
70 – 80	6	33
> 80	3	17
Sex		
Male	13	72
Female	5	28
Tumour Site		
Right colon	13	72
Left colon	5	28
Tumour Grade		
G1	0	0
G2	12	67
G3	6	33
Tumour Staging		
I	2	11
II	5	28
III	8	44
IV	3	17
Total	**18**	**100%**

Table 2

TP53 gene mutations distribution by exons

P 53	Exon 5	%	Exon6	%	Exon7	%	Exon8	%	Total	%
m/n	2/18	11	0/18	0	3/18	17	8/18	44	13/18	72

m: Number of mutations detected; **n**: Total number of cases

Table 3

TP53 gene mutations by TGGE in tumour xenografts and primary tumours

Case	Case Code	TP53 gene exon number			
		Exon 5	Exon 6	Exon 7	Exon 8
1	XHROC18P	0	0	0	**M**
	2T15	0	0	0	**M**
2	XHROC32	0	0	0	**M**
	841/07	0	0	0	**M**
3	XHROC39	0	0	0	**0**
	2T29	0	0	0	**0**
4	XHROC40	0	0	0	**M**
	2T30	0	0	0	**M**
5	XHROC46	0	0	0	0
	2T34	0	0	0	0
6	XHROC54	0	0	**M**	0
	1373/08	0	0	**M**	0
7	XHROC59	0	0	0	0
	2T52	0	0	0	0
8	XHROC60	0	0	0	**M**
	2T51	0	0	0	**M**
9	XHROC62	**M**	0	0	0
	7373/08	**M**	0	0	0
10	XHROC65P	0	0	**M**	0
	2T47	0	0	**M**	0
11	XHROC68	0	0	0	0
	11142/08	0	0	0	0
12	XHROC69	0	0	0	**M**
	2T55	0	0	0	**M**
13	XHROC70	0	0	0	**M**
	2T57	0	0	0	**M**
14	XHROC80	0	0	**M**	0
	2T66	0	0	**M**	0
15	XHROC82	ND	ND	ND	ND
	5109/09 *	0	0	0	**M**
16	HROC85	0	0	0	0
	2T64	0	0	0	0
17	HROC86	**M**	0	0	0
	2T74	**M**	0	0	0
18	XHROC107	0	0	0	**M**
	2T89	ND	ND	ND	ND

XHROC: Tumour xenograft; **2T**: Primary tumour; **HROC**: Tumour cell line; *****: LCM primary tumour; **M**: Mutation; **ND**: Not done

3.2.2. Gene locus allelotyping screening for AI and LOH

Three polymorphic microsatellite markers were used to test for AI and/or LOH at chromosome 17p, all the markers included the TP53 gene locus. AI was scored if the normal/tumour ratio exceeded 0.5 or 2.0, and LOH was recorded for cases with complete loss of one allele (see figure 2 for representative electropherograms). The remaining cases were determined to have retention of heterozygosity.

Study material for 18 cases included the primary tumours and adjacent normal mucosa as well as their matched xenografts, in 2 cases primary cell lines were used instead of xenograft. Because neither xenografts nor primary carcinoma cell lines were available for case no.15, DNA extracted from tumour tissue obtained by Laser-capture microdissection was used.

Overall, for all cases at least one of the three microsatellite markers was informative. Complete loss of one allele at 17p (i.e. LOH) by at least one microsatellite marker was observed for 13 of these cases (72%) when DNA from xenografts or cell lines was used. By contrast, with DNA extracted from the corresponding primary tumours LOH was observed in only 2 of the microsatellite analyses; for the remaining cases only AI was scored (10) or retention of heterozygosity (5). For case no. 15, the analysis resulted in AI for each of the three microsatellite markers. The results of the microsatellite analysis are set out in detail in table 4.

Table 4

TP53 gene allelic imbalance and LOH

Case	Case Code	Chromosomal locus			Overall
		D17S250	D17S1583	D17S13.1	
1	XHROC18P	NI	NI	LOH	LOH
	2T15	NI	NI	AI	AI
2	XHROC32	NI	NI	AI	AI
	841/07	NI	NI	ROH	ROH
3	XHROC39	ROH	ROH	ROH	ROH
	2T29	ROH	ROH	ROH	ROH
4	XHROC40	ROH	LOH	LOH	LOH
	2T30	ROH	ROH	AI	AI
5	XHROC46	ROH	LOH	LOH	LOH
	2T34	ROH	AI	AI	AI
6	XHROC54	ROH	LOH	ROH	LOH
	1373/08	ROH	ROH	ROH	ROH
7	XHROC59	ROH	NI	ROH	ROH
	2T52	ROH	NI	ROH	ROH
8	XHROC60	ROH	LOH	LOH	LOH
	2T51	ROH	ROH	ROH	ROH
9	XHROC62	LOH	LOH	LOH	LOH
	7373/08	AI	LOH	AI	LOH
10	XHROC65P	NI	AI	NI	AI
	2T47	NI	AI	NI	AI
11	XHROC68	ROH	NI	LOH	LOH
	11142/08	ROH	NI	AI	AI
12	XHROC69	NI	NI	LOH	LOH
	2T55	NI	NI	AI	AI
13	XHROC70	AI	LOH	AI	LOH
	2T57	ROH	AI	AI	AI
14	XHROC80	AI	LOH	LOH	LOH
	2T66	ROH	ROH	AI	AI
15	XHROC82	ND	ND	ND	ND
	5109/09 *	AI	AI	AI	AI
16	HROC85	ROH	LOH	LOH	LOH
	2T64	ROH	AI	LOH	LOH
17	HROC86	LOH	NI	AI	LOH
	2T74	AI	NI	AI	AI
18	XHROC107	AI	NI	LOH	LOH
	2T89	ROH	NI	AI	AI

XHROC: Tumour xenograft; **2T**: Primary tumour; **HROC**: Tumour cell line; *****: LCM primary tumour; **LOH**: loss of heterozygote; **AI**: Allelic imbalance; **ROH**: Retention of heterozygote; **NI**: Not informative (homozygote); **ND**: Not done

3.2.3. Gene mutation and LOH synoptic analysis

Combined TP53 LOH and point mutation of the gene was observed for 10 (56%) of the cases (chi-square p = 0.04), predicting a non-functional state of the gene. In three cases, TP53 mutations were found in combination with AI, and LOH without mutation was observed in three cases. For two tumours neither LOH nor a gene mutation were demonstrated. These results are summarised in table 5.

Table 5

Association of TP53 gene mutation and LOH

TP53	LOH	AI	ROH	Total
Mutation positive	10	3	0	**13**
Mutation negative	3	0	2	**5**
Total	**13**	**3**	**2**	**18**

LOH: loss of heterozygote **AI**: Allelic Imbalance **ROH**: Retention of Heterozygote

3.2.4. TP53 immunohistochemistry

By TP53 immunohistochemistry, staining was seen confined to the tumour cell nuclei, and was never found in the adjacent normal mucosa. Representative images are given in figure 3. Nuclear staining in at least 10% of the tumour cells was categorized as a positive reaction. Using this cut-off, 13 (72%) tumours were classified as TP53 positive by immunohistochemistry 11 of which had been demonstrated to have gene mutation. The results are given in table 6.

Table 6

Association of TP53 gene mutation state and immunohistochemistry

p53 IHC	TP53 gene mutation state		
	M +	**M -**	**Total**
Strong	6	1	**7**
Moderate	5	1	**6**
Weak	1	0	**1**
Negative	1	3	**4**
Total	**13**	**5**	**18**

* **IHC**: Immunohistochemistry

3.3. PTEN gene

3.3.1. Gene mutation sequence analysis

Direct sequencing of PTEN gene exon 1-9 was carried out for the 17 xenograft tumours. For case no. 15, DNA obtained by laser-capture microdissection of neoplastic glands from paraffin-sections of the primary tumour was used, this yielded sufficient, amplifiable DNA (see figure 4 for images of the laser-capture microdissection).

PTEN gene mutations were seen in only one of the tumours (case no. 12). However, this tumour harboured a total of 3 somatic point mutations in two different exons: a GAG>TAG mutation found in codon 7 of exon 1 as well as two additional mutations detected in exon 5 with a CGA>CAA at codon 130 and CGG>CAG at codon 142. The first mutation results in a stop codon whereas the other two mutations were point mutation that resulting in an amino acid changes from Arginine to Glutamine (see figure 5 for demonstration of sequencing analysis results). All sequencing results were confirmed by re-amplification and sequence analysis of an independent PCR product.

3.3.2. Gene locus allelotyping for AI and LOH

PTEN allelotyping was informative for all cases with at least one of the microsatellite markers. Complete loss of one allele (LOH) at the PTEN locus was observed in two tumours (11%). AI and ROH were recorded for 6 and 10 tumours, respectively. PTEN allelic status is set out in detail in table 7.

3.3.3. Gene mutation and LOH synoptic analysis

LOH or AI was not observed in the single case for which a PTEN gene mutation was found (table 8).

Table 7

PTEN allelic imbalance and loss of heterozygosity

Case	Case Code	PTEN chromosomal locus			
		D10S541	D10S579	D10S1765	Overall
1	XHROC18P	ROH	ROH	ROH	ROH
2	XHROC32	AI	NI	ROH	AI
3	XHROC39	ROH	NI	ROH	ROH
4	XHROC40	AI	AI	ROH	AI
5	XHROC46	ROH	ROH	ROH	ROH
6	XHROC54	ROH	ROH	ROH	ROH
7	XHROC59	LOH	NI	LOH	LOH
8	XHROC60	NI	ROH	AI	AI
9	XHROC62	ROH	NI	ROH	ROH
10	XHROC65P	NI	AI	ROH	AI
11	XHROC68	LOH	NI	AI	LOH
12	XHROC69	ROH	NI	ROH	ROH
13	XHROC70	ROH	ROH	ROH	ROH
14	XHROC80	ROH	NI	ROH	ROH
15	5109/09 *	ROH	NI	ROH	ROH
16	HROC85**	ROH	ROH	ROH	ROH
17	HROC86**	ROH	AI	ROH	AI
18	XHROC107	NI	AI	NI	AI

******: Tumour cell line; *****: LCM primary tumour; **LOH**: Loss of heterozygote; **AI**: Allelic imbalance; **ROH**: Retention of heterozygote; **NI**: Not informative (homozygote)

Table 8

Association of PTEN gene mutation and LOH

PTEN	LOH	AI	ROH	Total
Mutation Positive	0	0	1	**1**
Mutation negative	2	6	9	**17**
Total	**2**	**6**	**10**	**18**

LOH: loss of heterozygote **AI**: Allelic Imbalance **ROH**: Retention of Heterozygote

3.3. 4. PTEN immunohistochemistry

Staining of endothelial nuclei was a constant feature and is served as an internal control of the staining reactions. Intensities of immunostaining varied between the tumours. Scoring intensities, in 5 cases (28%) PTEN immunohistochemistry revealed strong staining of the cytoplasm and nuclei of the tumour cells, moderate staining was seen in 12 (67%) cases, and 1 of the cases (6%) was completely negative. Interestingly, this last case was the single tumour that harbored the PTEN gene mutations. An association between LOH/AI and intensities of immunostaining was not observed (see table 9 for details).

Table 9

Association of PTEN gene allelic status and immunohistochemistry

PTEN IHC	PTEN allelic state			
	LOH	**AI**	**ROH**	**Total**
Strong	0	1	4	**5**
Weak	2	5	5	**12**
Negative	0	0	1*	**1**
Total	**2**	**6**	**10**	**18**

* Case no. 12 with mutation

LOH: loss of heterozygote **AI**: Allelic Imbalance **ROH**: Retention of Heterozygote

Figure 1. TP53 gene mutation screening of exon 8 by PCR-TGGE. Line 1-7, homozygote wild type; line 8 heterozygous mutation; line 10 and 12 homozygous mutation. The abnormal bands (arrows) indicate the presence of a TP53 mutation. **C**: Mutated control specimen.

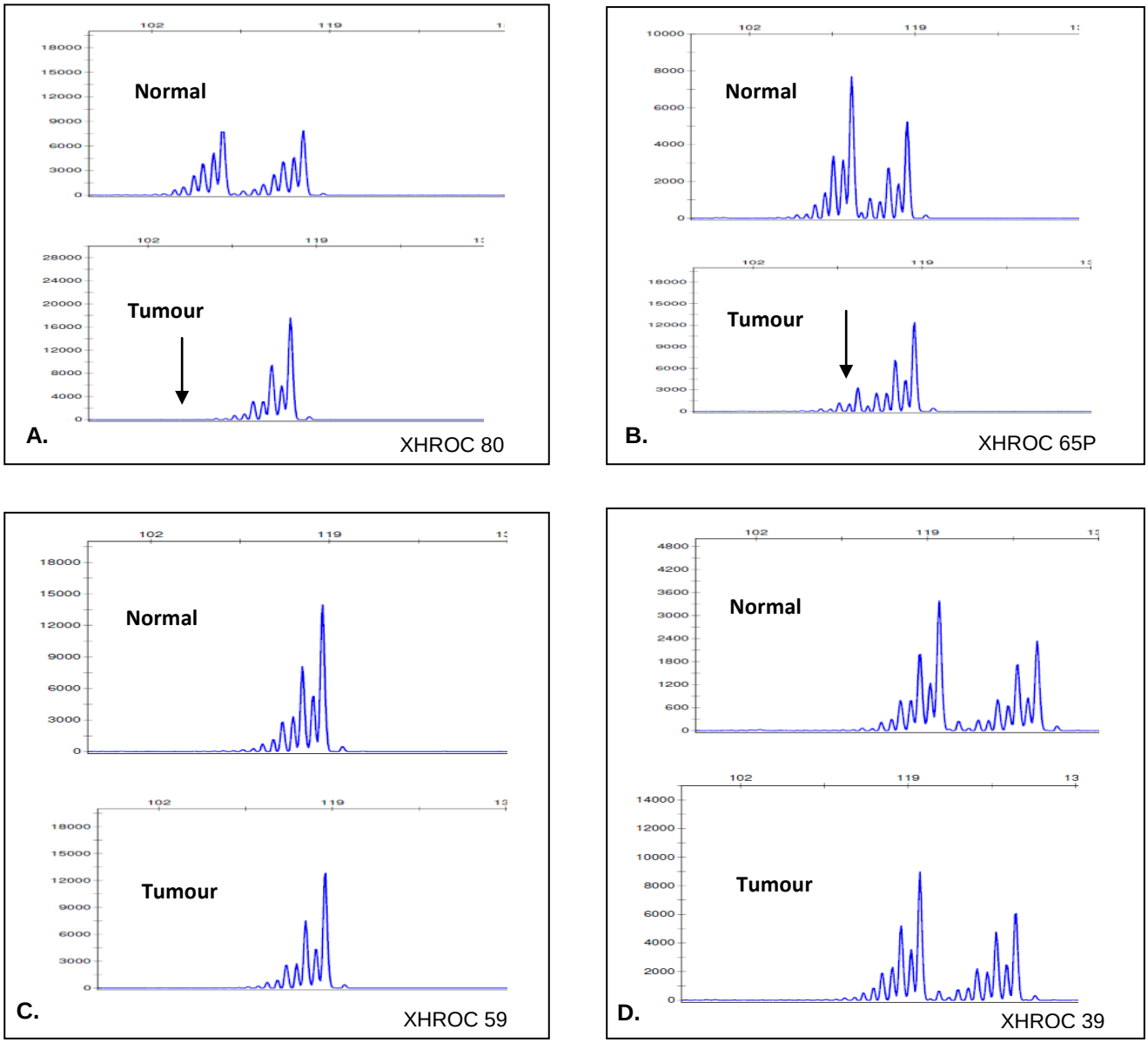


Figure 2. Examples of electropherograms from microsatellite analysis with D17S1583. Electropherograms are arranged in pairs with normal DNA in the upper lane and tumour DNA in the lower lane. **A.** Complete loss of one of the two alleles, corresponding to LOH. **B.** Partial loss of one allele, corresponding to AI. **C.** Homozygosity for D17S1583, this case is not informative. **D.** Both alleles present without significant differences in peak height ratios, corresponding to ROH.

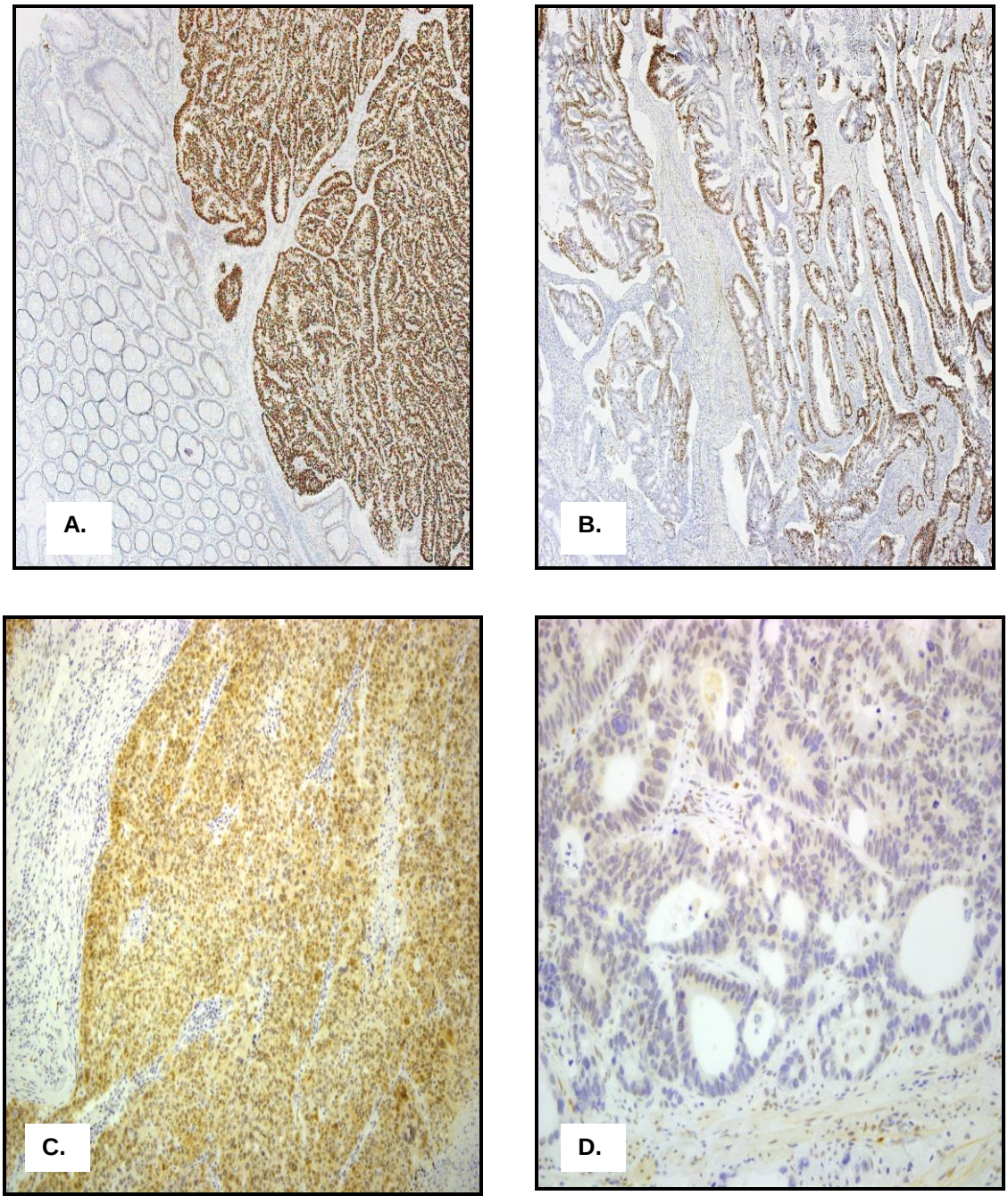
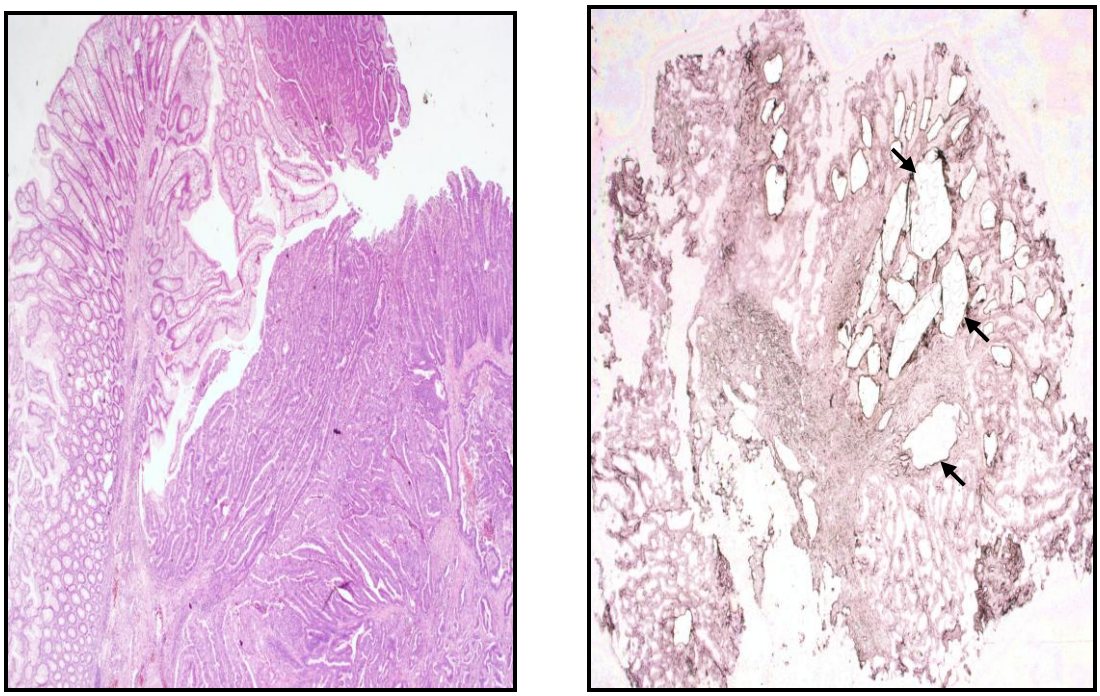


Figure 3. Examples of TP53 immunohistochemistry. **A.** Intense and widespread nuclear immunostaining in a case of colorectal carcinoma (tumour no. 5109/09), score +++. Note absence of immunostaining in the adjacent normal mucosa. **B.** Example of score ++, strong nuclear immunostaining of the majority of the tumour cells. **C.** Example of score +, weak immunostaining of a minority of tumour cells. **D.** Example of a tumour negative by TP53 immunohistochemistry.



A. **B.**

Figure 4. Illustration of laser capture microdissection (LCM) of neoplastic glands from cryostat sections of case no. 15. **A.** H&E stained paraffin section as scout slide. **B.** Example of a section after LCM.

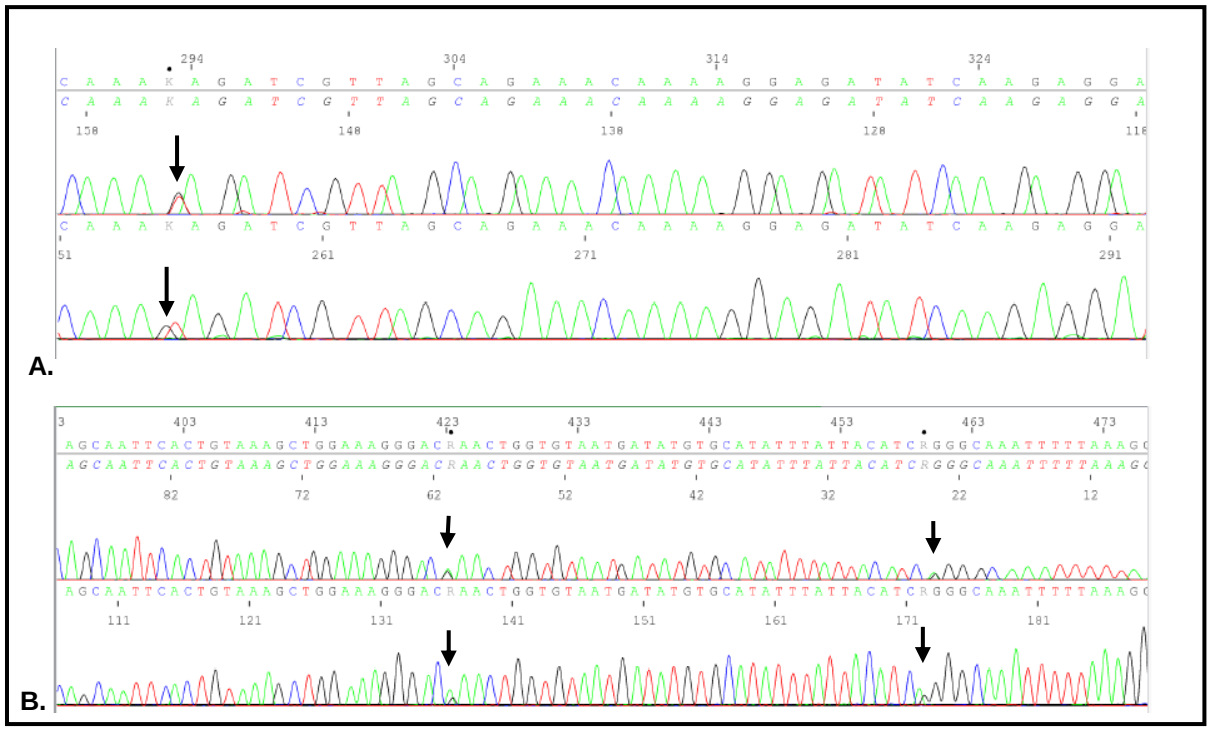


Figure 5. Demonstration of PTEN gene mutations by direct sequencing of the PCR product in case 12. **A.** Exon 1 GAG>TAG mutation codon 7 → stop codon. **B.** Exon 5 CGA>CAA codon 130 and CGG>CAG codon 142, Arginine → Glutamine.

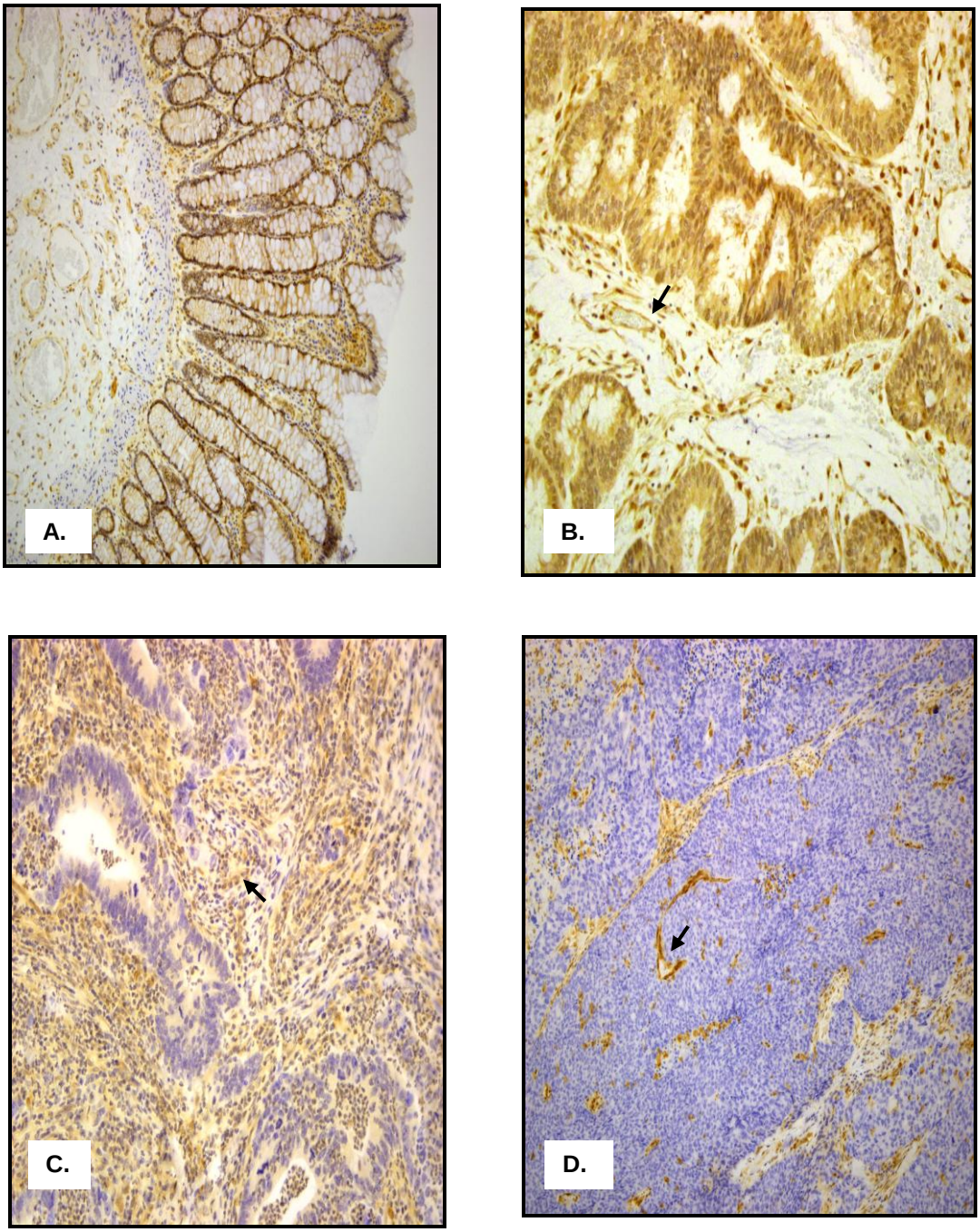


Figure 6. Examples of PTEN immunohistochemistry. **A**. Example of normal colonic mucosa showing strong (++) cytoplasmic and nuclear immunostaining of the colonic epithelium and endothelia. **B.** Example of a case with retention of strong (++) PTEN expression in tumour cells. **C.** Examples of weak PTEN expression (score +), and **D.** complete loss of PTEN expression. Arrows indicate endothelia that provided a convenient internal control of the immunoreactions.

4. Discussion

Tumour suppressor genes play an important role in restraining inappropriate cell growth and division, as well as in regulating apoptotic cell death. This helps net cell number in tissue to be kept in balance and protects against cancer. Suppressor function of genes is mainly lost through mutational gene inactivation affecting both alleles. According to Knudson's "two-hit" model, for a TSG inactivation the two gene alleles are functionally inactivated by a combination of genetic alterations, e. g. mutation of one allele and genome loss of the other allele.

In colorectal cancer, the p53 gene is a prototype for this, as shown by synoptic evaluations of gene mutational status and LOH in a number of published studies. Technically, these studies usually relied on direct DNA sequencing and microsatellite analysis with polymorphic markers. In the vast majority of these studies the tumour DNA used for the analyses derived from whole tumour homogenates, i.e. mixtures of tumour cells and stromal cells. Provided that a reasonable (usually $>$ 30%) fraction of tumour is included in the sample this type of study material is quite sufficient for finding a gene mutation by sequencing. However, documenting LOH by microsatellite analysis with polymorphic markers with DNA from such homogenates is not possible: this technique does not allow for a distinction between LOH and AI; in fact, the so-called LOHs reported for genes in most studies in reality are AI. This drawback of allelotyping analyses can only be overcome if tumour xeno-grafts, tumour cell lines or tumour cells isolated by laser-capture microdissection are used.

TP53 is a well known TSG involved in DNA repair process and it fulfils the cardinal criteria of TSGs participating in the suppressor pathway by loss of function during tumour initiation or progression. In contrast, much less is known about PTEN as a TSG. It is reported to be deleted in various types of tumours and known to contribute to the inhibition of the cell cycle and cell survival. However, presently it is not clear if PTEN inactivation in colorectal cancer indeed proceeds by cellular mechanisms of the suppressor pathway. Using DNA from 18 colorectal cancer specimens (15 tumour xenografts, 2 tumour cell lines, and 1 laser capture micro-dissected primary tumour) this study addresses this issue.

The significant impact of TP53 aberrations in colorectal cancer is highlighted by the observation of TP53 mutations in 72% of the tumours in this series and the fact that mutations mapped to the highly conserved gene domains that are important for gene product function. The prevalence of TP53 mutations frequency in this study is in line with previous reports on TP53 mutation in sporadic colorectal cancer (Goh HS et al, 1995; Russo A et al, 2005). Furthermore, consistent with the notion that inactivation of p53 function by both mutation and LOH is essential in the progress to CRC we found that p53 gene mutation was combined with LOH in 56% of the tumours.

Allelotyping by polymorphic marker analyses with the tumour material used in this study allowed to distinguish efficiently between true allelic loss and allelic imbalance: using this type of material, in the case of LOH (N=13 for TP53), complete loss of the PCR product corresponding to one allele was observed in the electropherograms, whereas only a shift of the tumour/normal ratios was seen in the case of AI (N= 3 for TP53). However, applying polymorphic microsatellite marker analyses to DNA from the whole tissue homogenates only AI was recorded for all the cases.

Thiagalingam S et al, 2001 analysed early passage cell lines and xenografts in a series of 62 colorectal carcinomas. In accordance with our observations “true” TP53 LOHs were detected in 73% of their tumours. Other published studies that addressed allelic loss by the usual technique of using DNA from whole tumour homogenates, had lower percentages of LOHs with a range from 50-68% (Kanazawa T et al, 2002; Sugai T et al, 2006). Conceivably, contaminating effects of non-neoplastic tissue masked the deletions and led to lower rates.

Taken together, the molecular features observed for TP53 in this study were, as expected, those of a tumour suppressor gene, i.e. an inactivating mutation of one allele was seen to combine with genomic loss of the other allele. These findings attest to the proficiency of the methods used. They also highlight the fact that the common approach of allelotyping with whole tumour homogenates results in a significant fraction of spurious LOH calls.

Traditionally, TP53 immunohistochemistry has been used as a surrogate method to test for TP53 mutations. The methods adopted in this study allowed, as a corollary, to assess if this is a valid assumption. Assuming unequivocal nuclear immunostaining of

>10% of tumour cells as a cut-off for classifying a case as positive by TP53 immunohistochemistry sensitivity and specificity for detecting gene mutation by immunohistochemistry was calculated as 85% and 60%, respectively. This is in accord with previous published studies on this issue (Nasierowska-Guttmejer A et al, 2000 and Slebos RJ et al, 1996). Many TP53 gene mutations are point mutations that do not truncate the protein but alter its conformation and inhibit its degradation (Iacopetta B, 2003). This provides an elegant explanation for the fact that a large proportion of mutated tumours can be detected by immunohistochemical TP53 overexpression. However, there remains a significant proportion of tumours escaping detection, and, overall, TP53 immunohistochemistry does not appear to be sufficiently sensitive and/or specific to be useful as a surrogate for gene sequencing.

Applying these methods to the PTEN gene a different picture emerged. First, the PTEN gene was affected by mutation only in a single tumour, although, remarkably, in this tumour a total of three different mutations were seen. Second, two tumours were found to have LOH, but the tumour with mutations was not among these. Third, AI was observed in 6 cases, making this a fairly common event. The obvious conclusions from these findings is that in this series of colorectal carcinomas the “canonical” tumour suppressor gene features were not observed, raising some doubt about the universal conception of PTEN as a common TSG in this type of cancer. However, the following points merit comment as follows:

1. Apparently, PTEN gene mutations are a rare event in microsatellite stable colorectal carcinomas, as investigated in this series. The low PTEN gene mutation frequency shown in this study is in accord with the results reported by Zhou XP et al, 2002 and Shin KH et al, 2001 who studied a series of 39 and 32 microsatellites stable colorectal cancers, respectively: not a single case studied by these authors was reported positive for a PTEN gene mutation. In another two series of 20 and 327 unselected microsatellite stable colorectal carcinomas, (Negoro K et al, 2000 and LI XH et al, 2009) PTEN gene mutations were found in 0 and 1 case, respectively.

However, PTEN gene mutations were reported as fairly frequent among CRCs with high degree microsatellite instability (Day FL et al, 2013; Dicuonzo G et al, 2001; Guanti G et al, 2000), and this is well explained by these cancers’ incapacity of the

mismatch repair system to remove misincorporated nucleotides in the nucleotide repeats that are frequent in the downstream coding regions of the PTEN gene. Accordingly, PTEN gene mutations were observed in 3 out of 9 microsatellite instable colorectal cancers in a complementary study of the PTEN gene by our group (Hühns M et al, 2014).

Taken together, PTEN gene mutation could be relevant for CRCs driven by microsatellite instability rather than those driven by chromosomal instability, arguing against the hypothesis that PTEN plays a tumour suppressor gene role in the classical sense.

2. Gene promoter methylation is an alternative mechanism of gene inactivation and may substitute for mutation or allelic loss in the suppressor pathway. PTEN gene promoter methylation in colorectal carcinoma has been demonstrated by methylation-specific PCR in previous studies (Goel A et al, 2004), giving rise to the hypothesis that a combination of promoter methylation with mutation or allelic loss could be the tumour suppressor gene mechanism. However, when PTEN gene promoter methylation was tested quantitatively by our group in an additional study of 42 cases by MethyLight technology, (Hühns M et al, 2014), epigenetic PTEN silencing was observed in a single case only, and this tumour did not have a PTEN gene mutation or PTEN allelic loss.

3. In our series of colorectal carcinomas, LOH was seen in 11% of the tumours, but AI was observed in 33%, the latter figure corresponding quite well to the “LOHs” reported for the PTEN gene locus in previous publications (Frayling IM et al, 1997; Nassif NT et al, 2004; Garcia JM et al, 2003; Karoui M et al, 2004). These previous studies used DNA from whole tissue homogenates. As cautioned by Devilee et al 2001, such type of study is not a study of LOH but of allelic imbalance, and, accordingly, what was reported as “LOH” in previous studies in reality was AI, with a doubtful functional role in tumorigenesis.

When studying PTEN expression in our tumours by immunohistochemistry, a significant reduction, or even loss of PTEN expression was a frequent event, seen in 13 of the 18 tumours amenable to this study (72%). Considering the well-established function of PTEN in cell cycle control and cell migration, this observation points to a

definite role of PTEN in colorectal carcinoma tumour biology, although apparently by mechanisms different from what is usually meant when referring to PTEN as a tumour suppressor gene: the most plausible mechanism to explain reduction or even loss of PTEN expression in colorectal carcinoma cells at this juncture is (dys) regulation by unknown factors from the microenviroment that act on the tumour cells. This hypothetical explanation also allow for plasticity, i.e. it can explain fluctuations in downregulation and re-expression, and thus accounts for the somewhat inhomogenous pattern of expression observed by immunohistchemistry in many of the cases.

Reduced PTEN expression has been reported as a negative predictor for EGF-R blocking agents like cetuximab or panitumumab in the setting of metastasizing colorectal carcinoma (Razis E et al, 2008; Perrone F et al, 2009), underscoring its functional importance. Given the difficulties surgical pathologists usually encounter when trying to adapt quantitative/semi quantitative immunohistochemistry for routine use, testing the gene by molecular pathology instead would have been desirable: but alterations of PTEN expression are not appreciably mirrored in genomic aberrations, precluding this approach.

In summary, the following main conclusions can be stated from these investigations:

1. Counter to the common concept of a tumour suppressor gene, our study showed that for colorectal carcinoma the PTEN gene (in contrast to TP53) apparently is not typically targeted by two hits: the molecular features classically ascribed to a tumour suppressor gene, i.e. mutation of one allele and loss of the other is not usually observed.

2. Nevertheless, as indicated by immunohistochemistry, PTEN gene expression apparently is dysregulated/downregulated, and likely has a role in colorectal cancer pathogenesis.

5. Appendix

5.1. Sequences of primers for PCR of TP53 gene exons 5, 6, 7, and 8 with the length of amplicons

Exon	Forward primer (5‘→3‘)	Reverse primer (5‘→3‘)	Amplic. (bp)
5	(GC40)TTCCTCTTCCTACAGTACTC	CTGGGCAACCAGCCCTGTCGT	282
6	(GC40)ACGACAGGGCTGGTTGCCCA	AGTTGCAAACCAGACCTCAG	227
7	(GC40)TCTCCTAGGTTGGCTCTGACTG	GCAAGTGGCTCCTGACCTGGA	184
8	CCTATCCTGAGTAGTGGTAATC	(GC40)CCGCTTCTTGTCCTGCTTGCTT	215

(GC40) = CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCG

5.2. TGGE temperature gradient and running time for the different exons of TP53 gene

Exon	Running Time at 20°C (min.)	Temperature Gradient (°C)	Running Time (min.)
Exon 5	15	55/75	225
Exon 6	100	35/68	170
Exon 7	30	40/75	150
Exon 8	180	40/78	180

5.3. Sequences of primers for PCR and sequencing for P53 and PTEN genes with the length of amplicons

Gene	Exon	Forward primer (5‘→3‘)	Reverse primer (5‘→3‘)	Amplicon (bp)
TP53	2 and 3	TGGGGCAGGAGTGCTTGGGT	TTGGGACGGCAAGGGGGACT	563
	4	GCCATGGAGGAGCCGCAGTC	AAGCCAGCCCCTCAGGGCAA	629
	9	GAGCCTCACCACGAGCTGCC	CCCCCTGATGGCAAATGCCCC	317
	10	TTGCTGCAGATCCGTGGGCG	AGGGCCAGGAAGGGGCTGAG	145
	11	CGTTGTCCCAGCCTTAGGCCC	TGGAGCCCCGGGACAAAGCA	337
PTEN	1	CAGCCGTTCGGAGGATTA	ATATGACCTAGCAACCTGACCA	484
	2	GTACTTTAGTTCTGTGATGTATAAACCGT	CTGAAGTCCATTAGGTACGGTAA	509
	3	ATGTTTGTGAGGGTCGAATG	GGACTTCTTGACTTAATCGGTTTAG	726
	4	TTGAAAAAGGTGATCGTTGG	ATTGTTATGACAGTAAGATACAGTCTATCG	657
	5	TTCTGAGGTTATCTTTTTACCACA	TCCAGGAAGAGGAAAGGAAAA	352
	6	AATGTATATATGTTCTTAAATGGCTACGA	TCATAAATATAATTTGGCTTCGACTAC	484
	7	TGACAGTTTGACAGTTAAAGG	GGATATTTCTCCCAATGAAAG	262
	8	TGTCATTTCATTTCTTTTTCTTTTC	AAGTCAACAACCCCCACAAA	305
	9	GTTCATCTGCAAAATGGA	TTTTCATGGTGTTTTATCCCTC	328

5.4. Sequences of primers, annealing temperatures, $MgCl_2$ concentrations and the length of amplicons for LOH analysis

Gene	Locus	Forward primer (5‘→3‘)	Reverse primer (5‘→3‘)	Annealing temprature (˚C)	$MgCl_2$ Conc. (mM)	Amplicon (bp)
TP53	D17S250	6-FAM-GGA AGA ATC AAA TAG ACA AT	GCT GGC CAT ATA TAT ATT TAA ACC	55	2.75	151-169
	D17S1583	6-FAM- TGC CCA TGC TGA CAT A	GAC CTG ACT AAA ANA CTC CA	57	1.5	103-139
	17p13.1	6-FAM- ACT GCC ACT CCT TGC CCC ATT C	AGG GAT ACT ATT CAG CCC GAG GTG C	60	1.5	72-118
PTEN	D10S541	6-FAM- AAG CAA GTG AAG TCT TAG AAC CAC C	CCA CAA GTA ACA GAA AGC CTG TCT C	60	1.5	247-273
	D10S579	6FAM- CCG ATC AAT GAG GAG TGC C	ATA CAC CCA GCC AAT GCT GC	60	1.5	260-276
	D10S1765	6FAM- ACA CTT ACA TAG TGC TTT CTG CG	CAG CCT CCC AAA GTT GC	60	1.5	166-184

N= A/C/G/T

5.5. Materials and buffer solutions for TGGE

- 8% Polyacrylamid Gel

21.6 g Urea

12 ml Acrylamid 30%

0.9 ml 50X MOPS

2.25ml Glycerol 40%

14.5ml aqua H_2o

0.34ml APS*

75 µl TEMED **

Gel Bond PAG-Film (BIOzym)

50 x Mops

9.3 g EDTA,*** 104.6 g MOPS ****

500 ml Aqua dest ; the PH is to adjust with 5M NaOH to 8.

- Silver staining

Solutions:

Puffer A: - 200 ml absolute Ethanol

- 10 ml glacial acid, fill up to 2 L with Aqua dest

Puffer B: 1g $AgNO_3$ silver nitrate in 1L Aqua dest

Puffer C: - 4.5g NaOH

- 0.05 g $NaBH_4$

- 1.2 ml Formaldehyde

fill up to 300 ml with Aqua dest

Puffer D: 15 g Na_2CO_3 in 2 L Aqua dest

Procedure

Gel was shaken twice for 3 minutes in buffer A and for 10 minutes in buffer B. After rinsing twice briefly by Aqua dest, it was incubated for 20 minutes with shaking in buffer C and for 10 minutes in buffer D.

* Ammonium persulfate.

** Tetramethylethylendiamin.

*** Ethylenediaminetetraacetic acid.

**** 3-(N-morpholino) propanesulfonic acid.

6. References

Albuquerque C, Breukel C, van der Luijt, et al. The 'just-right' signalling model: APC somatic mutations are selected based on a specific level of activation of the ß-catenin signalling cascade. Hum Mol Genet 11: 1549 - 1560, 2002.

Ali IU, Schriml LM & Dean M. Mutational Spectra of PTEN/MMAC1 Gene: A tumour suppressor with lipid phosphatase activity. J Nat Cancer Inst 91: 1922 - 1932, 1999.

Baast IO, Mulder JWR & Offerhaus GJ A. An evaluation of six antibodies for immunohistochemistry of mutant p53 gene product in archival colorectal neoplasms. J Path 172: 5 - 12, 1994.

Baker SJ & Fearon ER, et al. Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science 244: 217 - 221, 1989.

Baker SJ, Preisinger AC, Jessup JM, et al. p53 gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. Cancer Res 50: 7717 - 7722, 1990.

Bartsch D, Barth P & Bastian Dl. Higher frequency of DPC4/Smad4 alterations in pancreatic cancer cell lines than in primary pancreatic adenocarcinomas. Cancer Letters 139: 43 - 49, 1999.

Beach R, Chan AO, Wu TT, et al. BRAF mutations in aberrant crypt foci and hyperplastic polyposis. Am J Pathol. 166: 1069 - 1075, 2005.

Berti L, Medintz IL, Tom J & Mathies RA. Energy-transfer cassette labelling for capillary array electrophoresis short tandem repeat DNA fragment sizing. Bio-conjugate Chem. 12: 493 - 500, 2001.

Bhattacharyya NP, Skandalis A, Ganesh A, Groden J & Meuth M. Mutator phenotypes in human colorectal carcinoma cell lines. Proc Natl Acad Sci 91: 6319 - 6323, 1994.

Bonneau D & Longy M. Mutations of the human PTEN gene. Hum Mut 16:109 - 122, 2000.

Broderick P, Carvajal-Carmona L, et al. A genome-wide association study shows that common alleles of SMAD7 influence colorectal cancer risk. Nat Genet 39: 1315 - 1317, 2007.

Cacev T, Jokic M, Spaventi R, et al. Loss of heterozygosity testing using real-time PCR analysis of single nucleotide polymorphisms. J Cancer Res Clin Oncol 132: 200 - 204, 2006.

Cairns P, Okami K, Halachmi S, et al. Frequent inactivation of PTEN/MMAC1 in primary prostate cancer. Cancer Res 57: 4997 – 5000, 1997.

Calvert PM & Frucht H. The genetics of colorectal cancer. Ann Intern Med 137: 603 - 612, 2002.

Cardoso J, Boer J, et al. Expression and genomic profiling of colorectal cancer. Biochim. Biophys Acta 1775: 103 - 137, 2007.

Carracedo A, Alimonti A & Pandolfi PP PTEN Level in Tumour Suppression: How Much Is Too Little? Cancer Res 71: 629 - 633, 2011.

Carvalho B, Postma C, et al. Multiple putative oncogenes at the chromosome 20q amplicon contribute to colorectal adenoma to carcinoma progression. Gut 58: 79 - 89, 2009.

Chan TL, Yuen ST, Kong CK, et al. Heritable germline epimutation of MSH2 in family with hereditary nonpolyposis colorectal cancer. Nat Genet 38: 1178 - 1183, 2006.

Cheah PY. Recent advances in colorectal cancer genetics and diagnostics. Crit Rev Oncol Hematol 69: 45 - 55, 2009.

Cho KR & Vogelstein B. Suppressor gene alterations in the colorectal adenoma-carcinoma sequence. J Cell Biochem 166:137 - 141, 1992.

Cho KR, Oliner J.D, Simons JW, et al. The DCC gene: structural analysis and mutations in colorectal carcinomas. Genomics 19: 525 - 531, 1994.

Chow LM & Baker SJ. PTEN function in normal and neoplastic growth. Cancer Letters 241: 184 – 196, 2006.

Cui H, Cruz-Correa M, Giardello FM, et al. Loss of IGF2 imprinting: A potential marker of colorectal cancer risk. Science 299: 1753 - 1755, 2003.

Dahia PL. PTEN, a unique tumour suppressor gene. Endocrine-Related Cancer 7: 115 – 129, 2000.

Danielsen SA, Lind GE, Bjørnslett M, et al. Novel mutations of the suppressor gene PTEN in colorectal carcinomas stratified by microsatellite instability - and TP53 mutation status. Hum Mut 29: E252 – E262, 2008.

Day FL, Jorissen RN, Lipton L, et al. PIK3CA and PTEN gene and exon mutation-specific clinicopathologic and molecular associations in colorectal cancer. Clin Cancer Res. 19: 3285 – 3296, 2013.

Devilee P, Cleton-Jansen AM, Cornelisse CJ. Ever since Knudson. Trends Genet 17: 569 - 573, 2001.

Dicuonzo G, Angeletti S, Garcia-Foncillas J, et al. Colorectal carcinomas and PTEN/MMAC1 gene mutations. Clin Cancer Res 7:4049 - 4053, 2001.

Dongen JJ, Langerak AW, Brüggemann M, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell

receptors gene recombinations in suspect lymphoproliferations. Report of the BIOMED-2 concerted action BMH4-CT98-3936. Leukemia 17: 2301 - 2317, 2003.

Duff EK & Clarke AR. Smad4 (DPC4) a potent tumour suppressor? Br J Cancer 78: 1615 - 1619, 1998.

Easton DF & Eeles RA. Genome-wide association studies in cancer. Hum Mol Genet 17: 109 - 115, 2008.

Egger G, Liang G, Aparicio A et al. Epigenetics in human disease and prospects for epigenetic therapy. Nature 429: 457 - 463, 2004.

Ellegren H. Microsatellite: Simple Sequence with complete evolution. Nature Reviews 5: 435 - 445, 2004.

Ellsworth RE, Ellsworth DL, Lubert SM, et al. High-throughput loss of heterozygosity mapping in 26 commonly deleted regions in breast cancer. Cancer Epid Biomarkers & Prevention 1: 915 – 919, 2003.

Erhan Y, Korkut MA & Kara E. Value of p53 protein expression and its relationship with short term prognosis in colorectal cancer. Annuals of Saudi Medicine, 22: 377 - 379, 2002.

Eshleman JR, Casey G, Kochera ME, et al. Chromosome number and structure both are markedly stable in RER colorectal cancers and are not destabilized by mutation of p53. Oncogene 17: 719 – 725, 1998.

Esteller M. CpG island hypermethylation and tumour suppressor genes: a booming present, a brighter future. Oncogene 21: 5427 – 5440, 2002.

Fata JE, Debnath S, et al. Nongenomic mechanisms of PTEN regulation. Int J Cell Biol: 2012.

Fearon ER & Vogelstein B. A genetic model for colorectal tumorigenesis. Cell 61: 759 - 767, 1990.

Feinberg AP & Tycko B. The history of cancer epigenetics. Nat Rev Cancer 4: 143 - 153, 2004.

Feinberg AP, Ohlsson R & Henikoff S. The epigenetic progenitor origin of human cancer. Nat Rev Genet 7: 21 - 33, 2006.

Firestein R, Bass AJ, et al. CDK8 is a colorectal cancer oncogene that regulates beta catenin activity. Nature 455: 547 - 551, 2008.

Forrester K, Almoguera C, Han K, Grizzle WE & Perucho M. Detection of high incidence of K-ras oncogenes during human colon tumorgenesis. Nature 327: 298 - 303, 1987.

Frayling IM, Bodmer WF & Tomlinson IPM. Allele loss in colorectal cancer at the cowden disease/ Juvenile Polyposis locus on 10q. Cancer Genet Cytogenet 97: 64 - 69, 1997.

Garcia JM, Rodriguez R, Silva J, et al. Intratumoural heterogeneity in microsatellite alterations in BRCA1 and PTEN regions in sporadic colorectal cancer. Ann Surg Oncol 10: 876 - 881, 2003.

Geiersbach KB & Samowitz WS. Microsatellite instability and colorectal cancer. Arch Pathol Lab Med135:1269 – 1277, 2011.

Geigl JB, Obenauf AC, Schwarzbraun T & Speicher MR. Defining chromosomal instability. Trends in Genetics 24: 64 - 69, 2008.

Georgescu MM, Kirsch KH, Akagi T, Shishido T & Hanafusa H. The tumour-suppressor activity of PTEN is regulated by its carboxyl-terminal region. Proc Natl Acad Sci USA 96: pp. 10182 – 10187, 1999.

Goel A, Arnold CN, Niedzwiecki D, et al. Characterization of sporadic colon cancer by pattern of genomic instability. Cancer Res. 63:1608 - 1614, 2003.

Goel A, Arnold CN, Niedzwiecki D, et al. Frequent inactivation of PTEN by promoter hypermethylation in microsatellite instability-High sporadic colorectal cancers. Cancer Res 64: 3014 - 3021, 2004.

Goh HS, Yao J & Smith DR. P53 point mutation and survival in colorectal cancer patients. Cancer Res 55: 5217 - 5221, 1995.

Goldgar DE, Easton DF, et al. Systematic population-based assessment of cancer risk in first-degree relatives of cancer probands. J Natl Cancer Inst 86: 1600 - 1608, 1994.

Grady WM & Markowitz SD. Hereditary colon cancer genes. Methods Mol Biol 222: 59 - 83, 2003.

Grady WM, Myeroff LL, Swinler SE, et al. Mutational inactivation of transforming growth factor beta receptor type II in microsatellite stable colon cancers. Cancer Res 59: 320 - 324, 1999.

Grady WM, Rajput A, Myeroff L, et al. Mutation of the type II transforming growth factor-beta receptors is coincident with the transformation of human colon adenoma to malignant carcinomas. Cancer Res 58: 3101 - 3104, 1998.

Grady WM. Genomic instability and colon cancer. Cancer and Metastasis Reviews 23: 11 - 27, 2004.

Gruis NA, Abeln ECA, Bardoell AFJ, et al. PCR-based microsatellite polymorphisms in the detection of loss of heterozygosity in fresh and archival tumour tissue. Br J Cancer 68: 308 - 313, 1993.

Guanti G, Resta N, Simone C, Cariola F, Demma I, Fiorente P, Gentile M. Involvement of PTEN mutations in the genetic pathways of colorectal carinogenesis. Hum Mol Genet 9: 283 - 287, 2000.

Hahn SA, Seymour AB, Hoque AT, et al. Allelotype of pancreatic adenocarcinoma using xenograft enrichment. Cancer Res. 55: 4670 - 4675, 1995.

Harkes IC, Elstrodt F, Dinjens WNM, et al: Allelotype of 28 human breast cancer cell lines and xenografts. Br J Cancer 89: 2289 - 2292, 2003.

Harris CC. P53: at the crossroads of molecular carcinogenesis and risk assessment. Science 262: 1980 - 1881, 1993.

Heinmöller E, Dietmaier W, Zirngibl H, et al. Molecular analysis of microdissected tumours and preneoplastic intraductal lesions in pancreatic carcinoma. Am J Pathol 157: 83 - 92, 2000.

Heldin CH, Miyazono K & ten Dijke P. TGF-ß signalling from cell membrane to nucleus through SMAD proteins. Nature 390: 465 - 471, 1997.

Hitchins M, Williams R, Cheong K, et al. MLH1 germline epimuations as a factor in hereditary nonpolyposis colorectal cancer. Gastroenterology 129: 1392 - 1399, 2005.

Hoang JM, Cottu PH, Thuille B, et al. BAT-26, an indicator of the replication error phenotype in colorectal cancer and cell lines. Cancer Res 57: 300 - 303, 1997.

Hollander MC, Blumenthal GM & Dennis PA. PTEN loss in the continuum of common cancers, rare syndromes and mouse models. Nature 11: 289 - 301, 2011.

Huang CS, O'Brien MJ, Yang S, et al. Hyperplastic polyps, serrated adenomas, and the serrated polyp neoplasia pathway. Am J Gastroenterol 99: 2242 - 2255, 2004.

Hühns M, Salem T, Schneider B, Krohn M, Linnebacher M and Prall F. PTEN mutation, loss of heterozygosity, promoter methylation and expression in colorectal carcinoma: two hits on the gene? Oncol Rep: in press, 2014.

Hsu CP, Kao TY, Chang WL, et al. Clinical significance of tumor suppressor PTEN in colorectal carcinoma. EJSO 37: 140 - 147, 2011.

Iacopetta B. TP53 mutation in colorectal cancer. Hum Mut 21: 271 - 276, 2003.

Ionov Y, Peinado MA, Malkhosyan S, et al. Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. Nature 363: 558 – 561, 1993.

Issa JP: Colon Cancer. It's CIN or CIMP. Clin Cancer Res 14: 5939 - 40, 2008.

Jacob S & Praz F. DNA mismatch repair defects: role in colorectal carcinogenesis. Biochimie 84: 27 - 47, 2002.

Jass JR, Iino H, Ruszkiewicz A, et al. Neoplastic progression occurs through mutator pathways in hyperplastic polyposis of the colorectum. Gut 47: 43 - 49, 2000.

Jen J, Kim H, Piantadosi S, et al. Allelic loss of chromosome 18q and prognosis in colorectal cancer. N Engl J Med 331: 213 - 221, 1994.

Kanazawa T, Watanabe T, Kazama S**,** et al. Poorly differentiated adenocarcinoma and mucinous carcinoma of the colon and rectum show higher rates of loss of heterozygosity and loss of E-cadherin expression due to methylation of promoter region. Int J Cancer 102: 225 - 229, 2002.

Karoui M, Tresallet C, Julie C. Loss of heterozygosity on 10q and mutational status of PTEN and BMPR1A in colorectal primary tumours and metastases. Br J Cancer 90: 1230 - 1234, 2004.

Keino-Masu K, Masu M, Hinck L, et al. Deleted in colorectal cancer (DCC) encodes a netrin receptor. Cell 87: 175 - 185, 1996.

Kim EC & Lance P. Colorectal polyps and their relationship to cancer. Gastroenterol Clin North Am 26: 1 - 17, 1997.

Kinzler K.W. & Vogelstein B. Lessons from Hereditary colorectal cancer. Cell 87: 159 - 170, 1996.

Knudson AG. Antioncogenes and human cancer. Proc Natl Acad Sci 90: 10914 - 10921, 1993.

Korinek V, Barker N, Morin PJ, et al. Constitutive transcriptional activation by a b-catenin–Tcf complex in APC-/- colon carcinoma. Science 275: 1784 - 1787, 1997.

Kubbutat MH, Jones SN & Vousden KH. Regulation of p53 stability by Mdm2. Nature 387: 299 - 303, 1997.

Kurose K, Zhou XP, Araki T, et al. Frequent loss of PTEN expression is linked to elevated phosphorylated Akt levels, but not associated with p27 and cyclin D1 expression, in primary epithelial ovarian carcinomas. Am J Pathol 158: 2097-2106, 2001.

Lamlum H, Ilyas M, Rowan A, et al. The type of somatic mutation at APC in familial adenomatous polyposis is determined by the site of the germline mutation: a new facet to Knudson's 'two-hit' hypothesis. Nat Med 5: 1071 - 1075, 1999.

Lane DP. p53, guardian of the genome. Nature 358: 15 - 16, 1992.

Lasko D, Cavenee W, Nordenskjold M. Loss of constitutional heterozygosity in human cancer. Annual Rev Genet 25: 281 - 314, 1991.

Lee JO, Yang H, Georgescu MM, et al. Crystal structure of the PTEN tumour suppressor: implications for its phosphoinositide phosphatase activity and membrane association. Cell 99: 323 - 334, 1999.

Lengauer C, Kinzler KW & Vogelstein B. Genetic instabilities in human cancers. Nature 396: 643 - 649, 1998.

Leslie A, Carey FA, Pratt NR, et al. The colorectal adenoma-carcinoma sequence. Br J Surg 89: 845 - 860, 2002.

Levine AJ, Hu W, et al. The P53 pathway: what questions remain to be explored? Cell Death Differ 13: 1027 - 1036, 2006.

Li AG, Piluso LG, Cai X, Weiet G, et al. Mechanistic insights into maintenance of high p53 acetylation by PTEN. Molecular Cell 23: 575 - 587, 2006.

Li J, Yen C, Liaw D, et al. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science 275: 1943 - 1947, 1997.

Li XH, Zheng HC, Takahashi H, et al. PTEN expression and mutation in colorectal carcinomas. Onc Reports 22: 757 - 764, 2009.

Lin MS, Huang JX, Chen WC, et al. Expression of PPARγ and PTEN in human colorectal cancer: An immunohistochemical study using tissue microarray methodology Oncology Leteres 2: 1219 - 1224, 2011.

Linnebacher M, Maletzki C, Ostwald C, Klier U, Krohn M, Klar E, Prall F. Cryopreservation of human colorectal carcinomas prior to xenografting. BMC Cancer 10: 362, 2010.

Lo KW, Teo PML, Hui ABY, et al. High resolution allelotype of microdissected primary nasopharyngeal carcinoma. Cancer Res 60: 3348 - 3353, 2000.

Lothe RA, Nakamura Y, Woodward S, et al. VNTR (variable number of tandem repeats) markers show loss of chromosome 17p sequences in human colorectal carcinomas. Cytogenet Cell Genet 48:167 - 169, 1988.

Lynch HT & de la Chapelle A. Hereditary colorectal cancer. N Engl J Med 348: 919 - 932, 2003.

Lynch HT & Lynch J. Lynch syndrome: genetics, natural history, genetic counselling, and prevention. J Clin Oncol 18:19 - 31, 2000.

Macleod K. Tumour suppressor genes. Current opinion in genetics & development 10: 81 - 93, 2000.

Maehama T & Dixon JE. PTEN: a tumour suppressor that functions as a phospholipid phosphatase. Trends Cell Biol 9:125 - 128, 1999.

Malkin D, Jolly KW & Barbier N. Germline mutations of the p53 tumour-suppressor gene in children and young adults with second malignant neoplasms. N Engl J Med 326: 1309 - 1315, 1992.

McLure KG. & Lee PW. How p53 binds DNA as a tetramer. EMBO J 17: 3342 - 3350, 1998.

McQueen HA, Wyllie AH, Piris J, et al. Stability of critical genetic lesions in human colorectal carcinoma xenografts. Br J Cancer 63: 94 - 96, 1991.

Medintz IL, Lee CC, Wong WW, et al: Loss of heterozygosity assay for molecular detection of cancer using energy-transfer primers and capillary array electrophoresis. Gen Res 10: 1211 - 1218, 2000.

Michor F, Iwasa Y, Vogelstein B, et al. Can chromosomal instability initiate tumorigenesis? Seminars in Cancer Biology 15: 43 - 49, 2005.

Miyoshi Y, Nagase H, Ando H, et al. Somatic mutations of the APC gene in colorectal tumours: mutation cluster region in the APC gene. Hum Mol Genet 1: 229 - 233, 1992.

Monte NM, Webster KA, Neuberg D, et al. Joint loss of PAX2 and PTEN expression in endometrial precancers and cancer. Cancer Res 70: 6225 - 6232, 2010.

Morin PJ, Sparks AB, Korinek V, et al: Activation of ß catenin-Tcf signalling in colon cancer by mutation in ß-catenin or APC. Science 275:1787 - 1790, 1997.

Mutter GL, Lin MC & Fitzgerald JT. Altered PTEN expression as a diagnostic marker for the earliest endometrial precancers. J Natl Cancer Inst 92: 924 - 931, 2000.

Nasierowska-Guttmejer A, Trzeciak L, Nowacki MP, Ostrowski J. P53 protein accumulation and p53 gene mutation in colorectal cancer. Pathol Oncol Res 6: 275 - 279, 2000.

Nassif NT, Lobo GP, Wu X, et al. PTEN mutations are common in sporadic microsatellite stable colorectal cancer. Oncogene 23: 617 - 628, 2004.

Negoro K, Takahashi S, Kinouchi Y, et al. Analysis of the PTEN gene mutation in polyposis syndromes and sporadic gastrointestinal tumours in Japanese patients. Dis Colon Rectum 43: 29 - 33, 2000.

Nowak MA, Komarova NL, Sengupta A, et al. The role of chromosomal instability in tumour initiation. PNAS 99: 16226 - 16231, 2002.

Ogino S, Brahmandam M, Kawasaki T, et al. Epigenetic profiling of synchronous colorectal neoplasias by quantitative DNA methylation analysis. Modern Path. 19: 1083 -1090, 2006.

Parsons R, Li GM, Longley MJ, Fang WH, et al: Hypermutability and mismatch repair deficiency in RER + tumour cells. Cell 75:1227 - 1236, 1993.

Parsons R, Myerof LL, Liu B, et al. Microsatellite instability and mutations of transforming growth factor beta type II receptor gene in colorectal cancer. Cancer Res 55: 5548 - 5550, 1995.

Peltomaki P. Deficient DNA mismatch repair: a common etiologic factor for colon cancer. Hum Mol Genet 10: 735 - 740, 2001.

Perren A, Komminoth P, Saremaslani P, et al. Mutation and expression analyses reveal differential subcellular compartmentalization of PTEN in endocrine pancreatic tumours compared to normal islet cells. Am J Pathol 157: 1097 - 1103, 2000.

Perren A, Weng LP, Boag AH, et al: Immunohistochemical evidence of loss of PTEN expression in primary ductal adenocarcinomas of the breast. Am J Pathol 155:1253 - 1260, 1999.

Perrone F, Lampis A, Orsenigo M, et al. PI3KCA/PTEN deregulation contributes to impaired responses to cetuximab in metastatic colorectal cancer patients. Ann Oncol 20: 84 - 90, 2009.

Powell SM, Zilz N, Beazer-Barclay Y, et al. APC mutations occur early during colorectal tumorgenesis. Nature 359: 235 - 237, 1992.

Razis E, Briasoulis E, Vrettou E, et al. Potential value of PTEN in predicting cetuximab response in colorectal cancer: an exploratory study. BMC Cancer 8: 234 - 244, 2008.

Reyes G, Villanueva A, Garcia C, et al. Orthotopic xenografts of human pancreatic carcinomas acquire genetic aberrations during dissemination in nude mice. Cancer Res 56: 5713 - 5719, 1996.

Russo A, Bazan V, Iacopetta B, et al. The TP53 colorectal cancer international collaborative study on the prognostic and predictive significance of p53 mutation: Influence of tumour site, type of mutation, and adjuvant treatment. J Clin Oncolo 23: 2005.

Sugai T, Habano W, Jiao YF, et al. Analysis of molecular alterations in left- and right-sided colorectal carcinomas reveals distinct pathways of carcinogenesis: proposal for new molecular profile of colorectal carcinomas. J Mol Diagn 8:193 - 201, 2006.

Saha S, Bardelli A, Buckhaults P, et al: A phosphatase associated with metastasis of colorectal cancer. Science 294: 1343 - 1346, 2001.

Salmena L, Carracedo A & Pandolfi PP. Tenets of PTEN tumour suppression. Cell 133: 403 - 404, 2008.

Sawai H, Yasuda A, Ochi N, et al. Loss of PTEN expression is associated with colorectal cancer liver metastasis and poor patient survival. BMC Gastroenterology 8:56, 2008.

Scholz RB, Mllde-Langosch K, Jung R, et al. Rapid screening for Tp53 mutations by temperature gradient gel electrophoresis: a comparison with SSCP analysis. Hum Mol Genet 2: 2155 - 2158, 1993.

Shin KH, Park YJ & Park JG. PTEN gene mutations in colorectal cancer displaying MSI. Cancer letters 174:189 - 194, 2001.

Skotheima RI, Diepa CB, Kraggeruda SM, et al. Evaluation of loss of heterozygosity/allelic imbalance scoring in tumour DNA. Cancer Genet. & Cytogenet. 127: 64 - 70, 2001.

Slebos RJ, Baas IO & Clement M. Clinical and pathological associations with p53 tumour-suppressor gene mutations and expression of p21WAF1/CiP1 in colorectal carcinoma. Br J Cancer 74: 165 - 171, 1996.

Smits R, Kielman MF, Breukel C, et al. Apc1638T: a mouse model delineating critical domains of the adenomatous polyposis coli protein involved in tumorigenesis and development. Genes Dev 13: 1309 - 1321, 1999.

Solomon E, Voss R, Hall V, et al. Chromosome 5 allele loss in human colorectal carcinomas. Nature 328: 616 - 619, 1987.

Song MS, Carracedo A, Salmena L, et al. Nuclear PTEN regulates the APC-CDH1 tumour suppressive complex in a phosphatase-independent manner. Cell 144: 187 - 199, 2011.

Steck PA, Pershouse MA, Jasser SA, et al. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nat Genet 15: 356 - 362, 1997.

Suter CM, Martin DI & Ward RL. Germline epimutation of MLH1 in individuals with multiple cancers. Nat Genet 36: 497 - 501, 2004.

Taniyama K, Goodison S & Ito R, et al. PTEN expression is maintained in sporadic colorectal tumours. J Pathol194: 341 - 348, 2001.

Thiagalingam S, Laken S, Willson JK, et al. Mechanisms underlying losses of heterozygosity in human colorectal cancers. Proc Natl Acad Sci USA. 98: 2698 - 2702, 2001.

Thibodeau SN, Bren G & Schaid D. Microsatellite instability in cancer of the proximal colon. Science 260: 816 - 819, 1993.

Tomlinson IP, Lambros MB & Roylance RR. Loss of heterozygosity analysis: Practically and conceptually flawed?. Genes Chromosomes & Cancer 34: 349 - 353, 2002.

Uronis JM, Osada T, McCall S, et al. Histological and molecular evaluation of patient-derived colorectal cancer explants. PLos One 7: 2012.

Venkatachalam S, Shi YP, et al. Retention of wild-type p53 in tumours from p53 heterozygous mice: reduction of p53 dosage can promote cancer formation. EMBO J 17: 4657 - 4667, 1998.

Vilar E & Gruber SB. Microsatellite instability in colorectal cancer-the stable evidence. Nat Rev Clin Oncol advance online publication, 2010.

Vogelstein B & Kinzler KW. Cancer genes and the pathways they control. Nat Med 10: 789 - 799, 2004.

Vogelstein B, Fearon ER, Hamilton SR, et al. Genetic alterations during colorectal tumour development. N Engl J Med 319: 525 - 532, 1988.

Vogelstein B, Fearon ER, Kern SE, et al. Allelotype of colorectal carcinomas. Science 244: 207 - 211, 1989.

Vousden KH & Lu X. Live or let die: the cell's response to p53. Nat Rev Cancer 2: 594 - 604, 2002.

Vousden KH & Prives C. P53 and prognosis: new insights and further complexity. Cell 120: 7 -10, 2005.

Waite KA & Eng C. Protein PTEN: form and function. Am J Hum Genet. 70: 829 - 844, 2002.

Waniczek D, Snietura M, Liszka JM, et al. PTEN expression profiles in colorectal adenocarcinoma and its precancerous lesions. Pol J Pathol 1: 15 - 20, 2013.

Weber JC, Schneider A, Rohr S, et al. Analysis of allelic imbalance in patients with colorectal cancer according to stage and presence of synchronous liver metastases. An Surg 234: 795 - 803, 2001.

Whang YE, Wu X, Suzuki H, et al. Inactivation of the tumour suppressor PTEN/MMAC1 in advanced human prostate cancer through loss of expression. Proc Natl Acad Sci U S A 95: 5246 - 5250, 1998.

Wood LD, Parsons DW, Jones S, et al. The genomic landscapes of human breast and colorectal cancers. Science 318: 1108 - 1113, 2007.

Zhou XP, Gimm O, Hampel H, et al. Epigenetic PTEN silencing in malignant melanomas without PTEN mutation. Am J Pathol157: 1123 - 1128, 2000.

Zhou XP, Loukola A, Salovaara R, et al. PTEN mutational spectra, expression levels, and subcellular localization in microsatellite stable and unstable colorectal cancers. Am J Pathol 161: 439 - 447, 2002.

7. Declaration of original work

I hereby declare that the work presented here is, to the best of my knowledge and belief original and the results of my own investigations, except as acknowledged, and has not been submitted, either in part or whole, for a degree at this or any other University. Formulations and ideas taken from other sources are cited as such. This work has not been published.

Tareq Salem

8. Thesis for dissertation

1. The phosphatase and tensin homologue (PTEN) gene encodes a major tumour suppressor protein involved in the control of cell growth and proliferation by counteracting cellular growth signals. A combination of PTEN gene mutation with allelic loss (loss of heterozygosity, LOH) has previously been shown in various types of cancer, suggesting a role for PTEN as a tumour suppressor gene as specified by knudsons's classical "two-hit" concept.

2. Although the suggestion has been made in the literature, detailed studies of PTEN in colorectal carcinoma have not been made to test if PTEN actually meets the two-hit criteria for a tumour suppressor gene in the classical sense.

3. This study was conducted to find out if the patterns of PTEN gene molecular aberration in colorectal carcinoma are consistent with the classic molecular features ascribed to a tumour suppressor gene. To this end, PTEN gene mutations and LOH were assayed in a series of 18 colorectal carcinomas. Since loss of gene function by a tumour suppressor gene mechanism is a cardinal feature of tumours with chromosomal instability, this series was composed of non-familial microsatellite-stable colorectal carcinomas (so-called sporadic standard type).

4. Tumour DNA from colorectal carcinoma xenografts/primary cell lines (N = 17) or neoplastic glands isolated by laser capture microdissection (N = 1) was used for gene sequencing and allelotyping analyses; gene expression in primary tumour tissues was additionally tested by immunohistochemstry. TP53, a paradigm tumour suppressor gene, was studied in parallel for comparison and validation of the methods.

5. TP53 gene mutations were found in 13 (72%) of the tumours, 10 of which (56% of the total) also had a demonstrable loss of one TP53 allele. Overall, 11 tumours were classified as positive by TP53 immunohistochemistry. Sensitivities and specificities for detecting gene mutation by immunohistochemistry were 85% and 60%, respectively.

6. PTEN gene mutation was a rare event in this series. Sequencing of exons 1 - 9 revealed a total of 3 different somatic mutations, but restricted to a single tumour. Polymorphic microsatellite marker analyses showed LOH in 2 tumours and allelic imbalance (AI) in 6 tumours. LOH or AI did not target the 1 tumour with the PTEN gene mutations. By immunohistochemistry, reduced or even loss of PTEN expression was a frequent observation, found in 12 and 1 of the tumours, respectively.

7. This study confirms the universally accepted role of TP53 as a tumour suppressor gene in colorectal carcinoma. This result validates the methods in their extension to the study of the PTEN gene.

8. Contrary to TP53, PTEN gene mutations are an infrequent event in colorectal carcinomas with chromosomal instability, and, additionally, LOH in its strict sense also is not a common feature, although AI is not unusual. Contrary to the allelotyping studies in this work that relied on pure tumour DNA, previous studies addressing PTEN LOH in colorectal carcinoma used DNA from whole tumour homogenates which, technically, does not allow proper distinction between LOH and AI. Thus, the high rates of “LOH” reported in the literature are spurious and in reality were AI.

9. From these findings we conclude that PTEN gene is not typically targeted by two hits: the molecular features classically ascribed to a tumour suppressor gene, i.e. mutation of one allele and loss of the other is not usually observed. The widely held belief of PTEN as a classical tumour suppressor gene is not supported by the findings from this study.