The role of *PTEN* gene mutations in hyperplasia and carcinoma of the endometrium

BY

JOHANNA CATHARINA ALETTA JAMISON

Dissertation in fulfilment of the requirements for the degree of
Magister Scientiae in Human Genetics

In the
Department of Genetics (Human Genetics Section)
Faculty of Health Sciences
University of Pretoria

Supervisor: Professor E.J. van Rensburg

March 2004

ABSTRACT

Endometrial carcinoma, which is preceded by non-malignant hyperplasia, is the fifth most common cancer in women worldwide. Various genetic alterations appear to be early events in the pathogenesis of endometrial cancer. The *PTEN/MMAC1/TEP1* gene is most commonly mutated in endometrioid adenocarcinoma. This gene, on chromosome 10q23, codes for a tumour suppressor protein which displays lipid and dual-specific protein phosphatase activity. It has been implicated in several signal transduction pathways and seems to be involved in the negative regulation of the PI3K-, the MAPK- and the FAK pathways. Studies have shown that Caucasian Americans have a 4-fold higher frequency of *PTEN* mutations than African Americans. An association of *PTEN* mutation status with clinical outcome has been found, where patients with *PTEN* mutation-positive endometrial carcinoma had a better prognosis than those without *PTEN* mutations. It has been hypothesized that the molecular pathogenesis of endometrial carcinoma within Caucasians and Black African groups may be different.

The present study aimed to investigate the *PTEN* gene in Caucasians and Black South African women with endometrial hyperplasia and carcinoma. The correlation between the frequency and type of mutations and the pathological features of the cancers (stage and grade) were also assessed. Paraffin-embedded tissue samples from patients with endometrial hyperplasia [n=10] and cancer [n=47] were analysed for *PTEN* mutations using exon-by-exon PCR-SSCP.

Thirty-two mutations were detected of which 24 were pathogenic (23 in the adenocarcinomas, one in the hyperplasias). These included 10 frameshift, 7 nonsense, 4 missense and 3 splice site mutations. Pathogenic mutations were located throughout the gene with the highest frequency observed in exon 5 (39.1 %; 9/23), followed by exons 1 and 8 (both 17.4 %; 4/23). This data does not differ significantly from published findings ($P>0.05$; χ^2-test).

Pathogenic mutations were present in 54 % (20/37) of the endometrioid adenocarcinomas and 10 % (1/10) of the hyperplasias. No mutations were detected in the serous papillary cancers and poorly differentiated carcinomas. Fifty-five % (6/11) of tumours from Caucasians and 52 % (13/25) of the tumours from Black South Africans had genetic alterations. When comparing the African and Caucasian groups there were no significant differences with regards to *PTEN* mutation frequency ($P>0.05$; χ^2-test). Mutations occurred in early and advanced stage endometrial carcinomas, although the majority of the samples were stage I endometrioid adenocarcinomas.

In the present study no association between the frequency of *PTEN* mutations and the grade and stage of the endometrial cancer were found ($P>0.05$; χ^2-test). To validate these observations, however, a larger sample size representative of all the grades and stages of endometrial carcinoma needs to be analyzed.

ABSTRAK

Endometriële karsinoom, wat deur nie-kwaadaardige hiperplasie voorafgegaan word, is die vyfde mees algemene kanker in vroue wêreldwyd. Verskeie genetiese veranderinge blyk vroeë gebeurtenisse te wees in die patogenese van endometriële kanker. *Die PTEN/MMAC1/TEP1* geen is die mees gemuteerde geen in endometrioïede adenokarsinoom. Hierdie geen, op chromosoom 10q23, kodeer vir 'n tumoronderdrukker proteïen wat lipied- en twee-ledige proteïenfosfatase-aktiwiteit vertoon. Dit is betrokke by verskeie seinoordrag paaie waar dit blyk om die PI3K-, die MAKP- en die FAK-paaie negatief te reguleer. Studies het aangetoon dat Amerikaanse Kaukasiërs 'n 4-voudig hoër frekwensie van *PTEN* mutasies het as Swart Amerikaners. 'n Assosiasie tussen *PTEN* mutasies en kliniese uitkoms is gevind, waar pasiënte met *PTEN* mutasie-positiewe endometriële karsinoom 'n beter prognose toon as dié sonder mutasies. Dit is gepostuleer dat die molekulêre patogenese van endometriële karsinoom in Kaukasiërs en Swart Afrika-groepe verskillend mag wees.

Die doel van die huidige studie was om die *PTEN* geen in Kaukasiërs en Swart Suid-Afrikaanse vroue met endometriële hiperplasie en karsinoom te ondersoek. Die korrelasie tussen die frekwensie en tipe mutasies en die patologiese eienskappe van die kankers (stadium en graad) is ook bepaal. Paraffien-ingebedde weefselmonsters van pasiënte met endometriële hiperplasie [n=10] en kanker [n=47] is ondersoek vir *PTEN* mutasies deur gebruik te maak van ekson-vir-ekson PCR-SSCP.

Twee-en-dertig mutasies is gevind waarvan 24 patogenies was (23 in die adenokarsinome, een in die hiperplasieë). Hierdie mutasies sluit 10 leesraamverskuiwings-, 7 nonsens-, 4 fout- en 3 splytlasmutasies in. Patogeniese mutasies was deur die hele geen versprei met die hoogste mutasie frekwensie in ekson 5 (39.1 %; 9/23), gevolg deur eksons 1 en 8

(beide 17.4 %; 4/23). Hierdie data verskil nie betekenisvol van gepubliseerde bevindings nie ($P>0.05$; χ^2-test).

Patogeniese mutasies was teenwoordig in 54 % (20/37) van die endometrioïede adenokarsinome en in 10 % (1/10) van die hiperplasieë. Geen mutasies is opgespoor in die sereus papillêre kankers en swak gedifferensieerde karsinome nie. Vyf-en-vyftig % (6/11) van die tumore van Kaukasiërs en 52 % (13/25) van die tumore van Swart Suid-Afrikaners het genetiese veranderinge gehad. Vergelyking van die *PTEN* mutasie frekwensies tussen die Swart en Kaukasiese groepe het geen betekenisvolle verskille ($P>0.05$; χ^2-test) opgelewer nie. Mutasies was teenwoordig in vroeë- en laat stadium endometriële karsinome, hoewel die meerdeheid monsters stadium I endometrioïede adenokarsinome was.

Geen assosiasie tussen die frekwensie van *PTEN* mutasies en die graad en die stadium van die endometriële kanker ($P>0.05$; χ^2-test) is in die huidige studie gevind nie. 'n Groter aantal monsters, verteenwoordigend van al die grade en stadia van endometriële karsinoom, sal geanaliseer moet word om hierdie waarnemings te bevestig.

TABLE OF CONTENTS

ABBREVIATIONS

APC	Adenomatous polyposis coli
ASIR	Age standardized incidence rate
BAX	BCL-2-associated X protein
BP	Base pair
BRR	Bannayan-Riley-Ruvalcaba syndrome
CAH	Complex hyperplasia with atypia
CAV1	Caveolin-1
CDK	Cyclin-dependent kinase
CH	Complex hyperplasia without atypia
CS	Cowden syndrome
DDC	Deleted in colon carcinoma
E	Exon
ECM	Extracellular matrix
EEC	Endometrioid adenocarcinoma of the endometrium
EGF	Epidermal growth factor
EGFR	Epidermal (extracellular) growth factor receptor
ERK	Extracellular signal-regulated kinase
FAP	Familial adenomatous polyposis
FAK	Focal adhesion kinase
FIGO	International Federation of Gynaecology and Obstetrics
GRB2	Growth factor receptor-bound protein 2
GSK-3	Glucogen synthase kinase-3
HIF-1α	Hypoxia-induced factor-1α
HNPCC	Hereditary non-polyposis colorectal cancer syndrome
I	Intron
IGF-1	Insulin-like growth factor-1
IGFR	Insulin growth factor receptor
IGFIIR	Insulin-like growth factor II receptor
IR	Insulin receptor

IRS	Insulin receptor substrate
IRS-1	Insulin receptor substrate-1
LOH	Loss of heterozygosity
MAGI-2	Membrane associated guanylate kinase inverted-2
MAPK	Mitogen-activated protein kinase
MDE	Mutation detection enhancement
MMAC1	Mutated in Multiple Advance Cancers 1
MI	Microsatellite instability
NGF	Nerve growth factor
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDK1	Phosphoinositol 3,4,5-triphosphate-dependent protein kinase 1
PEST	(Peptide area rich in) proline, glutamic acid, aspartic acid, serine and threonine
PI3K	Phosphoinositol-3-phosphate kinase
PIP2	Phosphoinositol 4,5-diphosphate (PtdIns(4,5)P_3)
PIP3	Phosphoinositol 3,4,5-triphosphate (PtdIns(3,4,5)P_3)
PH	Pleckstrin homology domain
PHTS	*PTEN* hamartoma-tumour syndrome
PKB	Protein kinase B
PLC	Phospholipase-C
PNK	Polynucleotide kinase
$p130^{Cas}$	p130Crk-associated substrate
PS	Proteus syndrome
PSI (Ψ)	Pseudogene
PTP	Protein tyrosine phosphatase
PtdIns(3)P	Phosphoinositol 3-monophosphate
PtdIns(3,4)P_2	Phosphoinositol 3,4-diphosphate (PIP2)
PtdIns(3,4,5)P_3	Phosphoinositol 3,4,5-triphosphate (PIP3)
PTEN	Phosphatase and tensin homoloque deleted on chromosome 10

SAH	Simple hyperplasia with atypia
SD	Standard deviation
SH	Simple hyperplasia without atypia
SH2	Src homology-2
SH3	Src homology-3
SOS	Ras guanosine 5'-triphosphate exchange factor mSOS
SSCP	Single-stand conformation polymorphism
T_{ann}	Annealing temperature
TEP1	Transforming Growth Factor-β-regulated and Epithelial Cell-enriched Phosphatase 1
TGF-βRII	Growth factor β receptor type II
WHO	World Health Organization

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

1.1 UTERINE CANCER

1.1.1 Incidence of uterine cancer

Uterine cancer is the fourth most common cancer in women in the United States (Burke *et al.*, 1996; Rose, 1996). In South Africa cancer of the uterus is ranked third in Asian (5.4 %), fourth in African (3.7 %), fourth in Coloured (4.2 %) and seventh in Caucasian (2.3 %) women. Among the South African population the age standardized incidence rates (ASIR) of uterine cancer for white females (9.2/100 000) is higher than that of Black women (3.6/100 000). For Asian and Coloured females the incidence is 10.3 and 3.7 per 100 000 population respectively (Sitas *et al.*, 1998). In the United States the ASIR for uterine cancer among white and black women are proportionally larger, with the ASIR among white women at 19.2 per 100 000 and among African Americans at 9.7 per 100 000 (Schottenfeld, 1995). Uterine cancer includes cancers of the endometrium, myometrium and corpus uteri. Ninety-seven percent of all cancers of the uterus arise from the glands of the endometrium, and is known as endometrial carcinoma. The remaining 3 % of uterine cancers are sarcomas (Rose, 1996). Worldwide, endometrial carcinoma is the fifth most common cancer in women (Parkin *et al.*, 1989; Parazzini *et al.*, 1997). In the USA specifically, the incidence is 23.0 per 100 000 for Caucasians and 13.9 per 100 000 among black women (Rose, 1996).

1.1.2 Classification and histopathological features of endometrial carcinoma.

The development of endometrioid carcinoma from normal epithelium is preceded by pre-malignant hyperplasia. The histological presentation of endometrial hyperplasia is often typical. Although fewer than 10 % of hyperplasias progress to invasive cancer in the absence of atypia, the risk of malignant transformation rises to about 20 % to 30 % when atypia is present (Kurman *et al.*, 1985).

Endometrial hyperplasia forms a morphological continuum of abnormal epithelial and stromal proliferation ranging from simple hyperplasia to well-differentiated adenocarcinoma (Scully *et al.*, 1994; Prat, 1996). The new World Health Organization (WHO) classification of endometrial tumours classifies endometrial hyperplasia into four subtypes: simple hyperplasia without atypia (SH), simple hyperplasia with atypia (SAH), complex hyperplasia without atypia (CH), and complex hyperplasia with atypia (CAH) (Scully *et al.*, 1994). The term atypia refers to cellular atypia. The term complexity refers to severe architectural abnormality close to that seen in cases of well-differentiated adenocarcinoma (Kurman *et al.*, 1985; Prat, 1996). SH which is not significantly pre-cancerous, includes cystic hyperplasia with mild to moderate degrees of architectural abnormality. Similarly CH is also not demonstratebly pre-cancerous. Follow-up information about SAH is inadequate to indicate that it is pre-cancerous. When CAH is diagnosed in a biopsy specimen, well-differentiated adenocarcinoma is normally discovered in the hysterectomy specimen in 17 % to 20 % of the cases, or the lesion will eventually be followed by carcinoma in approximately 30 % of the patients (Kurman & Norris, 1982; Prat, 1996). Kurman *et al.* (1985) confirmed that progression to carcinoma occurred in 1 % of patients with SH, in 3 % of patients with CH, in 8 % of patients with SAH and in 29 % of patients with CAH. Although a trend was observed in the differences between the four subgroups it was not statistically significant.

The majority of malignant tumours of the endometrium are well differentiated adenocarcinomas and include various forms: endometrioid adenocarcinoma, serous papillary adenocarcinoma, clear cell adenocarcinoma and adenosquamous carcinoma. Mixed and undifferentiated carcinoma are infrequently observed. Endometrial adenocarcinoma invades the myometrium at an early stage and tends to be confined to the uterus until late in the course of the disease (Fox, 1992; Ronnet *et al.*, 2002). From a histological and clinical viewpoint, endometrioid adenocarcinoma of the endometrial epithelium (EEC) (Type 1) and serous papillary carcinoma (Type 2) are the two main forms of endometrial adenocarcinoma. The first type is low-grade adenocarcinoma that is estrogen-

related, occurring in a younger group of women. The second, more aggressive, estrogen-unrelated, non-endometrioid type, occur largely in older women (Ronnet *et al.*, 2002). Of all the women affected with endometrial cancer, 75 % are postmenopausal, 25 % are pre-menopausal of which 5 % is less than 40 years of age (Peterson, 1968; Gallup & Stock, 1984).

1.1.3 Epidemiology/Etiology of endometrial carcinoma

EEC is most commonly seen in patients between the ages of 50 and 75 years (Fox, 1992; Prat, 1996). Women with constant exposure to endogenous or exogenous estrogen not opposed by progesterone are at increased risk for this neoplasia. Increased exposure to unopposed estrogens due to obesity, early age at menarche, late menopause, nulliparity, history of menstrual disorders, problems with infertility and use of estrogen replacement therapy are associated with an increased risk (Ewertz *et al.*, 1988, Parazzini *et al.*, 1991; Fox, 1992; Burke *et al.*, 1996; Prat, 1996). Approximately 50 % of endometrial carcinomas occur in women with these particular risk factors (Parazzini *et al.*, 1989). Other predisposing factors include hypertension and diabetes (Ewertz *et al.*, 1988, Fox, 1992; Burke *et al.*, 1996; Prat, 1996). Many patients have an increased capacity for converting androstenedione (of adrenal origin) to estrone in the body fat and hence the association with obesity (Fox, 1992; Prat, 1996). Interestingly, risk factors such as obesity, menstrual irregularities and nulliparity seems to be predominant in women younger than 40 years of age (Peterson, 1968; Gallup & Stock, 1984) *versus* older women where factors like hypertension and diabetes seems to be more common (Gallup & Stock, 1984). Most women who develop endometrial cancer, do so, however, in the absence of such factors.

Although endometrioid adenocarcinomas evolve mainly from atypical hyperplasia, some tumours arise from an atrophic endometrium. Uterine serous papillary carcinoma, which accounts for 10 % of endometrial carcinomas, develops from a precursor lesion, endometrial intra-epithelial carcinoma, which arises from the atrophic endometrium (Fox, 1992; Ambros *et al.*, 1995).

1.2 THE GENETIC MECHANISMS IN CANCER DEVELOPMENT

1.2.1 The multi-step basis of cancer

Each cell carries a very complex and highly regulated genetic programme that controls normal cellular growth and differentiation. Cancer results from a disruption of this normal regulatory pattern, leading to uncontrolled cellular growth and proliferation that is recognized as a malignant tumour. Much progress has been made over the past twenty years in elucidating the molecular and cellular events that contribute to malignancy. Cancer has a clonal nature. It is now known that a normal cell can turn into a cancerous cell due to a multi-step process, where mutations accumulate in the same somatic cell during a patient's lifetime. These mutations are changes or damage to the DNA, affecting various genes. The genetic alterations also differ significantly from one type of cancer to another. The common view is that each step in the sequence creates an additional phenotypic aberration. Three categories of genes are known to be affected in various cancers. They are: proto-oncogenes, tumour suppressor genes and DNA mismatch repair genes, also known as mutator genes (Bishop, 1991; Cavenee & White, 1995; Weinberg, 1996; Van Rensburg, 1997). Susceptibility to cancer, however, can be inherited when the genetic changes are present in germ cells. The best example of the multi-step process is familial adenomatous polyposis (FAP) colon cancer. Mutation of the familial adenomatous polyposis coli (*APC*) gene, a tumour suppressor gene, is an early event, whether somatic or germline. This is followed sequentially by DNA methylation, activation of the k-*ras* oncogene, loss of the *DCC* (deleted in colon carcinoma) tumour suppressor gene and finally loss of p53 protein function (Fearon & Vogelstein, 1990; Kinzler & Vogelstein, 1996).

1.2.2 Genes involved in cancer

A variety of mutations such as frameshift, nonsense and missense mutations occur in proto-oncogenes, tumour suppressor genes and DNA mismatch repair genes in various cancers. Activation of oncogenes or inactivation of tumour suppressor genes can lead to irreversible activation of growth regulatory

pathways and uncontrolled growth. It is also crucial for cancer cells to acquire the ability to escape normal tissue architecture, to invade and to metastasize. Growth of cancer cells is generally independent of growth factors and adhesion to extracellular matrix, which normally regulates cell movement, growth and tissue remodelling (Schwartz, 1997; Giancotti & Ruoslahti, 1999).

Proto-oncogenes:

Proto-oncogenes are genes whose action promotes cell growth, cell proliferation and apoptosis (cell death). Four different groups of proto-oncogenes have been identified according to their function: extracellular growth factors, growth factor receptor and non-receptor tyrosine kinases, transduction proteins that relay signals down-stream and DNA-binding transcription factors. These genes play a role in cell signal transduction during cell growth, *via* the binding of a growth factor to its receptor at the cell surface that leads to a chain of events. These signals are transmitted to the cell nucleus, where transcription of genes involved in cell division is initiated (Figure 1.1A). Proto-oncogenes are therefore relays in the elaborate biochemical circuitry that governs the phenotype of mammalian cells. When mutated, these genes are called oncogenes that express excessive or inappropriate activity. Exsessive activity may occur due to inappropriate gene amplification to a high copy number as a result of abnormal chromosomal replication. Inappropriate activity may be due to mutations affecting protein structure or due to chromosomal translocation near a proto-oncogene locus. Mutations have a dominant effect, as only one of the alleles of a proto-oncogene needs to change to result in a gain of function. When an oncogene is activated it either causes uncontrolled over-stimulation of cell growth or prevention of cell death, both leading to an increase in cell numbers (Bishop, 1991; MacDonald & Ford, 1997; Van Rensburg, 1997).

Tumour suppressor genes:

The products of tumour suppressor genes inhibit cell proliferation. Cell growth is negatively regulated or inhibited by counteracting the effects of proto-oncogenes (Figure 1.1B). A very fine balance exists between the activity of proto-oncogenes

and tumour suppressor genes. In cancer cells, defective tumour suppressor genes have lost their function in regulating cell growth and proliferation. Both alleles of the genes need to be affected to result in the development of cancer. Mutations can be somatic in both alleles or could be a germline mutation inherited from one of the parents while the second allele is mutated later in life (Bishop, 1991; Marshall, 1991; Levine, 1995; MacDonald & Ford, 1997; Van Rensburg, 1997).

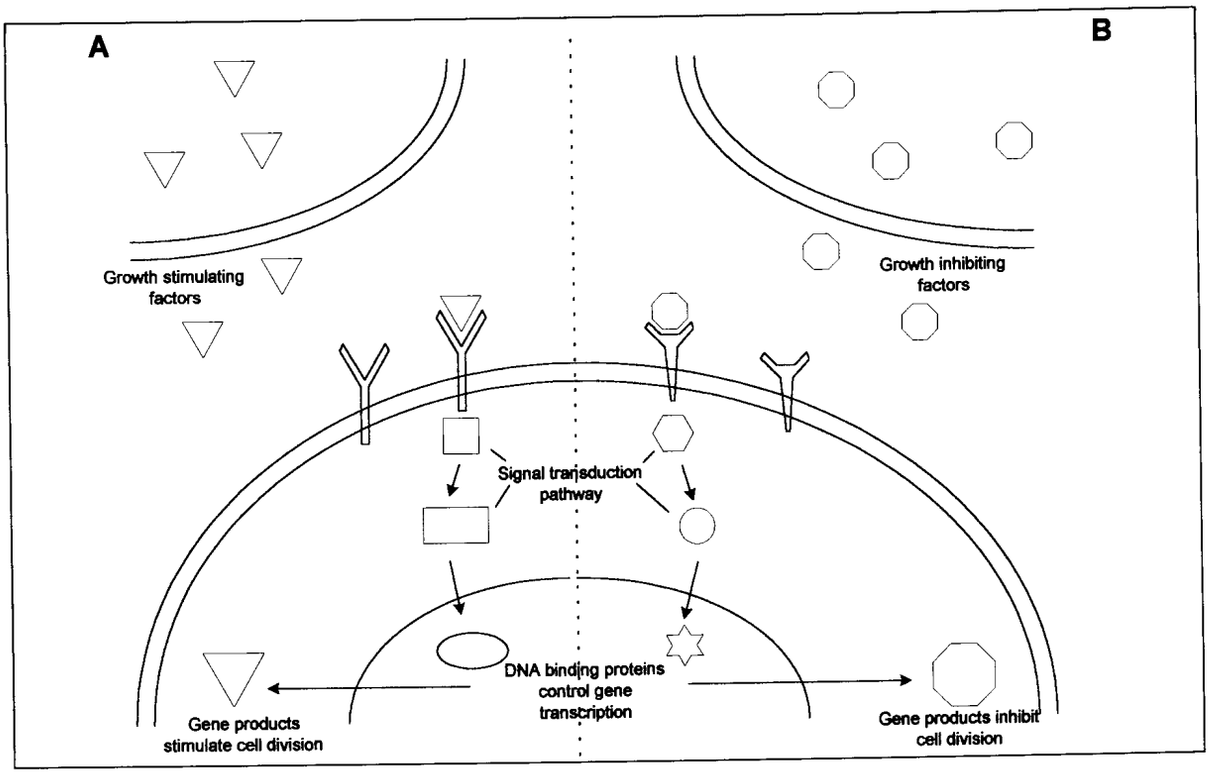


Figure 1.1: **Signal transduction pathway: A) positively regulated by proto-oncogenes B) negatively regulated by tumour suppressor genes** (Adapted from Cavenee & White, 1995; Van Rensburg, 1997)

Germline mutations in tumour suppressor genes seem to be closely linked with inherited predisposition to cancer. Familial cancer syndromes represent germline cancer susceptibility where an individual at birth may have a mutation in a specific gene. The cells still behave essentially normal. They are, however, one step

closer to malignancy than in the normal situation and hence a greater risk applies (Bishop, 1991; Easton, 1994). The cancer syndrome FAP is associated with three mutated tumour suppressor genes, namely *APC, DCC* and *p53* as compared to one changed oncogene, k-*ras* (Fearon & Vogelstein, 1990; Kinzler & Vogelstein, 1996). In most cases the APC gene carries a germline mutation. Affected individuals develop adenomatous polyps during their second and third decades of life. Some of these benign tumours will progress to invasive lesions. The median age of cancer diagnosis in untreated FAP patients is 42, 25 years earlier than the median age of sporadic colorectal cancer patients (Kinzler & Vogelstein, 1996).

Mismatch repair (Mutator) genes:

Mismatch repair genes are responsible for maintaining the integrity of the genome and the fidelity of information transfer. Most of these gene products act as enzymes that can recognize and correct mutations that occur during the normal DNA replication or due to environmental factors such as smoking and ultraviolet exposure. When these repair genes are perturbed, an accumulation of mutations in tumour suppressor genes and other growth-regulatory genes is observed. This may lead to an increase in mutation rate, thus inducing genomic instability, as in the case of hereditary non-polyposis colorectal cancer syndrome (HNPCC) (Loeb, 1991; MacDonald & Ford, 1997; Van Rensburg, 1997). This phenomenon is known as microsatellite instability (MI) or microsatellite mutator phenotype. Microsatellite DNA sequences are polymorphic, short-tandem repeats distributed throughout the genome. These repeats are particularly susceptible to mismatch repair alterations, but they are predominantly located in non-coding DNA sequences. Some repeats such as mononucleotide repeats are occasionally located within the coding sequence of important genes for example transforming growth factor β receptor type II (*TGF-βRII*), BCL-2-associated X protein (*BAX*), insulin-like growth factor II receptor (*IGFIIR*), and *hMSH3* and *hMSH6* that are mutated in colon and gastric cancers. These genes are involved in signal transduction pathways, DNA repair or apoptosis (Ouyang *et al.*, 1997; Ouyang *et al.*, 1998; Catasus *et al.*, 2000).

Mutations in the *hMSH2*, *hMLH1* and *hPMS2* mismatch repair genes account for the great majority of HNPCC cases. Adenomas form at approximately the same rate as in the general population. However, adenomas with the mismatch repair deficiency acquires mutations in other genes at a rate two to three magnitudes higher than in normal cells. This leads to a rapid progression to malignancy. Cancer in HNPCC patients occurs at a median age of 42, the same age as in FAP patients. Patients are often affected with tumours in other organs including the stomach, biliopancreatic system, urinary system and the endometrium. Notebly, endometrial carcinoma is the most common type of extracolonic malignancy in women of HNPCC families (Lynch *et al.* 1985; Kinzler & Vogelstein, 1996).

1.3 THE GENETICS OF ENDOMETRIAL CARCINOMA

The highest proportion of endometrial carcinoma cases is somatic. Hereditary endometrial carcinoma is generally associated with HNPCC. The specific genes involved in endometrial carcinoma can be divided into the three groups of genes known to be cancer causing.

1.3.1 Mismatch repair (Mutator) genes

MI has been detected in 75 % of endometrial cancer associated with HNPCC, but also in 17 % to 28 % of sporadic endometrial cancers (Risinger *et al.*, 1993; Burks *et al.*, 1994; Gurin *et al.*, 1999; Lax *et al.*, 2000). It is present in all the grades of endomterial carcinoma. It is also more frequent in EEC (28 % to 33 %) (Catasus *et al.*, 1998a; Lax *et al.*, 2000) than in non-endometrioid endometrial cancers (0 % to 11 %) (Catasus *et al.*, 1998a; Lax *et al.*, 2000). These tumours have either mutations in mismatch repair genes such as *hMLH-1, hPMS1, hMSH-2*, and *hPMS2* or hypermethylation of the *hMLH-1* promoter, leading to the disruption of DNA mismatch repair. MI was reported in the *hMLH1* and *hMSH2* genes in 25 % of endometrial tumour cases but the somatic mutations in coding region mononucleotide repeats in the *BAX, IGFIIR, TGF-βRII, hMSH3, hMSH6, BRCA1* and *BRCA2* genes were rare (Gurin *et al.*, 1999). However, in other

studies frameshift mutations were detected in *BAX* (12 % to 45.8 %), *IGFIIR* (12.5 % to 25 %) and *hMSH3* (25 %) (Ouyang *et al.*, 1997; Ouyang *et al.*, 1998; Catasus *et al.*, 2000). Catasus *et al.* (1998b) detected *BAX* frameshift mutations in 54 % of MI^+ endometrial tumours, but none in MI^- neoplasms. There were no significant differences between endometrial carcinomas with and without MI with regard to age of presentation, stage, evidence of estrogenic stimulation, estrogen receptor levels, c-erbB2 or p53 immunostaining (Caduff *et al.*, 1996; Gurin *et al.*, 1999).

Methylation of normally unmethylated CpG islands in the gene promoter regions may cause progressive inactivation of tumour suppressor genes or genes involved in DNA repair (Jones & Laird, 1999). It has recently been shown that the hypermethylation of the 5' CpG island of the *hMLH-1* promoter may be an early event in the pathogenesis of EEC, preceding the development of MI (Esteller *et al.*, 1998; Esteller, *et al.*, 1999). Esteller *et al.* (1998) reported hypermethylation in *hMLH1* in 45 % of EECs (92 % of MI^+ tumours and 6 % of MI^- cancers). Hypermethylation of *hMSH2* was not observed. Although the promotor methylation phenomenon is true for MI^+ colonic and gastric cancers, the mechanisms involved in MI^+ endometrial cancers do not necessarily follow the same process as some findings suggest. Colonic and gastric cancer with MI show correlation with promotor inactivation due to altered methylation, in genes *p16* (60% to 70 %), estrogen receptor α, *hMLH-1* (60 %) and insulin-like growth factor-2 (*IGF-2*) (60 %) (Ahuja *et al.*, 1997; Toyota *et al.*, 1999a; Toyota *et al.*, 1999b). No correlation could be found between promotor hypermethylation of genes *p16* and estrogen receptor α, and MI in endometrial carcinoma (Navari *et al.*, 2000; Salvesen *et al.*, 2000). Hypermethylation of the *hMLH-1* promoter region was identified in 71 % of MI^+ endometrial carcinoma cases by Gurin *et al.* (1999).

1.3.2 Oncogenes

The most commonly mutated proto-oncogene in endometrial carcinoma is k-*ras*. It is mutated in 10 % to 30 % of endometrial cancers, and mainly the endometrioid type (Enomoto *et al.*, 1990; Sasaki *et al.*, 1993; Varras *et al.*, 1996;

Lax *et al.*, 2000). Point mutations of the Ki-*ras* gene occur in low grade as well as high grade endometrial tumours, with the second nucleotide of codon 12 most commonly involved (Enomoto *et al.*, 1990; Ignar-Towbridge *et al.*, 1992; Sasaki *et al.*, 1993; Duggan *et al.*, 1994, Varras *et al.*, 1996; Lax *et al.*, 2000). A higher frequency of mutations in MI^+ endometrial cancers (42.8 %) has been observed compared to MI^- carcinomas (11.3 %), which seems to indicate that k-*ras* mutations are common in endometrial carcinoma with the microsatellite mutator phenotype. Methylation-related transitions were detected in MI^+ tumours (Lax *et al.*, 2000; Lagarda *et al.*, 2001; Matias-Guiu *et al.*, 2001). Ki-*ras* abnormalities seem to be involved in the hyperplasia-to-carcinoma sequence in human endometrium. The incidence of ras mutations in hyperplasia (16 %) is similar to the total percentage of carcinomas (18 %), and therefore, homogeneously present in pre-malignant (mainly atypical hyperplasia) and malignant endometrial tissues. This indicates that the changes may occur in the earliest clinically detectable stages of abnormal endometrial proliferation (Sasaki *et al.*, 1993; Duggan *et al.*, 1994).

Another oncogene that appears to play a role in the early steps of endometrial tumourogenesis, is the *CTNNB1* gene that codes for β-catenin. Beta-catenin seems to be important in the functional activities of E-cadherin and APC. It is a component of the E-cadherin-catenin unit that is essential for maintenance of normal tissue architecture, cell differentiation and cell growth. The main role of β-catenin in this unit appears to be to connect E-cadherin with α-catenin. It also plays a role in signal transduction by producing transcriptional activation through the β-catenin – T cell transcription factor pathway. APC and E-cadherin compete for mutually exclusive associations with β-catenin, although the binding sites are different (Kinzler & Vogelstein, 1996; Ilyas *et al.*, 1997; Morin *et al.*, 1997). The frequency of *CTNNB1* mutations in EEC ranges from 13 % to 44 % and mutations appear to be independent of MI. A low incidence of mutations in the *CTNNB1* gene (12.5 %) has been found in atypical hyperplasias. A large proportion of gene mutations are single-base missense mutations on serine/threonine residues, altering the glycogen synthase kinase-3β phosphorylation consensus motif, which

participates in the degradation of β-catenin (Fukuchi *et al.*, 1998; Mirabelli-Primdahl *et al.*, 1999; Saegusa *et al.*, 2001).

Overexpression of the HER-2/neu oncoprotein is associated with increased mortality from persistent or recurrent cancer and is observed in about one third of endometrial tumour specimens. High expression of this gene was found in 27 % of patients with metastatic disease compared with 4 % of patients with endometrial cancer confined to the uterus (Berchuck *et al.*, 1991).

Other specific genetic abnormalities have been found in endometrial adenocarcinoma and include the *c-myc* (Bai *et al.*, 1994) and *erb*B2 oncogenes (Berchuck *et al.*, 1991). In some cases these alterations appear to correlate with prognosis. These alterations, however, do not appear to be useful in the assessment of disease status or as a means of detecting early-stage disease.

1.3.3 Tumour suppressor genes

Mutation and overexpression of the *p53* tumor suppressor gene have been noted in 10 % to 17 % of cancer cases. Increased p53 levels correlated with the spread of disease outside the uterus in 40 % of patients as compared to the 9 % of cases with early stage cancers. Thus, inactivation of *p53* occurs as a later event in endometrial carcinogenesis (Okamoto *et al.*, 1991; Kohler *et al.*, 1992; Lukes *et al.*, 1994; Ambros *et al.*, 1994; Lax *et al.*, 2000). Notebly, p53 overexpression in cases with early and advanced stages of endometrial cancer is significantly lower in white Americans (11 % to 25 %) than African Americans (33 % to 55 %) (Kohler *et al.*, 1996; Clifford *et al.*, 1997). Overexpression of p53 may be one factor that contributes to racial disparity in the histological and survival outcome of the disease (Maxwell *et al.*, 2000).

The most commonly mutated gene identified in endometrial carcinoma is a tumour suppressor gene, *PTEN* (Phosphatase and Tensin homoloque deleted on chromosome 10 (ten)) on the chromosome region 10q23-24. It is also known as *MMAC1* (Mutated in Multiple Advance Cancers 1) or *TEP1* (Transforming Growth Factor-β-regulated and Epithelial Cell-enriched Phosphatase 1) (Steck *et al.*, 1997; Li & Sun, 1997; Li *et al.*, 1997). Somatic *PTEN* mutations have been detected in

26 % to 52 % of EEC in all three grades (Risinger *et al.*, 1997; Kong *et al.*, 1997; Tashiro *et al.*, 1997; Lin *et al.*, 1998; Simpkins *et al.*, 1998; Risinger *et al.*, 1998; Bussaglia *et al.*, 2000; Yaginuma *et al.*, 2000; Sun *et al.*, 2001; Konopka *et al.*, 2002). A concordance between MI status and *PTEN* mutations in EEC seems to exist, with *PTEN* mutations occurring in 60 % to 86 % of MI^+ EEC, but in only 24 % to 35 % of MI^- endometrial carcinomas (Steck *et al.*, 1997; Li & Sun, 1997; Risinger *et al.*, 1997; Kong *et al.*, 1997; Tashiro *et al.*, 1997; Risinger *et al.*, 1998; Bussaglia *et al.*, 2000; Mutter *et al.*, 2000a). Gurin *et al.* (1999) and Mutter *et al.* (2000a), however, could not confirm any relationship between a high *PTEN* mutation frequency and endometrial tumours with MI. Although MI, *PTEN* and k-*ras* mutations coexist in many cases, these alterations are not usually associated with *CTNNB1* changes (Matias-Guiu *et al.*, 2001). Findings also suggest that *PTEN* mutations may be involved directly in the development of atypical endometrial hyperplasia but may not facilitate the progression from hyperplasia to carcinoma (Sun *et al.*, 2001). Between 18 % and 27 % of mainly atypical hyperplasia cases displayed *PTEN* mutations (Maxwell *et al.*, 1998a; Levine *et al.*, 1998; Sun *et al.*, 2001).

CHAPTER 2: *PTEN* GENE AND ENDOMETRIAL CARCINOMA

2.1 INTRODUCTION

PTEN was identified in 1997 as a result of the mapping of the susceptibility gene for Cowden syndrome (CS) to 10q22-23 (Nelen *et al.*, 1996). CS is a rare autosomal dominant familial cancer prone syndrome also known as multiple hamartoma syndrome. Hamartomas are benign tumours containing differentiated but disorganized cells indigenous to the tissue of origin. In CS the multiple hamartomas affect derivates of all three germ layers. Pathognomonic criteria include trichilemmomas (benign tumours of the hair follicle infundibulum), papillomatous lesions and mucosal lesions. Macrocephaly, breast cancer (30 % to 50 % of patients), non-medullary thyroid cancer (10 % of patients) and dysplastic gangliocytoma of the cerebellum (known as Lhermitte-Duclos disease) are considered major criteria. Minor criteria include benign thyroid tumours, fibrocystic breast disease, gastrointestinal hamartomatous polyps, lipomas, fibromas and mental retardation (Starink *et al.*, 1986; Eng *et al.*, 1994). CS is a disease of adulthood where patients diagnosed with CS carry a lifetime risk for breast, thyroid and endometrial neoplasias (Starink *et al.*, 1986; Eng *et al.*, 1994; Nelen *et al.*, 1996). Since the initial identification of the *PTEN* gene, germline mutations have been identified in 80 % of probands with CS (Liaw *et al.*, 1997; Marsh *et al.*, 1998). It is estimated that 10 % to 50 % of CS cases are familial (Marsh *et al.*, 1999). The clinical spectrum of disorders that are associated with germline *PTEN* mutations has expanded to include seemingly disparate syndromes such as Bannayan-Riley-Ruvalcaba (BRR) syndrome (also known as Bannayan—Zonana syndrome) and Proteus syndrome (PS) (Marsh *et al.*, 1997; Arch *et al.*, 1997; Longy *et al.*, 1998; Marsh *et al.*, 1999; Smith *et al.*, 2002). BRR is also a predisposing syndrome, which presents most commonly in childhood. Identifying criteria includes macrocephaly, multiple lipomas, intestinal hamartomatous polyps, haemangiomas and speckled penis. Syndromes that are characterized by the presence of germline *PTEN* mutations may be grouped by

molecular definition and referred to as the *PTEN* hamartoma-tumour syndromes (PHTSs). PS and Proteus-like syndrome, with CS and BRR, may be considered PHTSs. The mosaic distribution of affected tissues in PS and its sporadic occurrence strongly suggest either somatic mutation or germline mosaic mutation in its etiology (Zhou *et al.*, 2000; Waite & Eng, 2002). PS is a complex disorder comprising malformations and overgrowth of multiple tissues. The disorder is highly variable and appears to affect patients in a mosaic way (Biesecker *et al.*, 1999; Zhou *et al.*, 2000). Some major PS criteria for diagnosis are disproportionate overgrowth of limbs, skull and vertebrae, connective tissue nevus, dysregulated adipose tissue and vascular malformations.

2.2 GENOMIC STRUCTURE AND cDNA OF THE *PTEN* GENE

The *PTEN* gene is composed of 9 exons spanning 128 336 bp of genomic DNA. The fourth ATG triplet in the sequence of exon 1 predicts a coding region of 1 212 base pairs (bp) (including the stop codon) for the polypeptide. This encodes a 403 amino acid protein with coding exons ranging in size from 44 to 239 bp (Table 2.1) (Steck *et al.*, 1997).

Table 2.1: The *PTEN* exons and corresponding codons.

Exon	Size of exon (bp)	Codon number
1	79	1-27
2	85	27-55
3	45	55-70
4	44	70-85
5	239	85-164
6	142	165-212
7	167	212-267
8	225	268-342
9	186	343-404

A pseudogene (Ψ) closely related to *PTEN* has been mapped to chromosome 9p21 and can be mistakenly identified as a mutant form of *PTEN* (Teng *et al.*, 1997; Dahia *et al.*, 1998; Whang *et al.*, 1998; Yokoyama *et al.*, 2000). The cDNA sequence contains a 1226 bp region with 98 % homology to the entire coding region of functional PTEN (Dahia *et al.*, 1998; Whang *et al.*, 1998). The Ψ*PTEN* seems to be actively transcribed, although at a lower rate than *PTEN,* in a number of tissues such as tissue from the brain, kidney, endometrium and lung (Fujii *et al.*, 1999; Yokoyama *et al.*, 2000). Western blot analysis, however, showed no translated protein of the pseudogene (Yokoyama *et al.*, 2000).

2.3 THE PTEN PROTEIN

2.3.1 Structure and domains

The PTEN protein is a single polypeptide that consists of 403 amino acids resulting in a protein with a predicted molecular weight of 47 122 Dalton and a pI of 5.86 (Steck *et al.*, 1997). PTEN shares sequence identity with the protein tyrosine phosphatases (PTPs) (Denu *et al*; 1996; Tonks & Neel, 1996; Neel & Tonks, 1997). PTEN also shows homology to tensin and auxilin, two cytoskeletal proteins. Tensin binds to actin filaments at focal adhesions and thus connecting the cell's internal skeleton of protein filaments to its external environment whereas auxilin involved in synaptic vesicle transport. The strongest region of homology between tensin and PTEN extends from amino acids 15 to 185 where the homology is 35%. Weaker homology extends to approximately amino acid 300 (Li *et al.*, 1997, Steck *et al.*, 1997; Parsons, 1998).

There are several domains in the protein that contribute to the activity and stability of PTEN.

N-Terminal Domain

The N-terminal domain (amino acids 1-185) contains the phosphatase domain of PTEN. The N-terminal side of the protein is composed of β-sheets surrounded by

α-helices. Analysis of the crystal structure revealed that PTEN is a unique phosphatase. Although PTEN has a phosphatase domain similar to those of other protein phosphatases, this active site is slightly larger in PTEN. This enlarged site, spanning from residues 90 to 142, allows for the accessibility of phospholipid substrates (Lee *et al.*, 1999). The domain contains a conserved catalytic domain from residues 123-131, which is an exact match for the signature motif HCXXGXXRS/T that defines all tyrosine phosphatases. This conserved catalytic domain is flanked by non-catalytic, regulatory sequences (Table 2.2) (Charbonneau & Tonks, 1992; Tonks & Neel, 1996).

Table 2.2: **The signature motifs of various protein tyrosine phosphatases** (Maehama & Dixon, 1999).

Tyrosine phosphatases	Signature motif
PTEN	H***CK***AG***K***G***R***TG
SopB*	N***CK***SG***K***D***R***TG
IpgD*	N***CK***SG***K***D***R***TG
4-ptase I*	S***CK***SA***K***D***R***TA
4-ptase II*	C***CK***SA***K***D***R***TS

* SopB = *Salmonella dublin* SopB; IpgD = *Salmonella flexneri* IpgD; 4-ptase I = Human inositol polyphosphatase type I; 4-ptase II = Human inositol polyphosphatase type II

The cysteine residue within the catalytic domain is essential for enzyme activity. This catalytic cysteine at codon 124 of PTEN at the centre of the signature core motif is contained in the loop between the β8-strand and α5-helix (Yuvaniyama *et al.*, 1996). The cysteine acts as a nucleophile by attacking the phosphorous atom in the phosphate moiety of its substrate. This results in the formation of a thiol-phosphate intermediate (Barford *et al.*, 1994). The arginine residue in the consensus active-site sequence plays a key role in binding of the phosphoryl group of the substrate and thus contributing to transition-state stabilization. The phosphatase reaction has two steps. The cysteine in the motif will accept the PO_3 moiety from the phosphorylated amino acid and generate a phosphocysteine

intermediate. The histidine preceding the cysteine and the serine/threonine following the arginine lowers the cysteine pK_a. In the next step, the PO_3 moiety is transferred to a water molecule, restoring the enzyme. Both steps are highly dissociative, with bond-breakage to the leaving group occurring faster than bond-formation to the nucleophile (Fauman & Saper, 1996; Denu *et al.* 1998). A third conserved residue in the signature core motif is an aspartic acid that acts as a general acid in the first step, enhancing catalysis by donating a proton to the leaving group oxygen, generating an uncharged hydroxyl (Fauman & Saper, 1996).

Three different research groups demonstrated the significance of the signature core motif for the functioning of PTEN in normal development. They demonstrated that loss of PTEN function could contribute to carcinogenesis. These groups generated *PTEN* -knockout mice. All three groups targeted exon 5, which contains the protein tyrosine phophatase signature core motif (Di Cristofano *et al.*, 1998; Suzuki *et al.*, 1998a; Podsypanina *et al.*, 1999). Suzuki *et al.* (1998a) deleted exons 3 to 5, Di Cristofano *et al.* (1998) deleted exons 4 and 5 and Podsypanina *et al.* (1999) targeted a frameshift mutation within the phosphatase core motif. These groups all reported embryonic death within a range of embryonic days 6.5 to 9.5 in the homozygous *PTEN* -negative mice. Altered ability to differentiate into endodermal, ectodermal and mesodermal derivates was displayed by the embryonic stem cells. In the heterozygous mice, the lack of significant similarity to CS and BRR was interesting, although some hyperplastic changes were seen in the prostate, skin and colon (Di Cristofano *et al.*, 1998). With long follow-up some mice developed breast and prostate cancer, pappilary thyroid tumours, atypical endometrial hyperplasia and/or endometrial cancer (Podsypanina *et al.*, 1999; Stambolic *et al.*, 2000).

Two other conserved areas flank the catalytic core and are necessary for PTEN function. These two α-helix motifs are α2 with the amino acid sequence NNDDVVRFLDS and α7 with sequence RDKKGVTIPSQRRYVYY. Although not directly involved in catalysis, the α2-helix helps with the formation of the overall secondary structure of the enzyme (Stuckey *et al.*, 1994; Yuvaniyama *et al.*,

1996). Mutations in this area result in complete loss or greatly reduced activity of PTEN (Myers *et al.*, 1997). The α7-helix is important for coordinating the water molecule necessary for regeneration of the active enzyme (Stuckey *et al.*, 1994; Yuvaniyama *et al.*, 1996).

C-Terminal Domain

The C-terminal domain consists of short α-helices that link antiparallel β-sheets together (Lee *et al.*, 1999). This domain (amino acids 186-403) contains important sub-domains for function that are common to other signal-transduction molecules. These sub-domains include the C2 domain (amino acids 186-351), two PEST domains (amino acids 350-375 and 379-396) and the PDZ domain. The CK2 phosphorylation sites, namely S380, T382 and T383, are situated in the second PEST domain (Figure 2.1) (Waite & Eng, 2002).

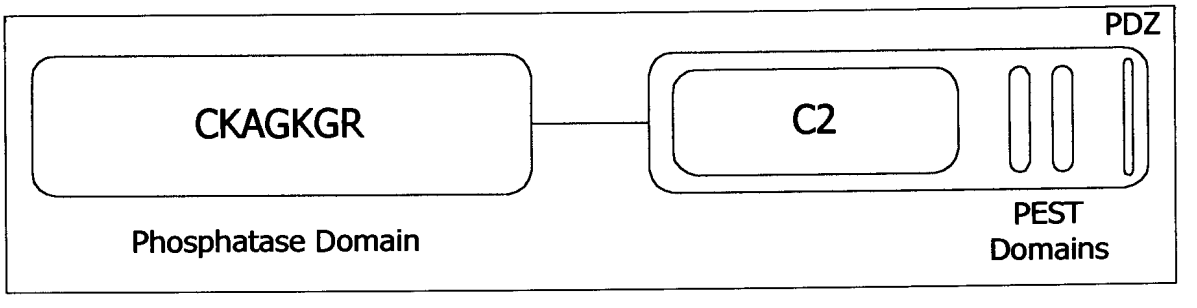


Figure 2.1: **The conserved domains of PTEN** (Waite & Eng, 2002).

The C2 domain, which is associated with lipid-binding regions, has shown to have an affinity for phospholipid membranes (Rizo & Südhof, 1998; Lee *et al.*, 1999). C2 domains have been identified in other proteins involved in signal transduction and membrane localization. Findings suggest that the catalytic domain of PTEN may be positioned on the membrane by the C2 domain (Lee *et al.*, 1999). These intracellular protein modules comprise approximately 130 residues. Its ability to bind phospholipids may or may not be regulated by Ca^{2+}, as Ca^{2+}-dependent and Ca^{2+}-independent forms of phosphatases exist (Rizo & Südhof, 1998). Apart from

the conserved phosphatase domain in exon 5, two potential conserved tyrosine phosphate acceptor sites have also been identified. One site, with sequence RREDFKMY, is at residues 233 to 240 in exon 7 and the other site (RADNDKEY) at residues 308 to 315 in exon 8 (Steck *et al.*, 1997).

Two PEST sequences are essential for the stability of the PTEN protein. These sequences are rich in proline, glutamic acid, aspartic acid, serine and threonine (PEST) residues. Proteins with short intracellular half-lives are targeted by the PEST sequences for proteolytic degradation (Georgescu *et al.*, 1999).

The PDZ domain of proteins contributes to protein stability by interacting with the phosphatase domain. This is done by hydrogen bonding and hydrophobic interactions (Lee *et al.*, 1999). The PDZ domain is also important for protein-protein interactions, which is of vital importance in cellular signal transduction. A great number of proteins with PDZ domains bind very specifically to four or more residues in the C-terminus of other proteins (Fanning & Anderson, 1999). PDZ proteins have been shown to direct the assembly of multiprotein complexes, often at membrane/cytoskeletal interfaces such as synapses (Craven & Bredt, 1998). It has also been shown that PTEN binds to MAGI-2 (membrane associated guanylate kinase inverted-2) through an interaction between the C-terminus of PTEN and the second PDZ domain of MAGI-2. MAGI-2 enhances PTEN stability, thereby increasing the efficiency of PTEN signalling. Removal of the PDZ domain reduces the ability of PTEN to inhibit one of its substrates, AKT (Wu *et al.*, 2000).

Several phosphorylation sites are located in the last 50 amino acids. Although this part of the protein does not play a role in phosphatase activity or cell-growth suppression, it is still critical for protein stability. Protein stability is dependant on the phosphorylation of S380, T382 and T383. When these sites are mutated both the PTEN protein half-life and PTEN levels are reduced (Vazques *et al.*, 2000). Dephosphorylated PTEN seems to be degraded by proteasome-mediated mechanisms (Torres & Pulido, 2001).

PTEN protein levels are also regulated by protein phosphorylation as this causes a conformational change that masks the PDZ domain. This reduces the ability of

PTEN to bind to other PDZ-domain-containing proteins (Vazquez *et al.*, 2000). The phosphatase enzyme that dephosphorylates PTEN has yet to be discovered and identified.

2.3.2 Mechanism of PTEN action

Distinct PTEN cell growth suppression mechanisms are impaired in different types of tumours. These mechanisms include producing G1 cell cycle arrest in some cancers such as glioblastoma cells and inducing apoptosis in a wide variety of carcinomas (Li & Sun, 1997; Myers *et al*, 1998). *PTEN* encodes a protein with two different functions (actions) that can dephosphorylate protein substrates; in addition, it is also able to dephosphorylate cellular phopholipids. It encodes a dual-specific phosphatase because it can dephosphorylate protein substrates as well as lipid substrates. The mechanism determining whether PTEN acts as a lipid or protein phosphatase remains unclear. As a dual-specific protein phosphatase PTEN can dephosphorylate protein substrates on their serine, threonine and/or tyrosine residues (Myers *et al.*, 1997).

PTEN has been implicated in several signal transduction pathways and seems to be involved in the phosphoinositide pathway, the mitogen-activated protein kinase (MAPK) pathway and the focal adhesion kinase (FAK) pathway, using various mechanisms.

Phosphoinositol Pathway

Phosphoinositide second messengers regulate cell growth and differentiation, apoptosis, metabolism, actin re-arrangements and membrane trafficking. These messengers are therefore essential agents in signal transduction pathways contributing to the well being of each cell in an individual (Waite & Eng, 2002). PTEN is a phosphoinositol 3-phosphatase acting as a negative regulator of the PI3K/AKT pathway (Li & Sun, 1997; Li *et al.*, 1997; Steck *et al.*, 1997; Maehama & Dixon, 1998; Stambolic *et al.*, 1998). *AKT*, coding for a protein serine/threonine kinase also known as protein kinase B (PKB), and phosphoinositol 3-phosphate kinase (*PI3K*) are two oncogenes, involved in cell

survival and proliferation, apoptosis and other effects such as cell cycle G1 arrest (Coffer *et al.*, 1998; Stambolic *et al.*, 1998).

Membrane receptors are stimulated by growth/survival factors such as insulin, platelet-derived growth factor (PDGF), nerve growth factor (NGF), insulin-like growth factor 1 (IGF-1) or other mitogens and cytokines. Following stimulation of a receptor such as the insulin receptor (IR) and insulin growth factor receptor (IGFR), signals are transduced in a cascade via the insulin receptor substrate protein (IRS) activating PI3K (Vuori & Ruoslahti, 1994; Kapeller & Cantley, 1994; Franke & Kaplan, 1997; Downward, 1998). This in turn generates the membrane-embedded phosphoinositide second messengers. PI3K does not only catalyses the production of phosphoinositol 3,4,5-triphosphate (PtdIns(3,4,5)P_3 /PIP3), but also phosphoinositol 3-monophosphate (PtdIns(3)P) and phosphoinositol 3,4-diphosphate (PtdIns(3,4)P_2/PIP2) (Auger *et al.*, 1988; Carpenter *et al.*, 1990; Serunian *et al.*, 1990). PtdIns(3,4,5)P_3 accumulates in cellular membranes due to PI3K action and activates AKT that plays a role in cell survival (Figure 2.4) (Coffer *et al.*, 1998; Vazquez *et al.*, 2000). It seems as if PTEN uses a mechanism of dephosphorylation similar to other PTP (Fauman & Saper, 1996; Denu & Dixon, 1998). PTEN exerts its effect upstream from PI3K. Maehama & Dixon (1998) observed that the overexpression of PTEN reduced the cellular levels of PtdIns(3,4,5)P_3 in response to insulin, by removal of phosphate from the D3 position of the inositol ring (Figure 2.2). Increased efficiency of this catalytic process is observed when PTEN associates with MAGI-2 (Wu *et al.*, 2000). Dephosphorylation occurs without affecting the activity of PI3K.

PI3K-dependant activation of AKT is mediated by high-affinity binding of the pleckstrin homology (PH) domain on the N-terminal of AKT to PtdIns(3,4,5)P_2. PtdIns(3,4,)P_3 can also bind with a slightly higher affinity. The PH group recognizes the phosphoinositide headgroup of the phosphatidylinositol phosphates. This results in the translocation of AKT from cytoplasmic stores to cellular membranes. On membrane docking, AKT undergoes a conformational change to expose an activation loop. The catalytic site of AKT is phosphorylated at Thr-308 by PDK1 (PtdIns(3,4,5)P_3-dependent protein kinase 1), another

protein-serine/threonine kinase that also has a PH domain that binds tightly to PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. This stimulates AKT, turning on the protein kinase activity, resulting in subsequent signalling via its downstream effectors such as transcription factors and glucose transporters, resulting in stimulated pathways required for cell survival and proliferation, suppressing apoptosis (Figure 2.2) (Vanhaesebroeck *et al.*, 1997; Franke & Kaplan, 1997; Coffer *et al.*, 1998). Activation of PI3K/AKT signalling promotes the phosphorylation and loss of function of pro-apoptotic targets and augments the function of anti-apoptotic targets. AKT compromises the function of factors such as BAD, human protease caspase-9, glycogen synthase kinase 3 (GSK3) and cyclin-dependent kinase (CDK) inhibitors $P27^{KIP1}$ and $p21^{WAF1}$ promoting cell proliferation, cell survival and cell cycle progression. Cell death pathways are also targeted. (Cross *et al.*, 1995; Datta *et al.*, 1997; Diehl *et al.*, 1998; Cardone *et al.*, 1998). The PI3K/AKT pathway also activates ribosomal protein-serine/threonine kinase $p70^{S6\ kinase}$, the Mdm2-p53 pathway and hypoxia-induced factor-1α (HIF-1α) which after a cascade of events will result in cell growth, angiogenic gene expression and inhibition of p53 tumour suppressor activity (Figure 2.2) (Chung *et al.*, 1994; Mazure *et al.*, 1997; Prives, 1998; Wang *et al.*, 1999; Zundel *et al.*, 2000; Mayo & Donner, 2001; Mayo *et al.*, 2002).

PTEN expression results in a decreased translocation of AKT to cellular membranes and causes down regulation of AKT activation by counteracting PI3K (Davies *et al.*, 1997; Davies *et al.*, 1998; Myers *et al.*, 1998; Weng *et al.*, 1999b). Through the regulation of PtdIns(3,4,5)P_3, one may also speculate that PTEN will play a role in the regulation of other PH-domain containing proteins such as phospholipase-C (PLC). PLC is activated following PtdIns(3,4,5)P_3 production by PI3K. PLC activation leads to the hydrolysis of PtdIns(4,5)P_2, forming the second messengers inositol(1,4,5)P_3 and diacylglycerol. This consequently leads to Ca^{2+} release (increased cellular calcium) from the intracellular stores such as the endoplasmic reticulum, as well as protein kinase C activation by PDK1 (Cantley & Neel, 1999; Besson *et al.*, 1999).

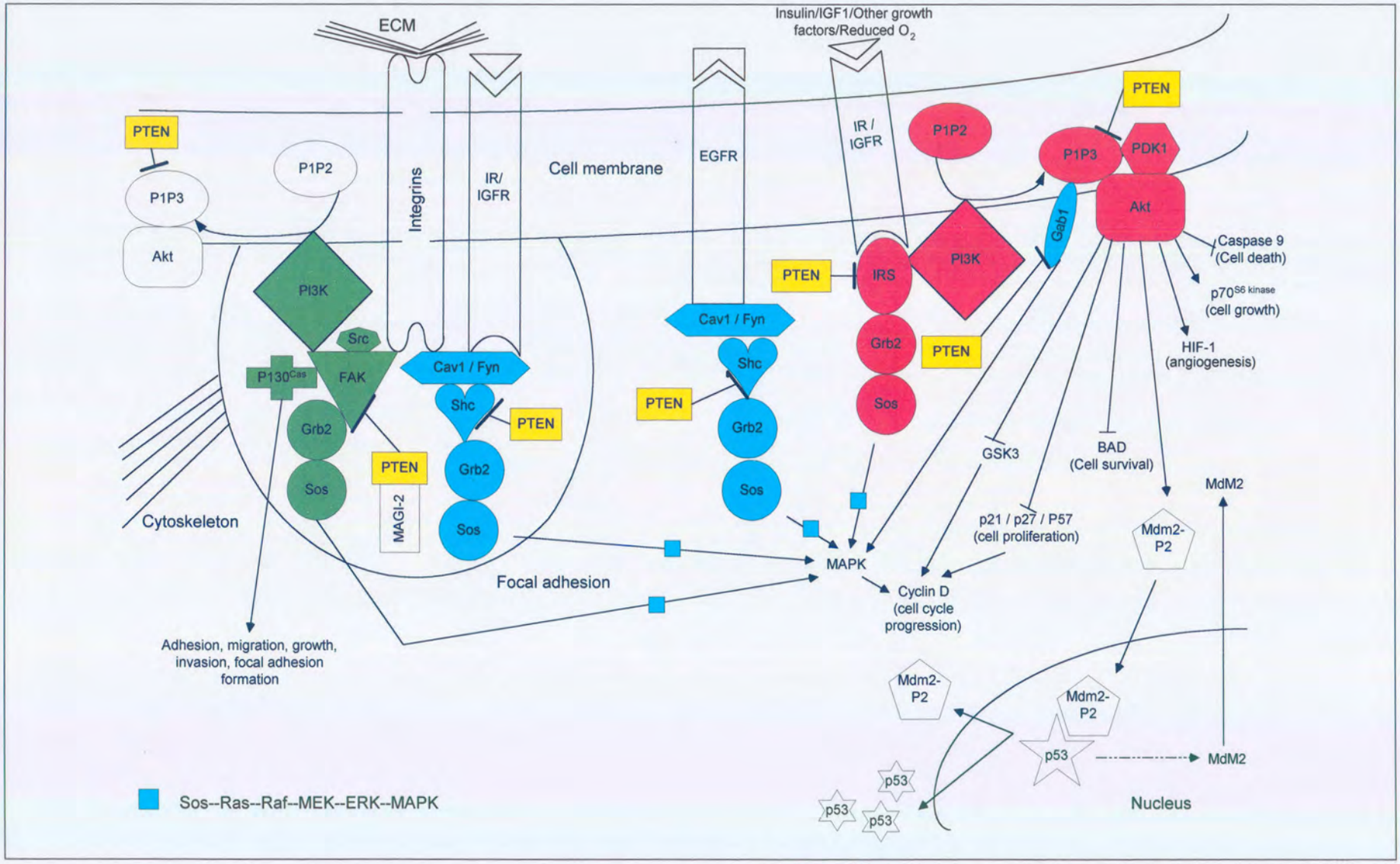


Figure 2.2: **A schematic model of the regulation of the PI3K, MAPK and FAK pathways by normal PTEN.**

MAPK Pathway

PTEN can inhibit the MAPK pathway that is critical for cell proliferation and differentiation. This can be done in either a Shc-dependent or Shc-independent manner (Figure 2.2). Shc is an SH2 (Src homology-2) phosphotyrosine-binding adapter protein that is phosphoralyzed following insulin, insulin growth factor or epidermal growth factor (EGF) stimulation (Gu *et al.*, 1998) *via* the IR, IGFR or the epidermal growth factor receptors (EGFR).

In the Shc-dependent pathway, Shc links Fyn tyrosine kinase to Ras signalling by recruiting the Grb2-Sos (growth factor receptor-bound protein 2 - Ras guanosine 5'-triphosphate exchange factor mSOS) complex. This is done by the membrane adaptor caveolin-1 (Cav-1) that links integrin α subunit to Fyn. Fyn is activated and binds, *via* its SH3 (Src homology-3) domain, to Shc. This sequence of events is necessary to couple integrins to the ras-ERK-MAPK pathway and promote cell cycle progression (Wary *et al.*, 1998). PTEN dephosphorylates the tyrosine of Shc, which results in the inhibition of Grb2 and Ras activity and ultimately, the down-regulation of MAPK, a serine/threonine kinase (Gu *et al.*, 1998; Weng *et al.*, 2001; Waite & Eng, 2002).

PTEN can regulate the MAPK pathway independent of Shc in two ways. First, PTEN by its tyrosine dephosphorylation activity may modulate the movement of Gab1 to the plasma membrane. Gab1 is an adapter molecule that transmits signals to ras-extracellular signal-regulated kinase (ERK)-MAPK for the cytokine receptor gp130. Gab1 forms a complex with PtdIns(3,4,5)P_3 *via* its pleckstin-homologous domain. When PTEN dephosphorylates PtdIns(3,4,5)P_3, Gab1 would be prevented from translocating to the membrane, thus decreasing the activation of MAPK (Takahashi-Tezuka *et al.*, 1998; Yart *et al.*, 2001; Ong *et al.*, 2001). Secondly, MAKP activation is prevented by inhibiting the insulin stimulation mechanism of the MAKP pathway. This inhibition results from the dephosphorylation of the insulin-receptor substrate-1 (IRS-1), which inhibits the formation of the IRS-1/Grb2/Sos complex that is required for MAPK activation, which leads to downregulation of cyclin D1, inhibition of cell cycle progression and suppression of cell growth (Weng *et al.*, 2001).

FAK pathway

Focal adhesions contain integrins, Src protein tyrosine kinases, paxillin, vinculin, tensin, FAK and localized phosphotyrosine- and growth factor receptors (Li *et al.*, 1997; Steck *et al.*, 1997; Li & Sun, 1997). This led to the hypothesis that PTEN may affect integrin function on the cytoskeleton. Integrins are major adhesion- and signalling cell surface protein receptors that are responsible for anchoring cells to the extracellular matrix (ECM). They also regulate intracellular signalling processes involved in migration, invasion, proliferation, differentiation and survival of normal and tumour cells (Clark & Brugge, 1995; Schwartz, 1997; Giancotti & Ruoslahti, 1999). The integrin family is composed of pairs of α and β membrane subunits to form more than 20 different αβ receptor complexes on the cell surfaces. Each unit contributes to ligand selectivity of the integrin (Clark & Brugge, 1995; Schwartz, 1997; Yamada & Geiger, 1997; Giancotti & Ruoslahti, 1999). Peptides derived from the β integrin cytoplasmic domain bind to the N-terminal of FAK. FAK may also interact with integrins through interactions with proteins such as paxillin and talin, that involve the focal adhesion-targeting region of the FAK C-terminal domain. This may contribute to cytoskeletal formation and signal transduction (Clark & Brugge, 1995; Schaller *et al.*, 1995; Hanks & Polte, 1997; Giancotti & Ruoslahti, 1999).

In response to integrin binding to the ECM, it has been noted that FAK binds to other intracellular signalling molecules such as PI3K, Src and Grb2, an adaptor protein. The FAK/Src association can lead to activation of the MAPK pathway through Grb2 binding to FAK. Activation of Src kinase leads to enhanced phosphorylation of FAK which creates a binding motif for Grb2 (Kaplan *et al.*, 1994; Schlaepfer *et al.*, 1994; Schlaepfer *et al.*, 1997; Hanks & Polte, 1997). Both FAK and Shc contribute to the activation of the ERK-MAPK cascade when Shc-linked integrins bind to the ECM (Schlaepfer & Hunter, 1997). The p130Crk-associated substrate ($p130^{Cas}$), a putative downstream target of FAK that promotes cell migration, invasion and adhesion, have been implicated directly in up-regulating cell migration through tyrosine phosphorylation of this molecule as well as FAK (Figure 2.2) (Clark & Brugge, 1995; Parsons, 1996; Hanks & Polte, 1997; Yamada & Geiger, 1997; Cary *et al.*, 1998).

Also, the SH2 domains of PI3K bind to tyrosine 397 of FAK and trigger the activation of PI3K and its signalling pathways (Figure 2.2) (Chen & Guan, 1994; Chen *et al.*, 1996).

Tamura *et al.* (1998) demonstrated that PTEN could significantly reduce cell migration by having effects on integrin-mediated cell spreading or on cytoskeletal processes required for cell spreading. It was also noticed that PTEN could down-regulate actin microfilament (stress fibre) formation, therefore reducing integrin-mediated focal adhesion formation and organization of the actin-containing cytoskeleton in a phosphatase-dependant manner (Tamura *et al.*, 1998). PTEN overexpression leads to change in cell migration and dephosphorylation of FAK. $P130^{Cas}$ is not phosphorylated directly by PTEN (Tamura *et al.*, 1998; Gu *et al.* 1998; Tamura *et al.*, 1999a). PTEN therefore, regulates the FAK structure, thus controlling FAK activity and the consequential regulation of cytoskeleton activity, cell spreading and invasion, cell surface interactions and cell motility.

In summary, PTEN consequently has complex but important effects on signalling and cell biology processes. PTEN has dual functions as a tumour suppressor: first, it helps to regulate apoptosis and growth through its lipid phosphatase activity, which regulates levels of $PtdIns(3,4,5)P_3$, activation of AKT/PKB and the processes of apoptosis; an secondly, it contributes to the regulation of cell adhesion, migration, tumour cell invasion, cytoskeletal organization and MAPK activation through its protein tyrosine phosphatase activity targeting FAK and Shc.

It therefore seems that PTEN acts as a physiological regulator of $PtdIns(3,4,5)P_3$ levels, thereby modulating cell growth and survival. Studies suggest that PTEN can act at two steps – at an upstream site involving FAK that can regulate PI3K activity and directly of $PtdIns(3,4,5)P_3$ itself as a substrate. The effect of PTEN on the AKT/PKB, which plays a key role in apoptosis, also contributes to the explanation of the tumour suppression function of PTEN. Other PTEN targets, such as FAK may also be relevant for tumour suppression. PTEN negatively regulates two distinct pathways regulating cell motility: one involves Shc, an MAPK pathway and random migration, whereas the other involves FAK, $p130^{Cas}$, more extensive actin cytoskeletal

organization, focal contacts and directional persistent cell motility (Gu *et al.*, 1998). Various types of cross-talk between components of these pathways exist (Tamura *et al.*, 1999b).

2.3.3 PTEN expression in tissue

PTEN is expressed in normal tissues such as neural crest/neuroendocrine, gastric, colon, breast, endometrial and other tissues (Weng *et al.*, 1999a; Perren *et al.*, 1999; Mutter *et al.*, 2000b; Yokoyama *et al.*, 2000; Taniyama *et al.*, 2001; Wang *et al.*, 2002; Fei *et al.*, 2002). Weng *et al.* (1999a) and Perren *et al.* (1999) reported uniform expression of *PTEN* in the myoepithelial cells of breast tissue while the level of PTEN protein in the epithelial cells was variable. Mutter *et al.* (2000b) and Yokoyama *et al.* (2000) found that PTEN was a constitutive cytoplasmic protein in the normal endometrium and in endometrial carcinoma.

Endometrial expression of normal PTEN is not constant throughout the menstrual cycle, but changes in response to the hormonal environment (Mutter *et al.*, 2000b). The menstrual cycle is divided into three stages - pre-ovulatory, post-ovulatory and menstrual. During the pre-ovularory stage, also known as the proliferating phase (day 5 to 15), the endometrium regenerates and grows under the influence of estrogen, which is secreted by the developing graafian follicle. After ovulation, the endometrium enters the secretory phase, which is divided into the early secretory (day 16 to 18), midsecretory (day 19 to 24) and late secretory (day 25 to 28) phases. When the corpus luteum begins to degenerate there is a rapid fall in the estrogen level and a sudden decrease in the thickness of the endometrium. Shortly before breakdown of the endometrium the endometrial cells becomes infiltrated by polymorphonuclear leucocytes and this leads to disintegration and haemorrhage. The menstrual phase (day 0 to 4) of the cycle has now been reached. Most of the functional layer is lost during menstruation but the basal layer persists to give rise to the regenerative phase of the next cycle (Fox, 1992; Prat, 1996).

PTEN expression in normal endometrium is ever-present in the estrogenic proliferative phase, but undergoes cell type-specific changes in response to progesterone. The post-ovulatory secretory phase has increased PTEN expression

relative to the estrogenic proliferative phase. Cytoplasmic PTEN protein is maintained in the epithelium of the early secretory phase. However, epithelial cells lose PTEN protein in the midsecretory phase, whereas stromal cells increase PTEN expression, especially in the cytoplasmic compartment. Epithelial PTEN function may be restricted to the mitotically active glandular epithelium. It is interesting that PTEN, with its growth-regulatory activity, has reduced expression in the latter post-ovulatory phase of the menstrual cycle. It is during this phase that progesterone levels are increased, which is known for its anti-neoplastic effects on endometrial tissues. Areas of the endometrium sheltered from cyclical hormone-driven changes like the endometrial basalis have low or absent PTEN levels, which remain stable throughout the cycle. It seems as if the functional requirement for PTEN-mediated tumour suppressor activity might be specific to a highly mitotic estrogenic environment and negated under progestin-dominated conditions that reduce cell division. *PTEN* mutation under unopposed estrogen conditions would then result in a high risk of developing carcinoma (Mutter *et al.*, 2000b).

2.3.4 Regulation of PTEN activity

Although PTEN appears to be constitutively active, the means of regulation of PTEN levels via transcription, translation and posttranslational events remains to be elucidated. PTEN seems to be inactivated upon growth factor stimulation or activated by growth factor depletion. It is interesting that all mammalian PTEN have a PDZ-binding domain at their C-termini that may interact with other biological factors to control expression and localization and activity of PTEN (Maehama & Dixon, 1999, Cantley & Neel, 1999). Vazquez *et al.* (2000) noted that the stability and activity of PTEN could be negatively regulated by CK2 that phosphorylates the C-terminal end of PTEN, mainly the three residues S380, T382 and T383. However, the phosphatase responsible for the phosphorylation of PTEN is still unknown (Waite & Eng, 2002).

It is known that the transcriptional activity of the gene, thus the *PTEN* mRNA levels, is rapidly down-regulated by the presence of transforming growth factor β (Li & Sun, 1997). In contrast, *PTEN* transcription is up-regulated by p53 (Mayo & Donner, 2002).

The presence of a long 5'-UTR may also indicate that *PTEN* expression may be translationally regulated (Steck *et al.*, 1997). DNA methylation could be one mechanism for gene silencing as multiple islands of CpGpG repeats are present in this region. This mechanism has been observed in tumour-specific, CpG methylation-induced transcriptional silencing of several human tumour susceptibility genes (Jones & Laird, 1999).

2.4 *PTEN* MUTATIONS

Loss of heterozygosity (LOH) was reported initially in chromosome 10 in 40 % of primary endometrial carcinomas (Peiffer *et al.*, 1995). Kong *et al.* (1997) and Lin *et al.* (1998) also performed LOH analysis to determine the role of *PTEN* as a classic tumour suppressor gene in endometrial cancer. Results demonstrated 48 % to 55 % LOH on the *PTEN/MMAC1* marker. Kong *et al.*, (1997) assessed LOH at three or more of the following seven loci: D2S123, D9S162, D9S165, S10S215, D10S197, D10S541 and D10S579. Four microsatellite markers were used by Lin *et al*, (1998); two flanking the gene (D10S185 and D10S215) and two intragenic (AFM086wg9 and D10S2491) to the gene. In the majority of samples only one allele had been deleted while the other allele contained point mutations or small deletions.

Mutations that occur in *PTEN* during tumourigenesis fall into three large classes: i) genomic deletions encompassing all or most of *PTEN*, ii) frameshift and nonsense mutations resulting in truncated PTEN proteins and iii) point mutations resulting in the substitution of one amino acid for another.

Various mutations will affect PTEN activity in different ways. Most mutations inactivate both the lipid- and protein-phosphatase action, although a minority results in lipid-phosphatase inactivation (protein-phosphatase activity is maintained). Examples of these two phenomenons are mutations at C124 within the catalytic core which render a lipid- and protein-phosphatase inactive protein, whereas mutations at G129, also in the catalytic core, result in a lipid-phosphatase-inactive yet protein-phosphatase-active PTEN (Li & Sun, 1997; Myers *et al.*, 1998; Maehama & Dixon, 1998; Han *et al.*, 2000; Waite & Eng, 2002). About 90 % of *PTEN* missense mutations eliminate or reduce lipid-phosphatase activity, without affecting the ability

of the protein to bind to the cellular membrane (Han *et al.*, 2000). These mutants demonstrate that the lipid phosphatase activity of PTEN is necessary for cell growth function and cell arrest. It also indicates that protein phosphatase activity (without lipid phosphatase effects) can suppress FAK-mediated cell spreading, migration, invasion and cytoskeletal formation (Tamura *et al.*, 1998; Tamura *et al.*, 1999a). Clinical and genetic analysis has revealed that *PTEN* mutations result in an ever-widening spectrum of phenotypic features.

2.4.1 Germline mutations of the *PTEN* gene

Germline mutations in *PTEN* have been detected mainly in patients affected with CS at an incidence of 64.5 % to 81 % (Lynch *et al.*, 1997; Tsou *et al.*, 1997; Liaw *et al.*, 1997; Marsh *et al.*, 1998; Tsou *et al.*, 1998; Bonneau & Longy, 2000). *PTEN* mutations have been seen in 23.6 % to 57 % of BRR cases (Marsh *et al.*, 1997; Arch *et al.*, 1997; Marsh *et al.*, 1998; Longy *et al.*, 1998; Marsh *et al.*, 1999; Bonneau & Longy, 2000). CS and BRR are allelic disorders at the *PTEN* locus on chromosome 10q (Arch *et al.*, 1997; Longy *et al.*, 1998).

Germline mutations are scattered along the whole gene, with the exception of exon 9. These mutations include missense, nonsense, frameshift and splice site mutations and gross deletions of the gene (Lynch *et al.*, 1997; Marsh *et al.*, 1997a; Tsou *et al.*, 1997; Arch *et al.*, 1997; Liaw *et al.*, 1997; Tsuchiya *et al.*, 1998; Marsh *et al.*, 1998; Tsou *et al.*, 1998; Longy *et al.*, 1998; Marsh *et al.*, 1999). A great number of the mutations (40 %) are found in exon 5, although this exon, which contains the signature core motif, represents only 20 % of the coding sequence. Approximately 23 % to 47 % of the mutations in exon 5 affect this motif, which encompasses codons 123 to 131, directly (Marsh *et al.*, 1998; Bonneau & Longy, 2000). Genotype-phenotype correlation analyses reveal that mutations in exon 5, including those affecting the core motif, significantly result more often in CS phenotype than BRR. The majority of germline mutations result either in truncation or in the total absence of the protein (Celebi *et al.*, 2000). Most of the germline mutations (70 %) have occurred in codons 130, 233 and 235 and can therefore be regarded as mutational hot spots (Bonneau & Longy, 2000). Mutations in these codons occurred in the CpG dinucleotides corresponding to a C→T/G→A transition. However, the

phenotypes associated with the same mutation can vary quite remarkably. This is particularly pronounced for the mutations, R233X, R235X and R335X (Marsh *et al.*, 1997; Marsh *et al.*, 1998; Zhou *et al.* 2000; Waite & Eng, 2000).

2.4.2 *PTEN* somatic mutations

PTEN mutations have been implicated in the development and/or progression of a wide range of sporadic cancers, when perturbed. The highest reported frequency of *PTEN* mutations in any type of primary tumour analyzed to date has been found in endometrial adenocarcinoma with an incidence of 26 % to 52 % (Figure 2.3) (Risinger *et al.*, 1997; Kong *et al.*, 1997; Tashiro *et al.*, 1997; Lin *et al.*, 1998; Simpkins *et al.*, 1998; Risinger *et al.*, 1998; Bussaglia *et al.*, 2000; Yaginuma *et al.*, 2000; Sun *et al.*, 2001; Konopka *et al.*, 2002). *PTEN* alterations have also been observed in other cancers at lower frequencies: brain tumours (15 % to 36 %) (Teng *et al.*, 1997; Liu *et al.*, 1997; Duerr *et al.*, 1998; Boström *et al.*, 1998; Zhou *et al.*, 1999; Davies *et al.*, 1999), prostate cancers (4.5 % to 43 %) (Cairns *et al.*, 1997; Suzuki *et al.*, 1998b; Gray *et al.*, 1998), ovarian endometrioid cancer (0 % to 21 %) (Maxwell *et al*, 1998b; Obata *et al.*, 1998; Lin *et al.*, 1998; Sato *et al.*, 2000), cervical cancer (2 %) (Yaginuma *et al.*, 2000), breast cancer (0 % to 14 %) (Rhei *et al.*, 1997; Steck *et al.*, 1997; Chen *et al.*, 1998), sporadic colon (1.4 %) (Wang *et al.*, 1998), malignant melanoma (0 % to 1.4 %) (Steck *et al.*, 1997; Teng *et al.*, 1997; Guldberg *et al.*, 1997), malignant lymphoma (2.4 % to 4.6 %) (Sakai *et al.*, 1998), head and neck tumours (10.3 %) (Okami *et al.*, 1998) and thyroid cancer (0.8 % to 1.1 %) (Dahia *et al.*, 1997; Bruni *et al.*, 2000).

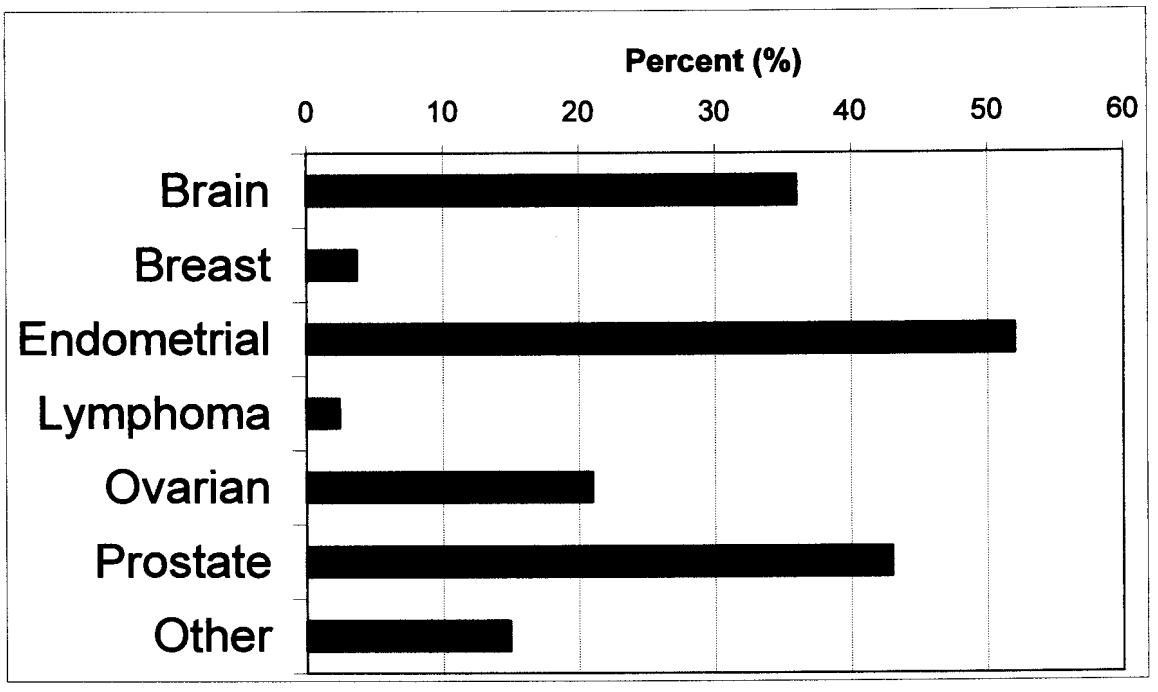


Figure 2.3: **The frequency of *PTEN* mutations in various cancers.**

2.4.3 *PTEN* mutations in endometrial carcinoma

Analysis of tumours such as glial and prostate cancers has shown *PTEN* gene mutations to be associated with high-grade tumours and/or metastasis and therefore, may be important in the progression of malignant tumours. However, mutations occur with equal frequency in all three stages of endometrial cancer with a lower frequency of mutations in hyperplastic tissue. *PTEN* mutations may therefore be an early event in endometrial carcinogenesis before the loss of normal differentiation or histology. Similarly to germline mutations, a great number (24 %) of somatic mutations occur in exon 5. Exon 5, particularly the phosphatase core motif, is also a mutational hotspot in endometrial carcinomas in addition to exons 7 and 8 (22 % and 38 %, respectively) (Bonneau & Longy, 2000; Yuginuma *et al.*, 2000; Bussaglia *et al.*, 2000; Sun *et al.*, 2001; Konopka *et al.*, 2002).

The frequency of *PTEN* mutations differs for MI^+- and MI^--associated endometrial tumours. A higher incidence of alterations was observed in the MI^+ cancer cases (Steck *et al.*, 1997; Li & Sun, 1997; Risinger *et al.*, 1997; Kong *et al.*, 1997; Tashiro

et al., 1997; Risinger *et al.*, 1998; Bussaglia *et al.*, 2000; Mutter *et al.*, 2000a) with the two short coding mononucleotide repeats $(A)_5$ and $(A)_6$ in exons 7 and 8 being targeted (Bussaglia *et al.*, 2000). These mutations are secondary to deficiencies in mismatch repair genes leading to the development of MI (Matias-Guiu *et al.*, 2001).

Two independent studies of patients diagnosed with mainly advanced endometrioid adenocarcinoma revealed a disparity between black and white Americans, where the mutation frequency was 22 % to 34 % in white Americans compared to 0 % to 5 % in black Americans (Risinger *et al.*, 1998; Maxwell *et al.*, 2000). Caucasian Americans therefore, have a 4-fold more frequent incidence of *PTEN* alterations relative to African Americans (Maxwell *et al.*, 2000).

An association of *PTEN* mutation status with clinical outcome has also been shown – patients with *PTEN* mutation-positive endometrial carcinoma had a better prognosis than those without *PTEN* mutations (Risinger *et al.*, 1998). Other complementary prognostic features that correlate with mutated *PTEN* are endometrioid histology, early stage and lower grade. African Americans have a higher frequency of poor prognostic factors such as non-endometrioid, poorly differentiated, advanced staged cancer that is deeply invasive. Their disease-related mortality is significantly higher than white Americans. It is hypothesized that the molecular pathogenesis of endometrial carcinoma within these two distinct ethnic groups may be different (Maxwell *et al.*, 2000). This may also explain the 2 to 2.5-fold difference in ASIR between the African and Caucasian populations in America as well as South Africa.

Carcinogenesis of the endometrium, however, is preceded by pre-malignant hyperplasia. *PTEN* mutations have been detected in endometrial hyperplasias with atypia and without atypia (19 % and 21 % respectively) (Maxwell *et al.*, 1998a). In one study *PTEN* gene mutations were found in 20 % to 27% of patients identified with complex atypical hyperplasia although the analysis was limited to four of the nine exons (exons 3, 5, 7, 8) of the *PTEN* gene (Levine *et al.*, 1998). The findings of Sun *et al.* (2001) somewhat conflicts with the findings of Maxwell *et al.* (1998a). *PTEN* mutations were detected in 18 % of atypical hyperplasias but only 2 % in hyperplasias without atypia. The prevalence of *PTEN* mutations was similar between

atypical hyperplasia and endometrial carcinoma (Sun *et al.*, 2001). Mutter *et al.* (2000a) investigated altered PTEN expression as a diagnostic marker for endometrial precancers. Somatic mutations were found in 83 % of endometrial cancers (associated with unopposed estrogen exposure) and in 55 % of precancers with the same risk factor. It was observed that most hyperplasias and cancers had a mutation in only one *PTEN* allele. A complete loss of PTEN protein expression was found in 61 % of endometrioid adenocarcinoma cases and 97 % showed at least some diminution in protein expression. Results from some studies also suggest than even in the absence of *PTEN* mutations, expression of the PTEN protein may be reduced due to aberrant promotor methylation, an additional mechanism in endometrial carcinoma (Matias-Guiu *et al.*, 2001).

2.5 AIM OF THE STUDY

The aim of this study is to investigate the *PTEN* gene in Caucasians and black African women with endometrial hyperplasia or carcinoma. Specifically, the frequency and nature of the mutations in relation to the pathological features of the disease are assessed.

CHAPTER 3: MATERIALS AND METHODS

3.1 REAGENTS

Chemical reagents were supplied by BDH Chemicals, Ltd. (AnalaR quality) and Merck, unless specified otherwise.

3.2 PATIENTS

The uterine material was obtained from paraffin-embedded tissues from 47 South African patients with endometrial cancer, who underwent hysterectomy at Kalafong Hospital and Pretoria Academic Hospital. These tissue samples were collected for routine diagnostic purposes by the Department of Anatomical Pathology at the University of Pretoria. Ten hyperplastic tissue samples were obtained from the Department of Anatomical Pathology, University of Leuven, Belgium. A pathologist reviewed the histological diagnosis of all cases. The pathological parameters of all the endometrial carcinoma and hyperplasia cases are presented in Appendix A.

3.3 METHODS

3.3.1 DNA Extraction

DNA was extracted from tumour, normal and hyperplastic paraffin-embedded tissues. Tissues were removed from micro-dissection slides. The pathologist indicated normal and cancerous endometrial tissue areas on one slide. This slide was the master slide, used to remove the relevant tissues from the other slides. Tissues were scraped from the slides using a sterile scalpel blade for each new specimen and transferred into sterile micro-centrifuge tubes. Each sample was treated with 200 µl extraction buffer (10mM Tris-HCL, pH 8.0; 0.45% Nonidet P40; 0.45% Tween-20) and 0.2 mg/ml Proteinase K (Roche). After overnight incubation at 55 °C, the enzyme was heat inactivated for 5 min. at 95 °C. The DNA solutions were cooled on ice for 5 minutes, centrifuged and the supernatant transferred to sterile micro-centrifuge tubes. The extracted DNA was used immediately or stored at 4 °C for later use.

3.3.2 Exon-by-exon PCR-SSCP analysis

Polymerase chain reaction (PCR)

The nine exons of *PTEN* including the intronic splice areas were amplified in eleven fragments using the primers described by Davies *et al.* (1999). Primers were optimised using a range of 1.0-3.0 mM $MgCl_2$ concentrations. The annealing temperature (T_{ann}) for each primer pair was calculated as follows:

T_{ann} (primer) = 0.41 (G+C / size of primer) + 34.9 °C (Eeles & Stamps, 1993)

The optimised conditions for each primer pair are summarized in Table 3.1.

Table 3.1: Optimized conditions for *PTEN* primer pairs

Exon	Primer name	Primer sequence*	Product length (bp)	Optimized T_{ann} (°C)	$MgCl_2$ (mM)
1	PTEN 1F PTEN 1R	caagtccagagccatttcc cccacgttctaagagagtga	233	58	2.0
2	PTEN 2F PTEN 2R	ttcttttagtttgattgctg gtatctttttctgtggcttag	239	50	2.0
3	PTEN 3F PTEN 3R	ctgtcttttggtttttctt caagcagataactttcactta	213	50	2.0
4	PTEN 4F PTEN 4R	tataaagattcaggcaatgtt cagtctatcgggtttaagtta	190	50	2.0
5a	PTEN 5AF PTEN 5AR	ttgttaattaaaaattcaagag gcacatatcattacaccagt	217	48	2.0
5b	PTEN 5BF PTEN 5BR	tgaccaatggctaagtgaa aaaagaaacccaaaatctgtt	248	50	2.0
6	PTEN 6F PTEN 6R	cccagttaccatagcaat taagaaaactgttccaataca	275	50	2.0
7	PTEN 7F PTEN 7R	ttgacagttaaaggcatttc cttattttggatatttctccc	264	50	2.0
8a	PTEN 8AF PTEN 8AR	ttcatttctttttctttctttt ggttggctttgtctttctt	238	53	2.5
8b	PTEN 8BF PTEN 8BR	ccaggaccagaggaaac cacatacatacaagtcaccaa	235	56	1.5
9	PTEN 9F PTEN 9R	agtcatatttgtgggtttt ttattttcatggtgttttatc	268	48	3.0

* Primer sequences obtained from Davies *et al.*, 1999

Two rounds of PCR were performed. The first round was a cold run but in the second round additional radioactively labelled primers were used. The first round PCR reactions consisted of 4 µl isolated DNA template, PCR buffer (20 mM Tris-HCl, pH 8.4; 50 mM KCl) (Invitrogen Life Technologies), the appropriate $MgCl_2$

concentration (Table 3.1), 0.25mM of each dNTP (Invitrogen Life Technologies), 2 µg bovine serum albumin (Roche), 4 pmole of each primer (Integrated DNA Technologies) and 0.5 Units of *Taq* DNA polymerase (Invitrogen Life Technologies) in a 20 µl reaction volume. Amplification was performed in a PTC100 thermocycler (MJ Research, Inc.) using the following protocol: 94 °C for 3 min, then followed by 35 cycles of 94 °C for 1 min, appropriate T_{ann} for 1 min, 72 °C for 1 min. The last cycle was followed by a final extension step of 7 min at 72 °C.

5'-End labelling

In preparation for second round PCR the primers were end-labelled with γ-^{32}P ATP. Each reaction contained T4 Polynucleotide kinase (PNK) buffer (50 mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$; 10 mM 2-mercaptoethanol)(Roche), 20 pmole primer, 10 Units T4 PNK (Roche) and 42 µCi γ-^{32}P ATP. Stock γ-^{32}P ATP (7000 Ci/mM) was obtained from ICN Biomedical. Each reaction mixture was covered with mineral oil and incubated at 37 °C for 60 min. The enzyme was then inactivated by incubating at 95 °C for 5 min after which the reactions were cooled on ice. Second round PCR was carried out using 2 µl of first round PCR product in a 10 µl reaction which contained 4 pmole each primer, as well as 0.4 pmole ^{32}P-labelled forward and reverse primer respectively, and 0.5 Units *Taq* DNA polymerase.

MDE gel electrophoresis:

PCR products were diluted 1:10 with formamide loading buffer (95 % formamide; 12.5 mM EDTA, pH 8; 0.25 % bromophenol blue; 0.25 % xylene cyanol), then denatured at 95 °C for 5 min. and quenched on ice. Three µl was loaded onto a 0.5X mutation enhancement detection (MDE) gel (Cambrex Bioscience, Rockland, USA) for single-stand conformation polymorphism (SSCP) analysis. Electrophoresis was carried out in a cold room (4 °C) in 0.6X TBE buffer at 8 Watts for 15 - 17 h (Table 3.2). After electrophoresis, the gels were transferred to 3 MM Whatman paper, dried under vacuum for 2 hours and exposed to medical X-ray film (Fuji Super RX) at –70 °C using an intensifying screen.

Table 3.2: SSCP electrophoresis duration times.

Exon	1	2	3	4	5A	5B	6	7	8A	8B	9
Hours	16	16	15	15	15	16	16	16	15½	16	17

3.3.3 DNA sequencing

Samples that displayed abnormal SSCP patterns were sequenced. Each sample was amplified in a new 20 µl PCR reaction using stock DNA. Prior to sequencing 5 µl of this product was pre-treated with 10 Units exonuclease I and 2 Units shrimp alkaline phosphatase at 37 °C for 15 min and thereafter inactivated at 80 °C for 15 min.

DNA sequencing was performed using the T7 Sequenase v2.0 PCR product sequencing kit (USB, Life Sciences) as prescribed by the manufacturer. The sequenced samples were diluted 1:10 loading buffer (95 % formamide; 12.5 mM EDTA, pH 8; 0.25 % bromophenol blue; 0.25 % xylene cyanol), denatured for 5 min at 75 °C and cooled on ice. Thereafter, 3 µl of each sample was loaded onto a 6 % (19:1) denaturing polyacrylamide gel, containing 7 M urea, in 1X TBE buffer. Electrophoresis was performed at room temperature at 60 Watts in 1X TBE buffer. After electrophoresis, the gels were transferred to 3 MM Whatman paper, dried under vacuum for 2 hours and exposed to medical X-ray film (Fuji Super RX).

CHAPTER 4: RESULTS AND DISCUSSION

4.1 PATIENTS AND TUMOURS

Ten hyperplasias and 47 endometrial carcinomas were investigated. All of the hyperplasia samples were from Caucasian patients (ages unknown) and were atypical, with 20 % (2/10) displaying complex atypical hyperplasia (Appendix A).

Tumour samples were obtained from 32 Black, 14 Caucasian and one Indian woman (Appendix A). The age of the black patients ranged from 43 to 92 (mean age 65 years; SD=11.5) and the white patients from 42 to 82 years (mean age 65 years; SD=13.5). The Indian patient was 47 years at diagnosis. Endometrioid adenocarcinoma was present in 89 % (42/47) of the tumour specimens whereas 4 patients had serous papillary carcinoma (4/47; 9 %) and one had poorly differentiated carcinoma (1/47; 2 %). Three of the four (75 %) patients with serous papillary carcinoma had grade III lesions. The fourth patient had grade II papillary carcinoma. Of the 42 patients with endometrioid adenocarcinoma 7 % (3/42) had architectural/nuclear grade I lesions, 81 % (34/42) had grade II lesions, 7 % (3/42) had grade II-III lesions and 5 % (2/42) had grade III lesions (Table 4.1; Appendix A). According to FIGO staging (Appendix B) 62 % (26/42; 16 Africans and 10 Caucasians) of the endometrioid carcinomas were stage I carcinoma, 10 % (4/42; 3 Africans and one Caucasian) were stage II carcinoma, 24 % (10/42; 7 Africans, 2 Caucasians and one Indian) were stage III carcinoma and 5 % (2/42; 2 African patients) were stage IV carcinoma (Table 4.1; Appendix A and B). Thus, the majority of the endometrioid tumours were early stage cancers (grade I/II; FIGO stage I/II).

Table 4.1: **Grade and FIGO stage of the 42 endometrioid adenocarcinomas.**

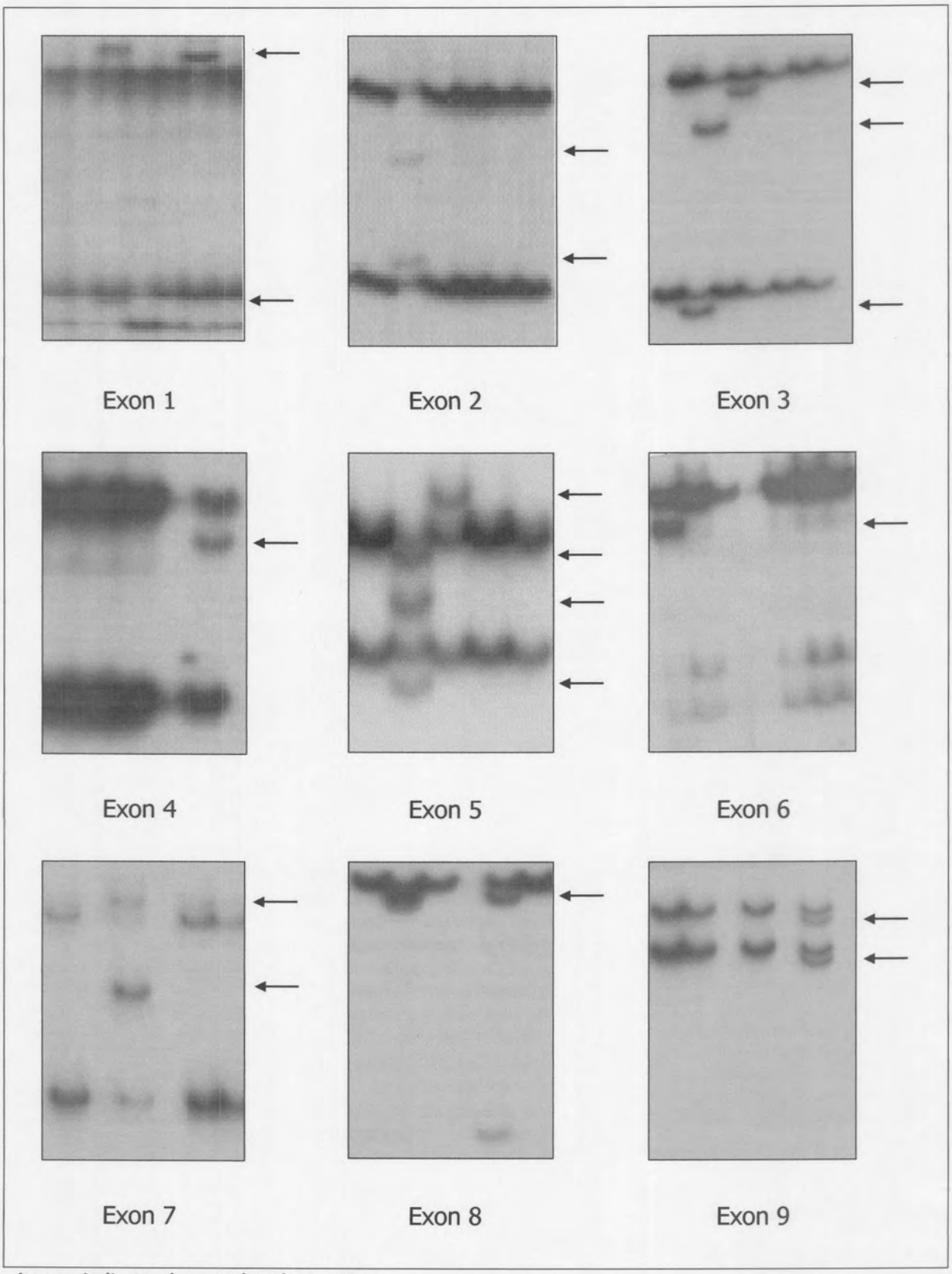
Patients	Architectural grading					FIGO staging				
	I	II	II/III	III	Total	I	II	III	IV	Total
African	2	22	3	1	28	16	3	7	2	28
Caucasian	1	11	0	1	13	10	1	2	0	13
Indian	0	1	0	0	1	0	0	1	0	1
Total	3	34	3	2	42	26	4	10	2	42

4.2 SSCP ANALYSIS

Paraffin-embedded tissue samples from patients with endometrial hyperplasia and cancer were analysed for *PTEN* mutations using exon-by-exon PCR-SSCP. *PTEN* was amplified using primers that included the intronic splice sites, therefore excluding the risk of amplifying the *PTEN* pseudogene.

Twenty % (2/10) of the hyperplasias and 57 % (27/47) of the endometrial carcinomas exhibited aberrant migrating patterns. In the tumours, shifts were observed only in endometrioid adenocarcinomas (64 %; 27/42). None of the serous papillary and weak differentiated carcinomas exhibited any aberrant migrating bands. Representative autographs of SSCP shifts are presented in figure 4.1.

Aberrant migrating samples were sequenced. Five of the adenocarcinomas with shifts (END6, END17, END21, END27, END30) could not be amplified adequately for sequencing and were therefore not included in further analysis. Thus, results from 37 adenocarcinomas were obtained.



Arrows indicate aberrant band migration.

Figure 4.1 **Representative examples of SSCP shifts in the various exons.**

4.3 MUTATIONS IN *PTEN*

Twenty-four different sequence alterations were detected; these include 19 different pathogenic mutations, four mutations of unknown significance and one novel polymorphism. Five of these mutations were observed in more than one tumour.

4.3.1 PATHOGENIC MUTATIONS

Frameshift mutations

Seven different frameshift mutations were detected in 10 endometrioid adenocarcinomas. The sequencing results of these mutations are summarized in table 4.2 and shown in figure 4.2, 4.3 and 4.4.

Table 4.2: Summary of *PTEN* frameshift mutations.

FRAMESHIFT MUTATIONS				
Patient	**Exon**	**Nucleotide change***	**Codon**	**Designation***
END4	1	c.13insA	5	c.13insA
END19	1	c.16delAA	6	c.16delAA
END8	5	c.389delG	130	c.389delG
END34	5	c.389delG	130	c.389delG
END35	5	c.389delG	130	c.389delG
END19	7	c.701delG	234	c.701delG
END26	8	c.863delA	288	c.863delA
END40	8	c.955delACTT	319	c.955delACTT
END43	8	c.955delACTT	319	c.955delACTT
END2	8	c.968insA	323	c.968insA

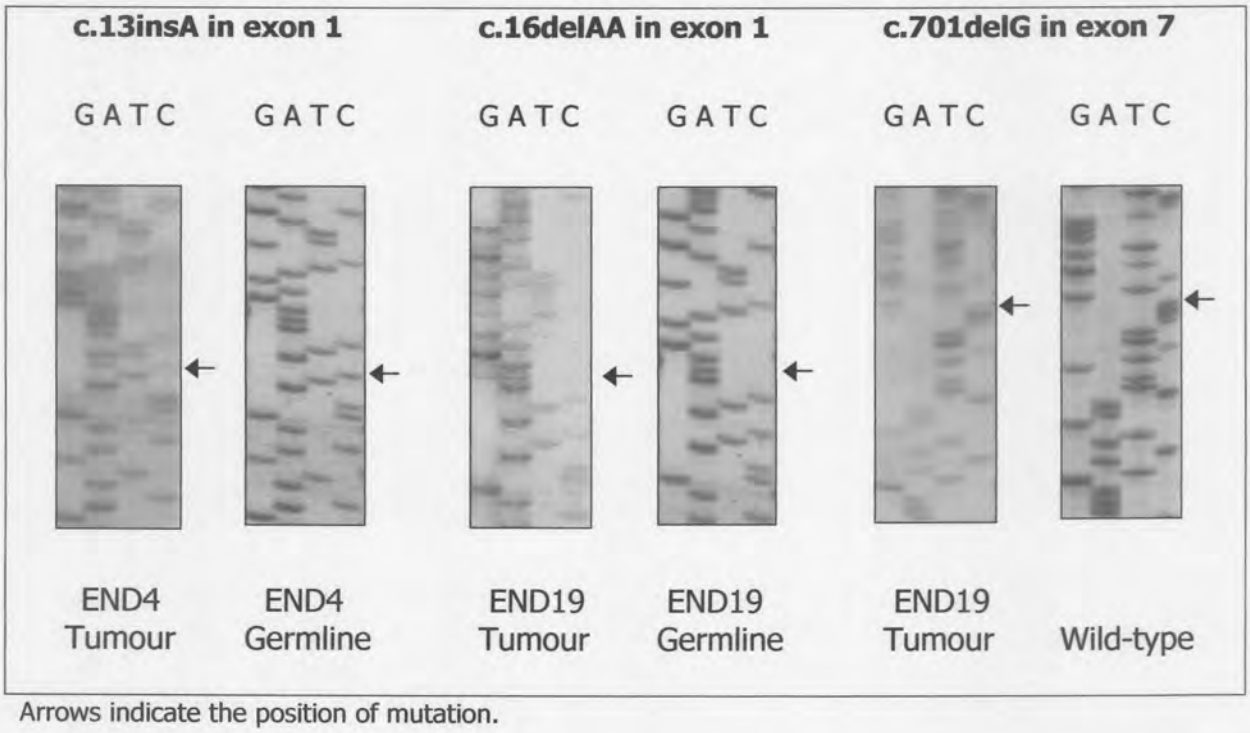
*Recommended nomenclature system (Antonarakis & the Nomenclature Working Group, 1998)

c.13insA in END4:

This patient is a 58-year old Caucasian woman diagnosed with grade II, stage IIb adenocarcinoma of the endometrium. The somatic insertion of an adenosine monophosphate at codon 5 in exon 1 (Figure 4.2) causes a stop at codon 10. Very early termination (near the 5' end) will most likely lead to total absence of mRNA due to instability (Cooper & Krawczak, 1993). This is a novel mutation.

c.16delAA in END19:

The 2 bp deletion (Figure 4.2) in codon 6 (exon 1) leads to a stop at codon 9. This somatic alteration will probably also result in a complete deficiency of PTEN due to the degradation of the very short mRNA (Cooper & Krawczak, 1993). The 53-year old African patient was diagnosed with grade I, stage Ic adenocarcinoma. Konopka *et al.* (2002) reported this mutation in endometrial cancer.



Arrows indicate the position of mutation.

Figure 4.2: **Sequence analysis of frameshift mutations in exons 1 and 7.** (Mutations in exon 1 are indicated on the sense strand and mutation in exon 7 on the antisense strand).

c.701delG in END19:

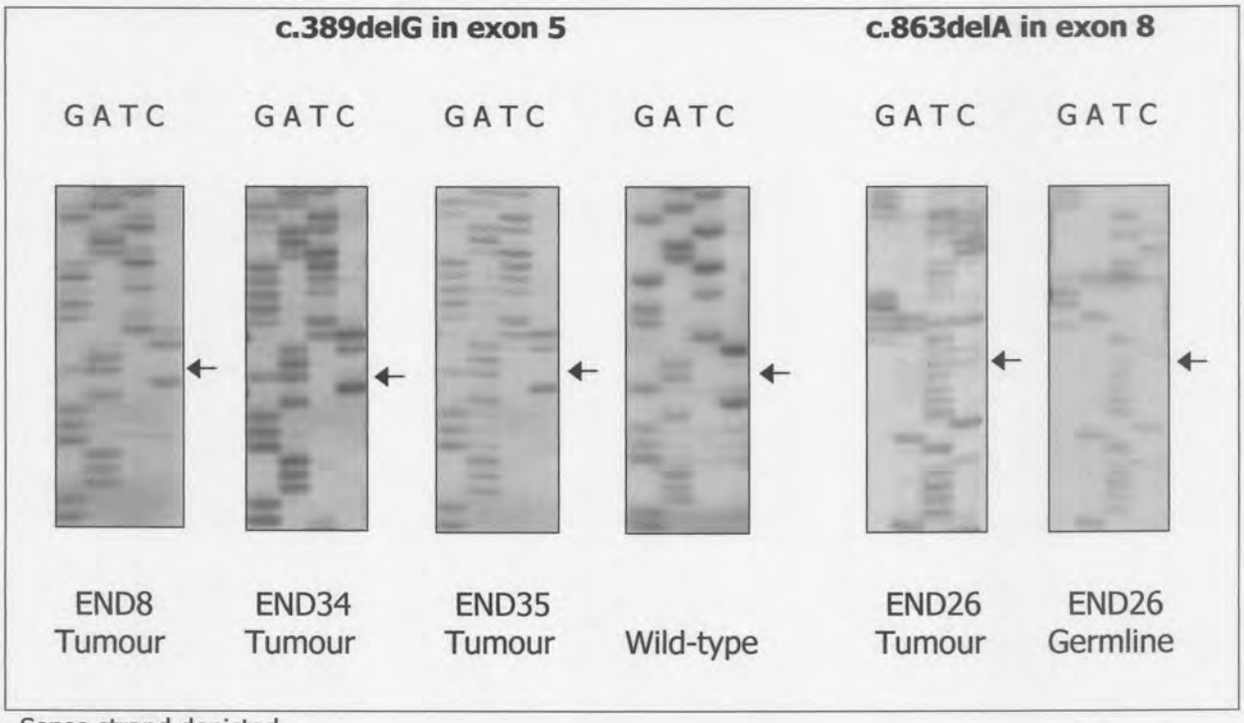
This novel mutation in exon 7, codon 234 (Figure 4.2) occurred in a conserved tyrosine phosphate acceptor site (sequence RREDFKMY - residues 233 to 240) within the C2 domain of the protein. This will affect the ability of the catalytic domain of PTEN to position itself on the lipid membrane (Lee *et al.*, 1999). It could not be determined whether this mutation was germline or somatic, as the germline tissue of this patient could not be amplified adequately for sequencing. This is the second frameshift mutation detected in this patient. The first mutation was c.16delAA in exon 1.

c.389delG in END8, END34 and END35:

The novel frameshift mutation, c.389delG (codon 130) was detected in three patients (Figure 4.3). The mutation was somatic in origin in all three cases. END8 is an Indian patient diagnosed with grade II, stage III carcinoma at the age of 47, END34 is a black African woman, age 57 with grade II, stage III carcinoma and patient END35 is a black African patient that had grade II-III, stage Ic adenocarcinoma at the age of 66. This mutation leads to a stop at codon 133 in exon 5 that results in termination within the phosphatase active centre.

c.863delA in END26:

This 92-year old African patient had grade II, stage Ic adenocarcinoma. The somatic mutation (c.863delA at codon 288) in exon 8 causes a stop in codon 290 (Figure 4.3). This alteration affects the C2 domain of PTEN (amino acid 186 to 351), which would result in a changed ability of PTEN to orientate itself on the membrane for positive activity (Lee *et al.*, 1999). The two PEST domains that contribute to PTEN protein stability are also absent. This mutation has previously been reported in endometrial carcinoma (Tashiro *et al.*, 1997).



Sense strand depicted

Figure 4.3: DNA sequence analysis of mutations in exons 5 and 8.

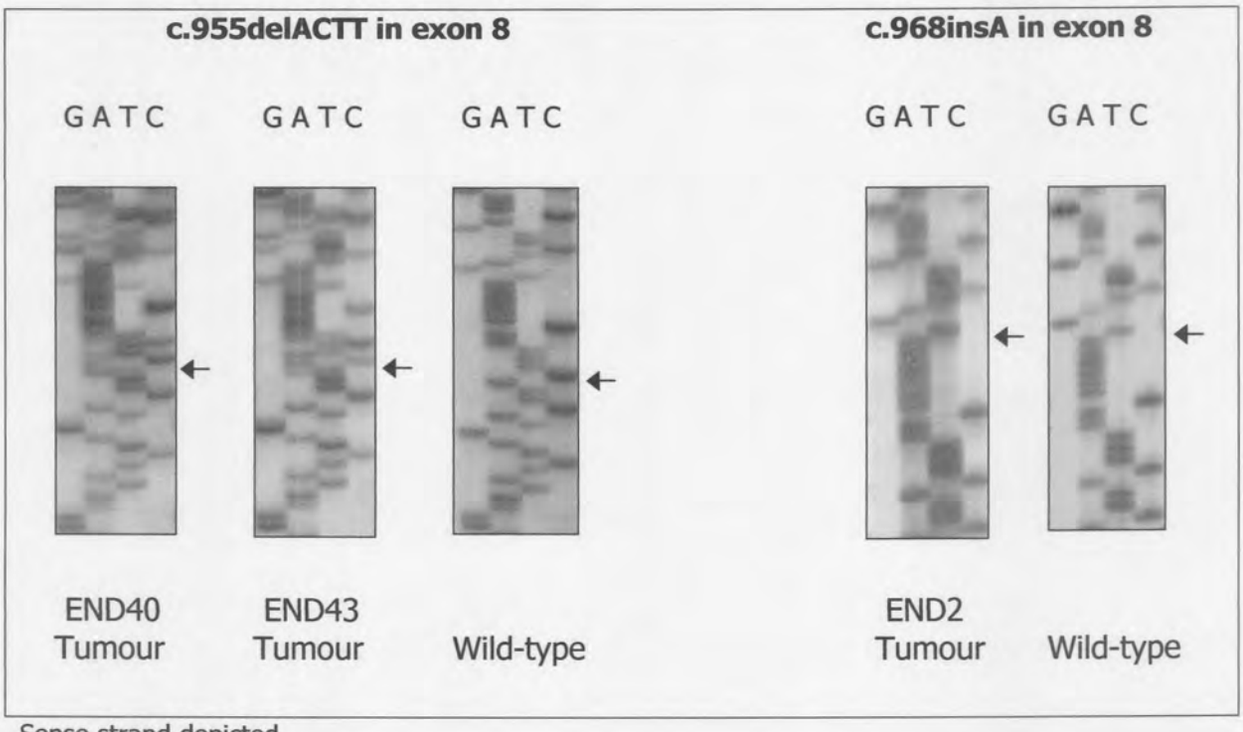
c.955delACTT in END40 and END43:

A 4 bp deletion occurred in two black African patients, END40 and END43. Patient END40, a 66-year old woman was diagnosed with grade II, stage Ib adenocarcinoma and patient END43, a 54-year old woman, with grade II, stage III adenocarcinoma. The deletion occurred in codon 319/320 (exon 8) changing it into a nonsense codon (Figure 4.4). This somatic alteration occurs within the C2 domain, close to a second conserved tyrosine phosphate acceptor site (amino acid sequence RADNDKEY) at codons 308 to 315. The protein-stabilizing PEST domains are also absent. This frameshift has been reported in endometrial carcinoma (Bonneau & Longy, 2000; Sun *et al.*, 2001).

c.968insA in END2:

END2, a Caucasian woman of 82 years old was diagnosed with grade II, stage Ib disease. The frameshift mutation, c.968insA (exon 8) at codon 323 (Figure 4.4)

creates a stop at codon 324 leading to the formation of a truncated protein, lacking the C2 domain of the protein. This is a novel mutation. Sequencing of the normal endometrial tissue to determine whether it is a somatic or germline mutation, was not successful.



Sense strand depicted

Figure 4.4: DNA sequence analysis of mutations in exon 8.

Nonsense mutations

Six different nonsense mutations were detected in seven patients (six adenocarcinoma and one hyperplastic tissue). The sequencing results of the mutations are summarized in table 4.3 and shown in figure 4.5, 4.6 and 4.7.

Table 4.3: Summary of nonsense mutations detected in endometrioid adenocarcinoma and hyperplasia patients.

NONSENSE MUTATIONS				
Patient	**Exon**	**Nucleotide change***	**Codon**	**Designation***
END22	1	c.19G→T	7	E7X
END12	1	c.49C→T	17	Q17X
END10	3	c.176C→A	59	S59X
END11	5	c.388C→T	130	R130X
END22	5	c.388C→T	130	R130X
END9	5	c.445C→T	149	Q149X
EHYP5	7	c.766G→T	256	E256X

*Recommended nomenclature system (Antonarakis & the Nomenclature Working Group, 1998)

E7X in END22:

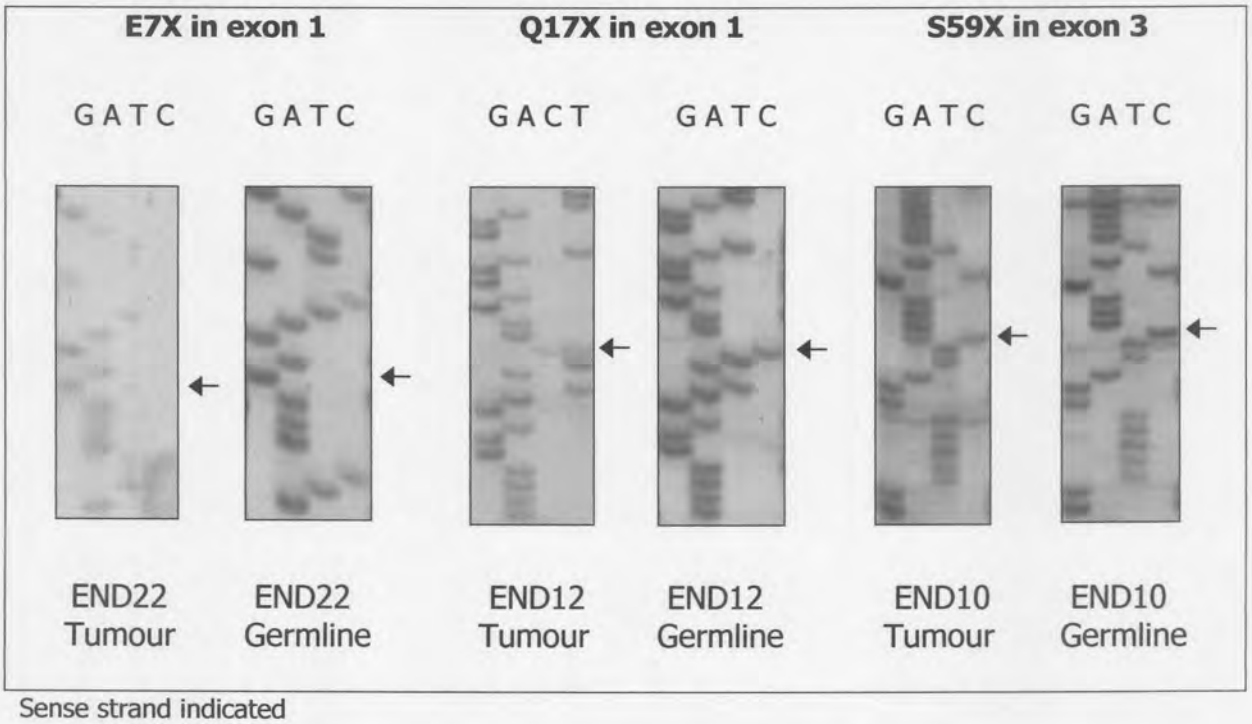
The somatic mutation was detected in a 56-year old African woman with grade II-III, stage Ib adenocarcinoma. This alteration in exon 1 results from a G→T substitution at nucleotide c.19 (Figure 4.5). Due to nonsense-mediated decay of RNA no protein will be produced (Cooper & Krawczak, 1993). Bussaglia *et al.* (2000) reported this mutation in endometrial carcinoma.

Q17X in END12:

Endometrial adenocarcinoma (grade I, stage Ic) was diagnosed in this 72-year old Caucasian woman. This somatic mutation, a C→T substitution (Figure 4.5), occurred at nucleotide c.49 (exon 1) resulting in a stop codon. The mutation had been reported before in endometrial cancer and lymphoma (Simpkins *et al.*, 1998; Dahia *et al.*, 1999).

S59X in END10:

This Caucasian woman was 49 years at the age of diagnosis when she presented with grade II, stage Ia endometrioid adenocarcinoma. The somatic mutation (c.176C→A) occurred in exon 3 (Figure 4.5). Tashiro *et al.* (1997) have reported this mutation in endometrial carcinoma.



Sense strand indicated

Figure 4.5: **DNA sequence analysis of exons 1 and 3 showing three different nonsense mutations.**

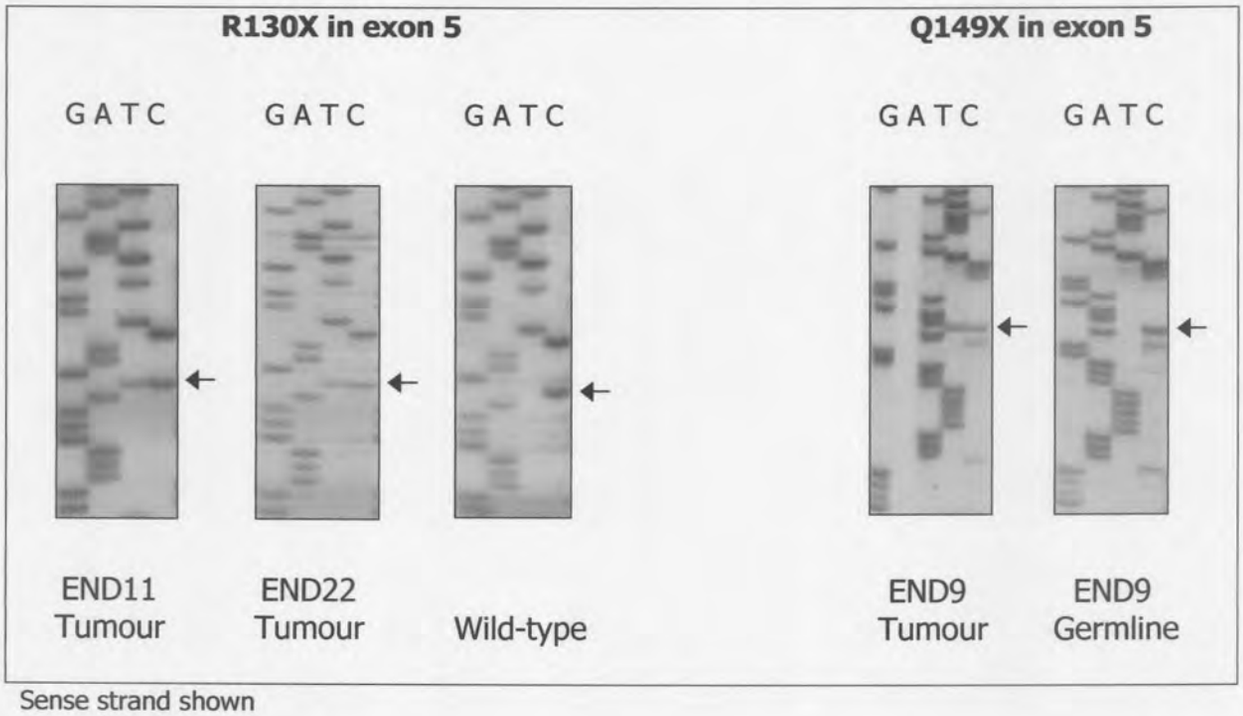
R130X in END11 and END22:

This nonsense mutation was detected in two African patients, END11 and END22, and was somatic in both cases. END 11 is a 65-year old patient with grade II, stage I adenocarcinoma. END 22 is a 56-year old woman with grade II-III, stage Ib adenocarcinoma. The nucleotide change c.388C→T at codon 130 in exon 5 (Figure 4.6) has been reported both as a germline mutation in Cowden syndrome and as a somatic mutation in endometrial carcinoma and hyperplasia (Kong *et al.*, 1997;

Rasheed *et al.*, 1997; Steck *et al.*, 1997; Marsh *et al.*, 1998; Obata *et al.*, 1998; Risinger *et al.*, 1998; Maxwell *et al.*, 1998a; Marsh *et al.*, 1999). This codon, which codes for a conserved arginine residue in the consensus active-site sequence – and thus essential for phosphatase activity - is a mutational hotspot. Various types of mutations in this codon have been observed, all of them rendering the protein completetly inactive (Han *et al.*, 2000). This is the second nonsense mutation observed in patient END22, the first mutation was reported as E7X.

Q149X in END9:

The somatic alteration was observed in a 53-year old African patient diagnosed with grade II, stage III adenocarcinoma. The mutation (c.445C→T) at codon 149 in exon 5 has not been reported before (Figure 4.6).



Sense strand shown

Figure 4.6 Sequence results of the nonsense mutations in exon 5.

E256X in EHYP5:

This novel mutation was observed in the atypical hyperplastic tissue of a Caucasian patient. No normal tissue was available to determine whether this mutation was germline or somatic. The mutation, c.766G→T occurred in exon 7 (Figure 4.7).

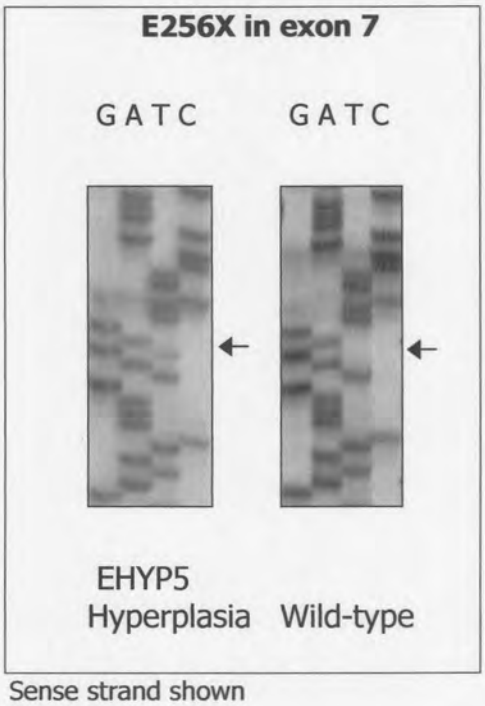


Figure 4.7 **DNA sequence results of the nonsense mutation in hyperplastic tissue.**

Splice site mutations:

Three different splice site mutations were detected in 3 patients with endometrioid adenocarcinoma. The sequencing results are shown in figure 4.8.

IVS4+5G→A in END48:

The novel 5′ donor splice site mutation (AGgtaggta→AGgtagata) in intron 4 was detected in a 78-year old Caucasian woman with grade II, stage Ic adenocarcinoma

(Figure 4.8). The guanosine monophosphate at the position IVS4+5 in the consensus splice site occurs with a frequency of 84 % in genes in general. This mutation, however, results in the unusual presence of adenosine monophosphate in this position, which is generally observed at a low frequency (5 %) (Cooper & Krawczak, 1993). It is therefore likely that this is a pathogenic mutation. Although the most common outcome of 5′ splice donor defects is skipping of the upstream exon (Nakai & Sakamoto, 1994), intron retention has also been observed (Celebi *et al.*, 2000). This alteration could therefore result in the skipping of exon 4 or the inclusion of partial intronic sequences due to the activation of possible cryptic donor sites. Whether this mutation was somatic or germline could not be determined as amplification of the normal endometrial tissue was inadequate for sequencing.

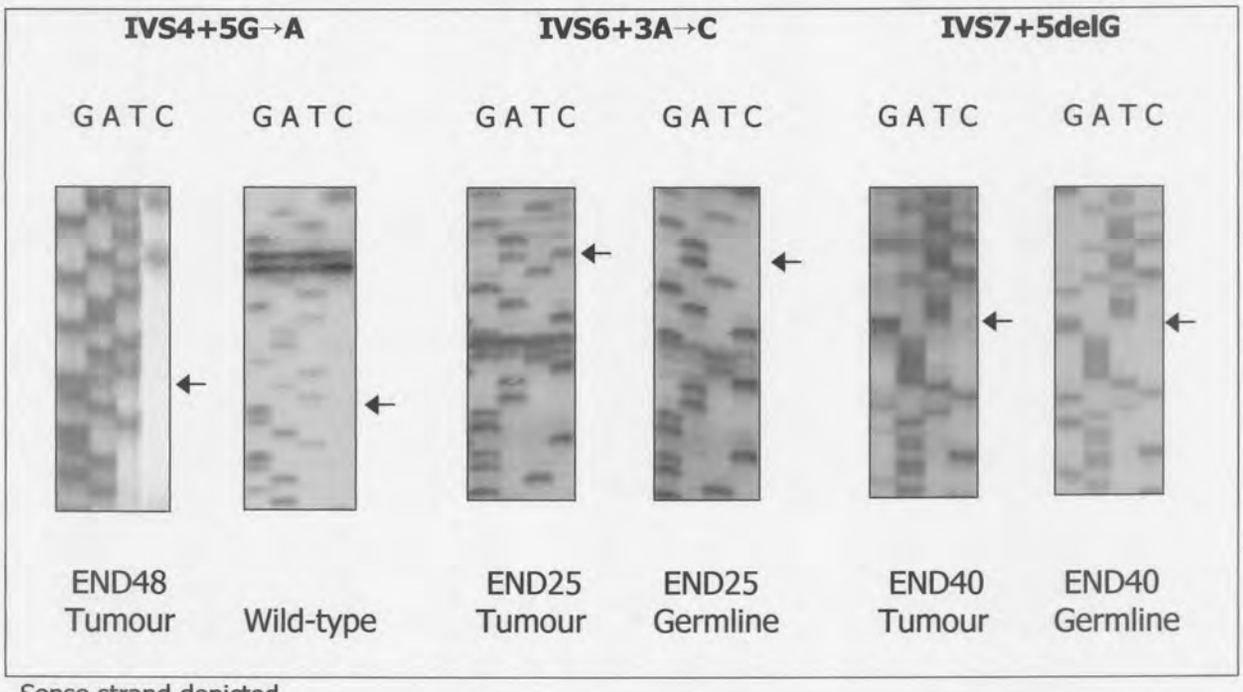
IVS6+3A→C in END25:

A somatic donor splice site mutation IVS6+3A→C (CAgtaa→CAgtca) (Figure 4.8) was detected in the tumour of this 59-year old Caucasian woman, diagnosed with grade III, stage III adenocarcinoma. The adenosine monophosphate at the intronic position +3, which normally occurs with a nucleotide frequency of 57 %, is substituted for a cytidine monophosphate which rarely (2 %) occurs in this position (Cooper & Krawczak, 1993). This alteration may lead to the skipping of exon 6 or the inclusion of intronic sequence, therefore corrupting the PTEN message. The mutation is novel.

IVS7+5delG in END40:

This 66-year old African patient, diagnosed with grade II, stage Ib adenocarcinoma, had a novel somatic mutation in the splice donor of intron 7 due to the deletion of a guanine in the position IVS7+5 (Figure 4.8). This mutation causes the thymidine monophosphate that follows the guanosine monophosphate to move into this position. The guanosine monophosphate occurs with a frequency of 84 % in consensus splice sites, but is replaced with a thymidine monophosphate that generally occurs with a frequency of 2 % in this position (Cooper & Krawczak, 1993). It can therefore be expected that the mutation is disease-causing. The consequence of this mutation may be skipping of exon 7 or the retention of partial intronic

sequence. This is the second mutation detected in this patient. The other mutation is the frameshift c.955delACTT in exon 8.



Sense strand depicted

Figure 4.8: DNA sequence results of splice site mutations.

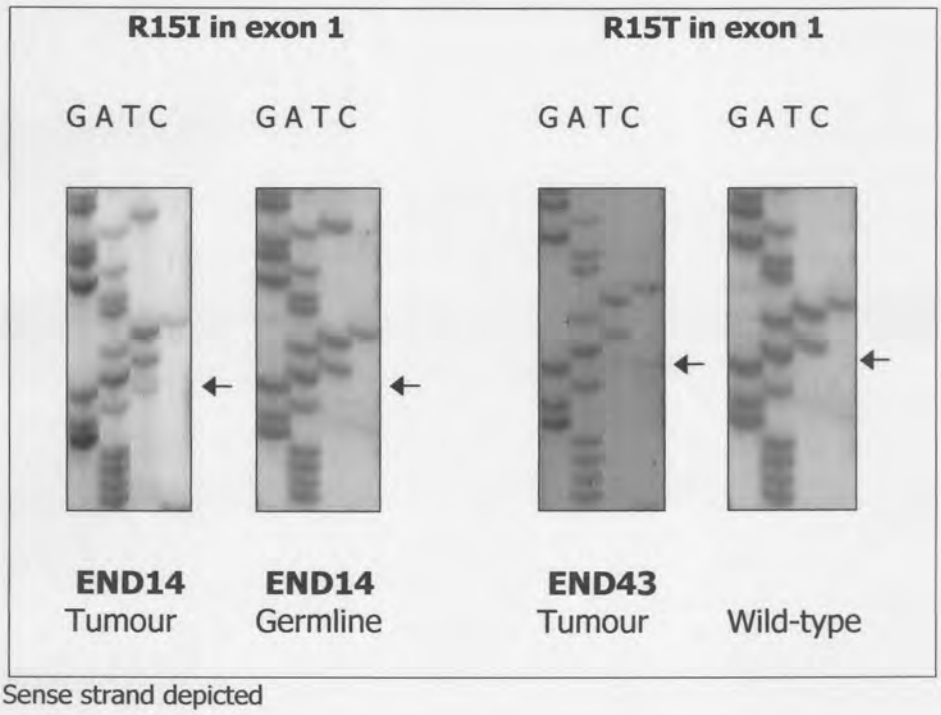
Missense mutations:

Six different missense mutations were detected in one hyperplastic tissue and six tumours. These mutations result in four non-conservative, one conservative and one synonymous amino acid substitution. The sequencing results are shown in figure 4.9, 4.10 and 4.11.

R15I in END14:

This somatic missense mutation occurred in the tumour of a 47-year old Caucasian woman diagnosed with grade II, stage Ib adenocarcinoma. The G→T substitution at c.44 in exon 1 changes the basic arginine to aliphatic isoleucine (Figure 4.9). Bussaglia *et al.* (2000) reported this mutation in two patients with endometrial cancer, where each of these patients was a compound heterozygote for another

pathogenic mutation. In addition to the R15I missense mutation one patient had a nonsense mutation (E7X) and the other patient a missense mutation (R173C), which was shown to be pathogenic (Han *et al.*, 2000). As both of these previously reported cases had other disease-causing mutations it is unclear whether the R15I mutation is contributing to the development of the cancer. In the case of END14 only R15I was detected.



Sense strand depicted

Figure 4.9: Sequence analysis of missense mutations in exon 1.

R15T in END43:

This novel somatic missense mutation (c.44G→C) was observed in a 54-year old African woman with grade II, stage III adenocarcinoma (Figure 4.9). The non-conservative substitution was observed in exon 1 where the basic arginine changes to neutral threonine. The significance of this mutation is unknown. However, other missense mutations in codon 15 (R15S and R15I) have been described in one glioblastoma and two endometrial carcinomas (Steck *et al.*, 1997; Bussaglia *et al.*, 2000). This codon therefore seems to be targeted for mutation in some cancers. No

functional studies have been performed on any of the codon 15 mutations and it is therefore unclear whether mutations in this codon contribute to disease. END43 already has another mutation, c.955del4 that plays a role in the pathogenesis of endometrial cancer.

H61H in EHYP9:

This novel synonymous mutation (c.183T→C) in exon 3 was detected in a Caucasian woman with atypical hyperplasia (Figure 4.10). It could not be determined whether the alteration was somatic or germline as no normal tissue was available. At this time the significance of the mutation is unknown but it has been reported that synonymous mutations may influence splicing accuracy and efficiency (Cartegni *et al.*, 2002). Until functional studies are carried out the effect of this synonymous change remains unknown.

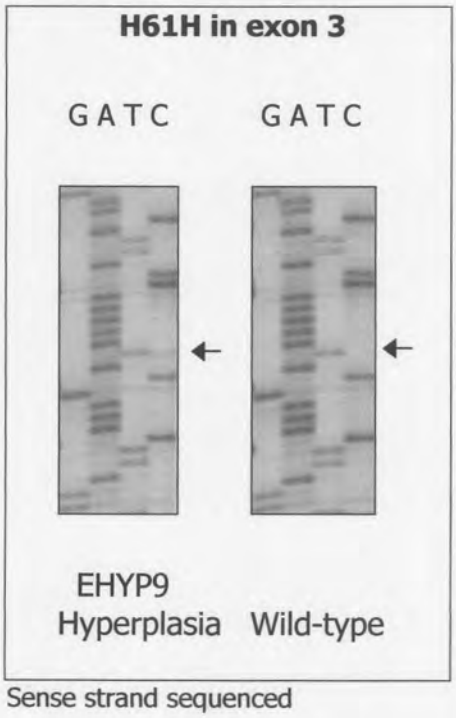


Figure 4.10: **DNA sequence analysis of a synonymous mutation.**

R130Q in END23 and END39:

Patients END23 and END39 are black Africans, both 68 years old, with grade II adenocarcinoma (stage III and Ib respectively). This previously reported missense mutation (Rasheed *et al.*, 1997; Simpkins *et al.*, 1998; Levine *et al.*, 1998; Yaginuma *et al.*, 2000; Bussaglia *et al.*, 2000; Konopka *et al.*, 2002) occurred within the core signature motif of the phosphatase domain at nucleotide c.389 in exon 5, by substituting G→A (Figure 4.11). It is somatic in both tumours. This non-conservative change removes the evolutionary conserved arginine residue in the consensus active-site sequence, which is essential for PTEN phosphatase activity. Han *et al.* (2000) has shown that this mutation results in a completely inactive PTEN protein with no lipid phosphatase activity.

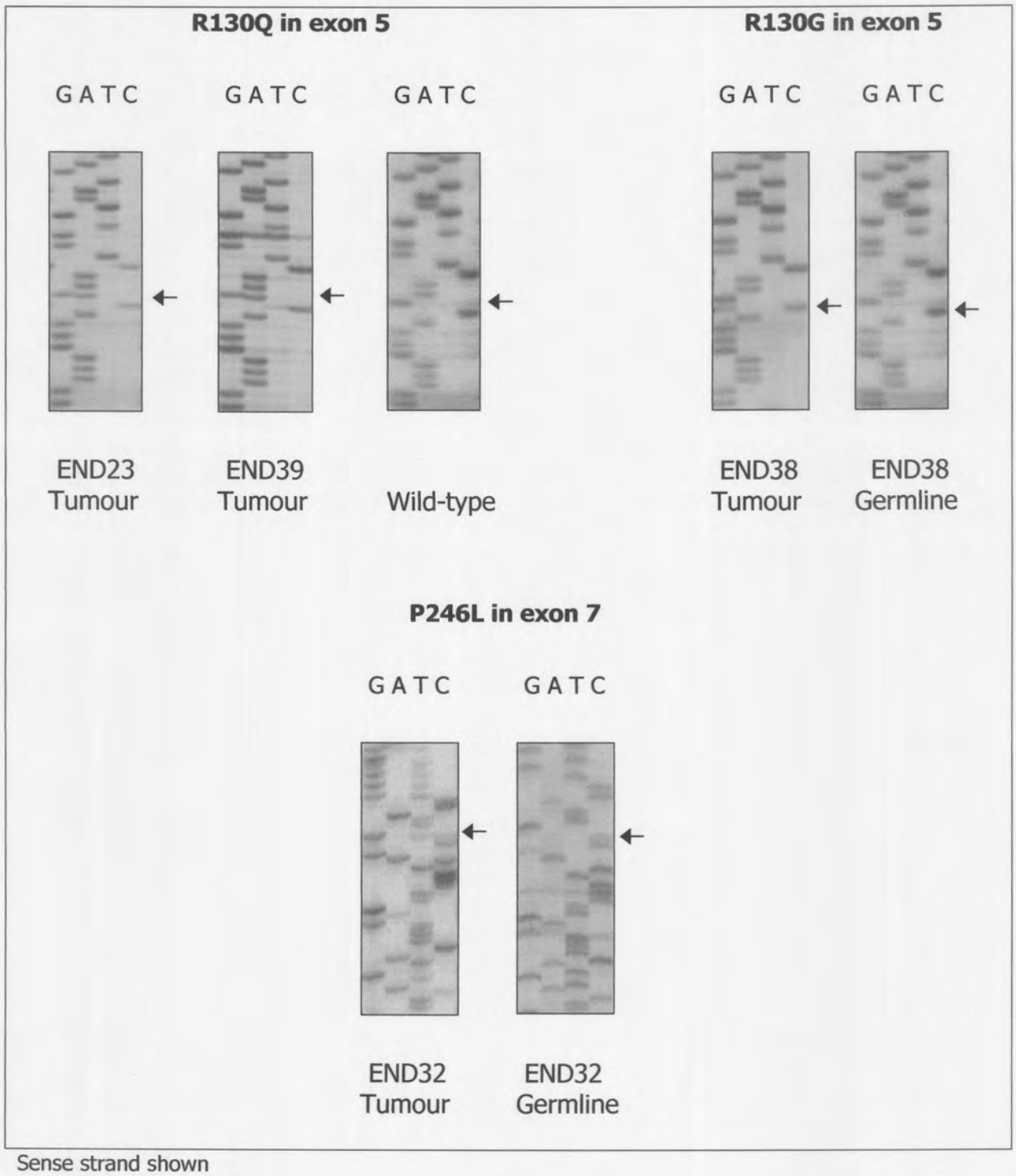
R130G in END38:

Grade II, stage Ib adenocarcinoma was diagnosed in this 64-year old African patient. The somatic missense mutation (c.388C→G) occurred in codon 130, a mutational hotspot in the phosphatase domain (Figure 4.11). The basic polar conserved arginine is changed to aliphatic non-polar glycine affecting PTEN function negatively by rendering it completely inactive (Han *et al.*, 2000). This mutation has previously been observed in ovarian and endometrial carcinoma (Risinger *et al.*, 1997; Kong *et al.*, 1997; Tashiro *et al.*, 1997; Obata *et al.*, 1998; Konopka *et al.*, 2002).

P246L in END32:

A nucleotide change in exon 7 was detected in a 43-year old African woman with grade II, stage IVa adenocarcinoma. This somatic mutation c.737C→T (Figure 4.11) results in a conservative amino acid change, from proline to leucine, close to a conserved tyrosine phosphate acceptor site (codon 233 to 240). The mutation also lies within the C2 domain of the protein, which is responsible for the positioning of the catalytic domain of PTEN on the phospholipid membranes (Lee *et al.*, 1999). This alteration was previously reported as a germline mutation in the BRR syndrome and as a somatic mutation in a glioblastoma (Liu *et al.*, 1997; Marsh *et al.*, 1999).

Genotype-phenotype analysis indicates that this mutation is pathogenic as it contributes to the development of BRR in an autosomal dominant manner (Marsh *et al.*, 1999).



Sense strand shown

Figure 4.11: **Sequence analysis of missense mutations in exons 5 and 7.**

b) Assessment of amino acid substitution includes position (protein region), functional importance (conserved versus non-conserved) and physical changes likely to affect polypeptide structure and function;

c) Prevalence of the mutation: A mutation that is found on less than 1 % of alleles in a control population may be disease-causing where a mutation occurring at higher frequency may be a neutral polymorphism;

d) Expression (functional) analysis of the mutant gene using preferably genomic DNA.

Applying these criteria to the six different missense mutations detected in this study showed the following:

a) No germline mutations were detected in the present study. Therefore, segregation analysis is not indicated.

b) Codon 130 is evolutionary conserved and occurs within the core signature motif of the phosphatase domain. This codon has been shown to be essential for phophatase activity (Han *et al.*, 2000). Thus the R130Q and R130G mutations can be considered to be pathogenic.

Codon 246 lies within the C2-domain of the protein, which is involved in the positioning of the catalytic domain of PTEN. As the P246L mutation has also been shown to contribute to the development of BRR this mutation is likely to be disease-causing.

c) As all missense mutations were somatic, the prevalence in the general population is not relevant.

d) No functional studies could be carried out.

Taking all of the above into account, three of the six different mutations appear to be pathogenic (Table 4.4).

Table 4.4: Pathogenic significance of the missense mutations.

MISSENSE MUTATIONS					PATHOGENIC SIGNIFICANCE
Patient	**Exon**	**Nucleotide change***	**Codon**	**Designation***	
END14	1	c.44G→T	15	R15I	Unknown
END43	1	c.44G→C	15	R15T	Unknown
EHYP9	3	c.183T→C	61	H61H	Unknown
END23	5	c.389G→A	130	R130Q	Yes
END39	5	c.389G→A	130	R130Q	Yes
END38	5	c.388C→G	130	R130G	Yes
END32	7	c. 737C→T	246	P246L	Yes

*Recommended nomenclature system (Antonarakis & the Nomenclature Working Group, 1998)

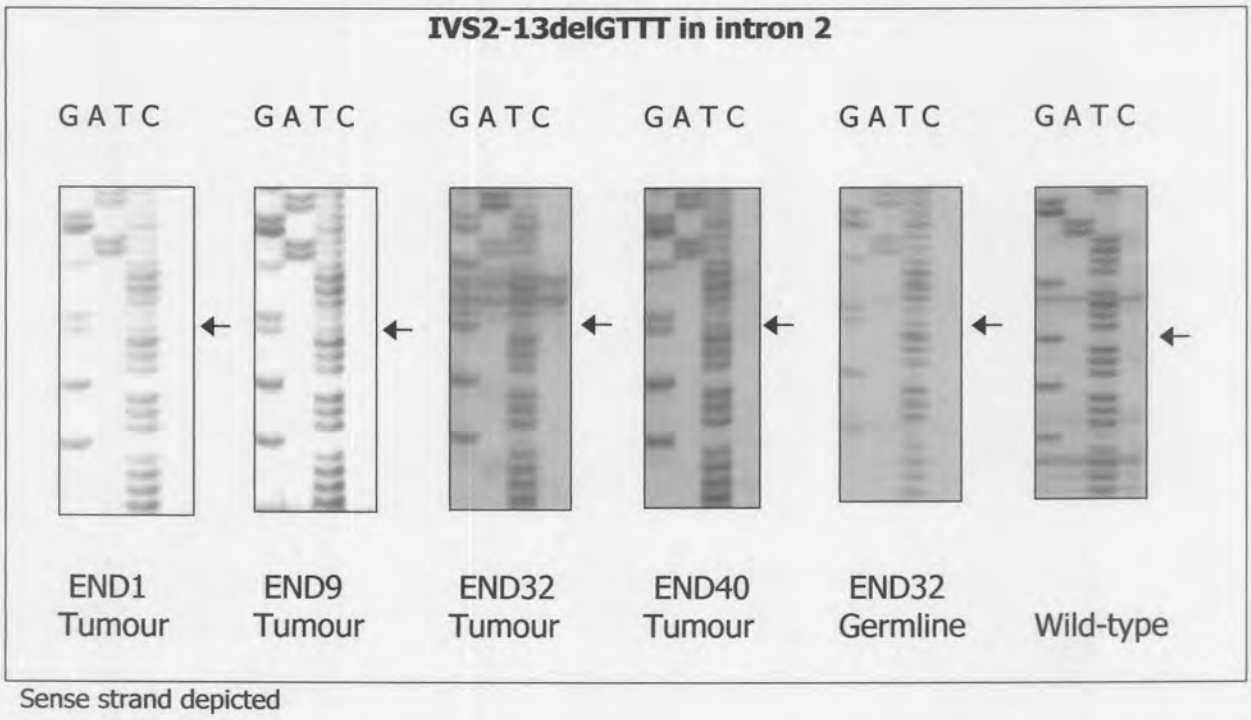
4.3.2 POLYMORPHISMS AND MUTATIONS OF UNKNOWN SIGNIFICANCE

One polymorphism and one in-frame deletion were detected.

Polymorphism:

IVS2-13delGTTT in END 1, END9, END32 and END40:

Four black African patients with grade II adenocarcinoma, END 1 (56 years), END9 (53 years), END32 (43 years) and END40 (66 years) were found to have a germline 4 bp deletion in intron two, 13 bp upstream of exon 3 (Figure 4.12). This deletion appears to be a polymorphism unique to black South Africans. It was present in 2 % of the 49 control Black patients, with an allele frequency of 0.02 (2/98) whereas none of the 50 control Caucasians exhibited this change. Furthermore, END9 (Q149X), END32 (P246L) and END40 (c.955delACTT and IVS7+5delG) have pathogenic mutations that indicate that this polymorphism is not associated with the disease.



Sense strand depicted

Figure 4.12: **Sequence results of the 4 bp deletion in intron 2.**

In-frame deletion:

Y176del in END26:

This somatic in-frame deletion (c.526delTAT) occurred in a 92-year old African patient with grade II, stage Ic adenocarcinoma (Figure 4.13). The mutation in exon 6 results in the deletion of tyrosine at codon 176 in the N-terminal domain of the protein. Duerr *et al.* (1998) reported this alteration in glioblastoma without indicating whether this mutation was considered pathogenic or not. The effect of this mutation is thus unknown according to the criteria of Cotton and Scriver (1998). Functional studies have to be performed in order to clarify the effect of the mutation.

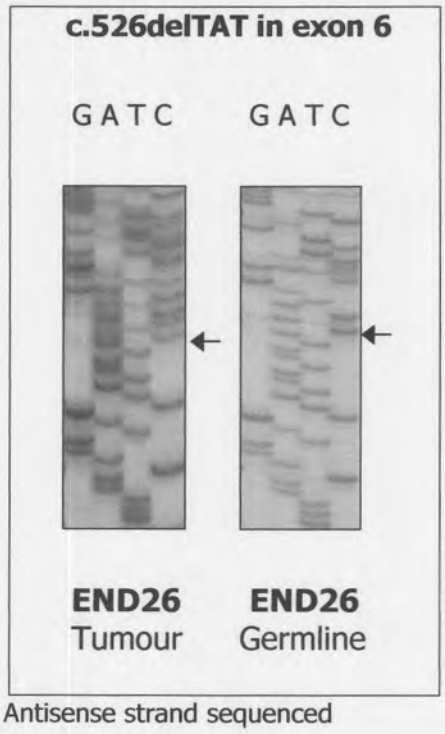


Figure 4.13: **Sequence result indicating the 3 bp in-frame deletion in exon 6.**

4.4 TUMOURS WITH COMPOUND HETEROZYGOSITY

Five of the endometrioid adenocarcinomas (5/37; 14 %) carried two distinct *PTEN* mutations each (Table 4.5). Three of these tumours (END19, END22, END 40) had two pathogenic mutations each whereas two tumours (END26, END43) had one disease-causing mutation and one mutation of unknown significance each. At this time it is uncertain whether these mutations are in *cis* or *trans*. Tumours with two *PTEN* mutations each (29/351; 8.3 %) have been observed in other studies (Kong *et al.*, 1997; Tashiro *et al.*, 1997; Simpkins *et al.*, 1998; Risinger *et al.*, 1998; Yaginuma *et al.*, 2000; Bussaglia *et al.*, 2000; Sun *et al.*, 2001; Konopka *et al.*, 2002).

Table 4.5: Tumours with compound heterozygosity.

Patient	Mutation
END19	c.16delAA c.701delG
END22	E7X R130X
END26	Y176del c.863delA
END40	c.955delACTT IVS7+5delG
END43	c.955delACTT R15T

4.5 ANALYSIS OF COMBINED MUTATIONAL DATA

Twenty-four different mutations, five of which occurred more than once, were observed (Tables 4.6A and B) in two hyperplastic (2/10; 20 %) and 22 (22/37; 59.5 %) endometrioid carcinoma specimens. No mutations were detected in the four serous papillary cancers and the one poorly differentiated carcinoma. Pathogenic mutations were present in 54 % (20/37) of the endometrioid carcinomas and 10 % (1/10) of the hyperplasias (Table 4.6B). These data correlate with published findings where mutations have been observed in 26 % to 52 % of endometrioid adenocarcinomas (Risinger *et al.*, 1997; Kong *et al.*, 1997; Tashiro *et al.*, 1997; Lin *et al.*, 1998; Simpkins *et al.*, 1998; Risinger *et al.*, 1998; Bussaglia *et al.*, 2000; Sun *et al.*, 2001; Konopka *et al.*, 2002) and in 0 % to 14 % of serous papillary carcinomas (Tashiro *et al.*, 1997; Obata *et al.*, 1998; Bussaglia *et al.*, 2000; Sun *et al.*, 2001). *PTEN* mutations were observed in other studies in 18 % to 27 % of mainly atypical hyperplasia cases (Maxwell *et al.*, 1998a; Levine *et al.*, 1998; Sun *et al.*, 2001).

Table 4.6A: Polymorphisms and *PTEN* mutations of unknown significance.

Patient	Ethnic group*	Exon/Intron	Nucleotide change**	Codon	Designation**	Effect
END14	C	1	c.44G→T	15	R15I	Arg→Iso
END43	A	1	c.44G→C	15	R15T	Arg→Thr
END1	A	2	IVS2-13delGTTT	N/A	IVS2-13delGTTT	Polymorphism
END9	A	2	IVS2-13delGTTT	N/A	IVS2-13delGTTT	Polymorphism
END32	A	2	IVS2-13delGTTT	N/A	IVS2-13delGTTT	Polymorphism
END40	A	2	IVS2-13delGTTT	N/A	IVS2-13delGTTT	Polymorphism
EHYP9	C	3	c.183T→C	61	H61H	His→His
END26	A	6	c.526delTAT	176	Y176del	Tyr del

* A = African; C = Caucasian
**Recommended nomenclature system (Antonarakis & the Nomenclature Working Group, 1998)
(Reference sequence: GenBank [accession number U93051 / U92436])

Table 4.6B: Pathogenic *PTEN* mutations observed in endometrial hyperplastic and tumour tissues.

Patient	Ethnic group*	Exon/Intron	Nucleotide change**	Codon	Designation**	Effect***
END4	C	1	c.13insA	5	c.13insA	FS ter 10
END19	A	1	c.16delAA	6	c.16delAA	FS ter 9
END22	A	1	c.19G→T	7	E7X	Glu→stop
END12	C	1	c.49C→T	17	Q17X	Gln→stop
END10	C	3	c.176C→A	59	S59X	Ser→stop
END48	C	4	IVS4+5G→A	N/A	IVS4+5G→A	Exon skipping
END11	A	5	c.388C→T	130	R130X	Arg→stop
END22	A	5	c.388C→T	130	R130X	Arg→stop
END38	A	5	c.388C→G	130	R130G	Arg→Gly
END8	I	5	c.389delG	130	c.389delG	FS ter 133
END34	A	5	c.389delG	130	c.389delG	FS ter 133
END35	A	5	c.389delG	130	c.389delG	FS ter 133
END23	A	5	c.389G→A	130	R130Q	Arg→Gln
END39	A	5	c.389G→A	130	R130Q	Arg→Gln
END9	A	5	c.445C→T	149	Q149X	Gln→stop
END25	C	6	IVS6+3A→C	N/A	IVS6+3A→C	Exon skipping
END19	A	7	c.701delG	234	c.701delG	FS ter 255
END32	A	7	c.737C→T	246	P246L	Pro→Leu
EHYP5	C	7	c.766G→T	256	E256X	Glu→stop
END40	A	7	IVS7+5delG	N/A	IVS7+5delG	Exon skipping
END26	A	8	c.863delA	288	c.863delA	FS ter 290
END40	A	8	c.955delACTT	319	c.955delACTT	FS ter 319
END43	A	8	c.955delACTT	319	c.955delACTT	FS ter 319
END2	C	8	c.968insA	323	c.968insA	FS ter 324

* A = African; C = Caucasian; I = Indian
**Recommended nomenclature system (Antonarakis & the Nomenclature Working Group, 1998)
*** FS = Frameshift
(Reference sequence: GenBank [accession number U93051 / U92436])

Maxwell *et al.* (2000) reported a 4-fold disparity in the frequency of *PTEN* mutations occurring in African Americans (5 %; 3/62) and Caucasian Americans (22 %; 17/78). In the present study no significant differences between the African (52 %; 13/25) and Caucasian (55 %; 6/11) groups were found ($P>0.05$; χ^2-test).

4.5.1 Distribution and frequency of mutations in the *PTEN* gene

In total 32 mutations (24 different mutations of which five occurred more than once) were observed. Thirteen of the 24 different mutations (54 %) were novel. With the exception of exons 2, 4 and 9 alterations were scattered along the gene (Figure 4.14). The frequency with which these mutations occurred in the exons (E) and introns (I) are as follows: E1=19 % (6/32), E3=6 % (2/32), E4=3 % (1/32), E5= 16 % (5/32), E6=6 % (2/32), E7=13 % (4/32), E8=9 % (3/32), I2=13 % (4/32), I4, I6 and I7=3 % (1/32) each.

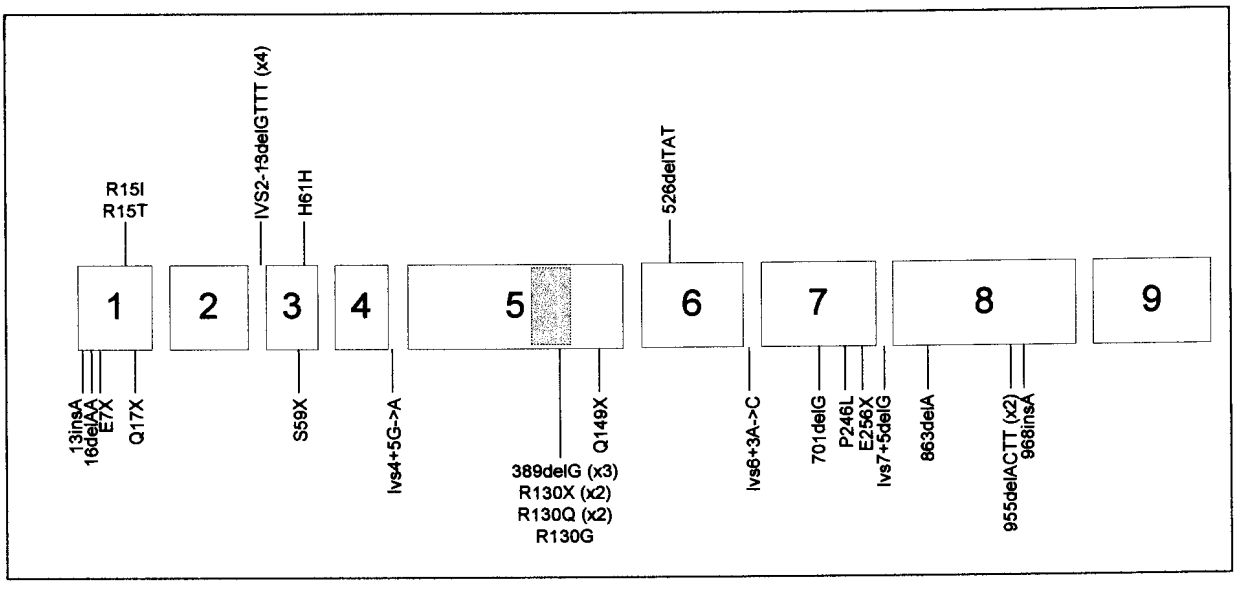


Figure 4.14: **Distribution of *PTEN* mutations in endometrial hyperplasia and carcinoma.** Polymorphisms and mutations of unknown significance indicated above and pathogenic mutations indicated below the gene.

4.5.2 Distribution and frequency of pathogenic mutations.

Twenty-four of the 32 mutations were pathogenic, with 23 occuring in adenocarcinomas and one in the hyperplastic tissue (E256X) (Figure 4.14). The frequency of pathogenic mutations in the respective *PTEN* exons present study is largely similar (P>0.05; χ^2-test) to published findings (Table 4.7). Exon 5 (39.1 %; 9/23) had the highest mutation frequency, followed by exons 1 and 8 (17.4 %; 4/23 each).

Table 4.7: Frequency of pathogenic *PTEN* mutations in individual exons (occurring in endometrial tumours).

Exon	Mutation frequency			
	Present study		Published findings*	
	Times mutated	%	Times mutated	%
1	4	17.4	15	7
2	0	0	15	7
3	1	4.4	13	6
4	0	0	7	3
5	9	39.1	48	21
6	0	0	10	4.5
7	2	8.7	42	19
8	4	17.4	63	28
9	0	0	1	0.5
Splice sites	3	13	8	4
Total	23	100	224	100

* (Bonneau & Longy, 2000; Yuginuma *et al.*, 2000; Bussaglia *et al.*, 2000; Sun *et al.*, 2001; Konopka *et al.*, 2002)

Of the nine mutations occurring in exon 5, codon 130 was mutated eight times. This codon has previously been found to be a mutational hotspot in endometrial tumours (Bonneau & Longy, 2000; Yuginuma *et al.*, 2000; Bussaglia *et al.*, 2000; Sun *et al.*, 2001; Konopka *et al.*, 2002).

The frameshift mutations occuring in exon 8 targets poly-A sequences or a 4-bp deletion in a duplicated motif. These regions/motifs have previously been found to be associated with MI^+-endometrial tumours (Kong *et al.*, 1997; Risinger *et al.*, 1997; Tashiro *et al.*, 1997; Lin *et al.*, 1998; Risinger *et al.*, 1998; Bonneau & Longy, 2000; Bussaglia *et al.*, 2000).

4.5.3 *PTEN* mutation types and their frequencies

The 32 sequence alterations comprised seven mutation types (Figure 4.15). The majority (63 %; 20/32) were truncating mutations, i.e. frameshift, nonsense and splice site mutations.

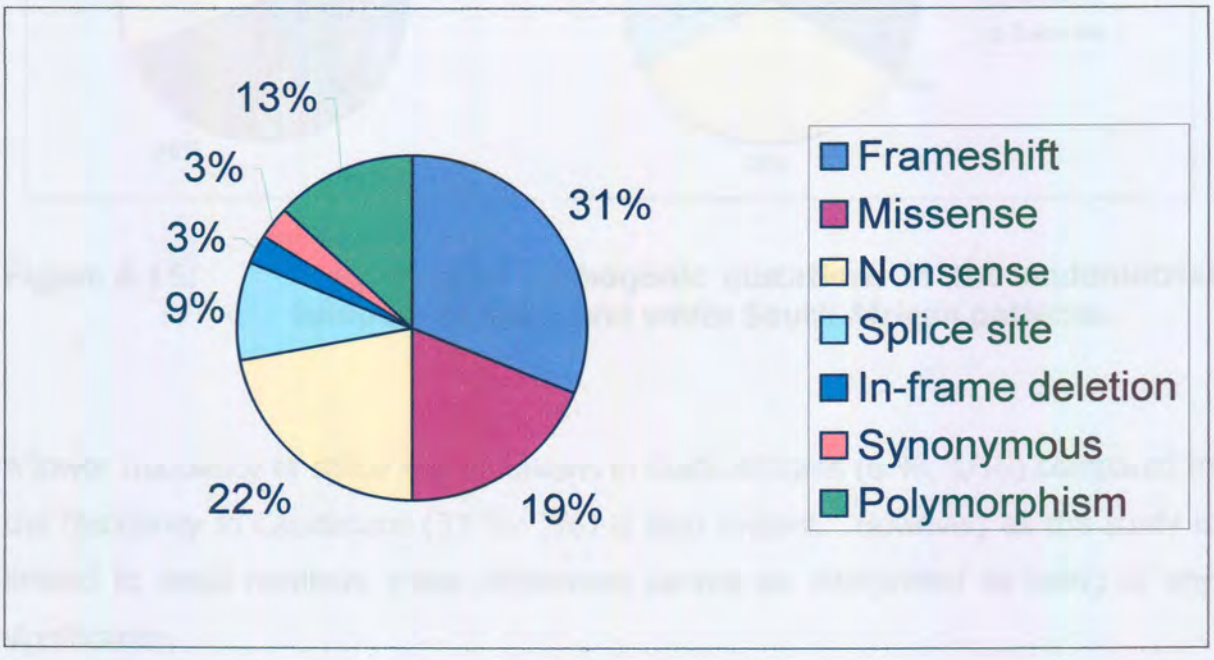


Figure 4.15 **Frequency of the different types of *PTEN* mutations in endometrial hyperplasias and endometrioid carcinomas.**

Of the 24 pathogenic mutations one nonsense mutation occurred in the hyperplastic tissue of a Caucasian patient. In the endometrioid tumours 10 frameshift (10/23; 43 %), six nonsense (6/23; 26 %), four missense (4/23; 18 %) and three splice site mutations (3/23; 13 %) were observed. Of these alterations 16 mutations were detected in black Africans, six in Caucasians and one in an Indian patient.

When comparing the mutations that occurred in endometrioid tumours from black African and Caucasian patients (Figure 4.16), it is clear that the frameshift, nonsense and splice site mutations in the Caucasians' tumours occurred at equal frequency. Missense mutations occurred only in the tumours of black Africans (25 %; 4/16) whereas the Caucasian tumours had none.

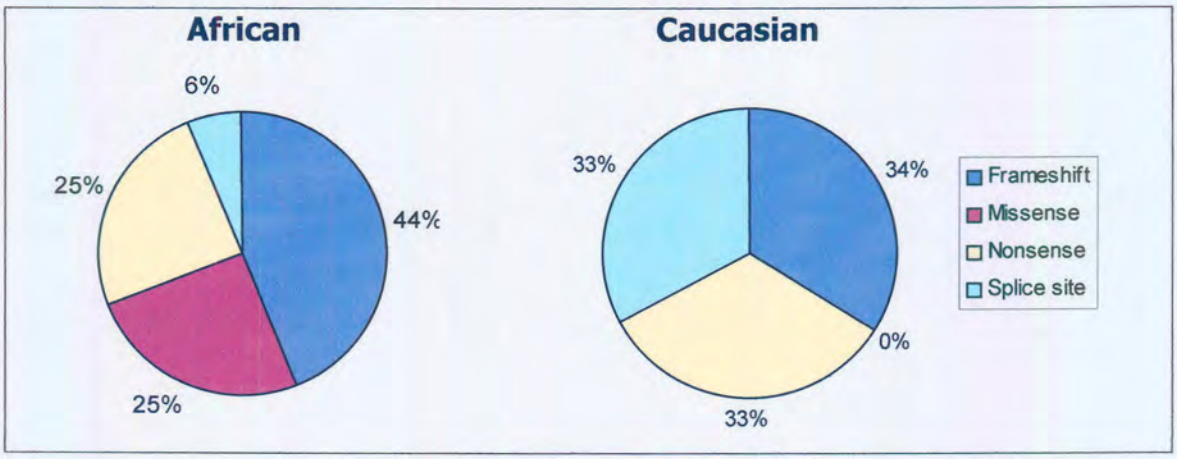


Figure 4.16: **Frequency of pathogenic mutations in the endometrial tumours of black and white South African patients.**

A lower frequency of splice site mutations in Black Africans (6 %; 1/16) compared to the frequency in Caucasians (33 %; 2/6) is also evident. However, as the study is limited to small numbers these differences cannot be interpreted as being of any significance.

4.5.4 Pathologic features of tumours with *PTEN* pathogenic mutations

Twenty-three pathogenic mutations were observed in the endometrioid adenocarcinomas. Three of these tumours had two disease-causing alterations. Thus, of the tumours that were screened for mutations, 20 harboured pathogenic mutations (20/37, 54 %). In the present study it was shown that *PTEN* gene mutations occurred in all three grades of endometrial adenocarcinoma (Table 4.8).

Table 4.8: **Relationship between pathogenic mutations and the architectural/nuclear grade of endometrioid adenocarcinomas according to ethnic groups.**

Grade	Tumours with mutations*							
	African		Caucasian		Indian		Total	
I	1 (2)	50 %	1 (1)	100 %	0 (0)	0 %	2 (3)	67 %
II	10 (19)	53 %	4 (9)	44 %	1 (1)	100 %	15 (29)	52 %
II-III	2 (3)	67 %	0 (0)	0 %	0 (0)	0 %	2 (3)	67 %
III	0 (1)	0 %	1 (1)	100 %	0 (0)	0 %	1 (2)	50 %
Total	13 (25)	52 %	6 (11)	55 %	1 (1)	100 %	20 (37)	54 %

* No. of mutations (Total no. of cases)

When comparing the African and Caucasian groups, the majority of the tumours were low grade, with no significant differences with regards to *PTEN* mutation frequency ($P>0.05$; χ^2-test). Mutations also occurred in early and advanced stage endometrial carcinomas, although the majority of the samples were stage I endometrioid adenocarcinoma (Table 4.9).

Table 4.9: **Relationship between pathogenic mutations and the FIGO stage of endometrioid adenocarcinomas according to ethnic groups.**

Stage	Tumours with mutations*							
	African		Caucasian		Indian		Total	
I	8 (15)	53 %	4 (8)	50 %	0 (0)	0 %	12 (23)	52 %
II	0 (2)	0 %	1 (1)	100 %	0 (0)	0 %	1 (3)	33 %
III	4 (6)	67 %	1 (2)	50 %	1 (1)	100 %	6 (9)	67 %
IV	1 (2)	50 %	0 (0)	0 %	0 (0)	0 %	1 (2)	50 %
Total	13 (25)	52 %	6 (11)	55 %	1 (1)	100 %	20 (37)	54 %

* No. of mutations (Total no. of cases)

The small sample size of advanced endometrial tumours in the present study complicates comparative analyses. Thus, meaningful investigation of a relationship between the presence of a pathogenic mutation and disease progression could not be carried out.

Similar to the present study, Konopka *et al.* (2002) also found no correlation between the frequency of *PTEN* mutations and the stage of endometrial cancer in 59 carcinoma samples. However, Konopka *et al.* (2002) observed a statistically significant correlation between the occurrence of mutations and the morphological grade of endometrial cancer, where mutations in less-differentiated adenocarcinomas (grade 2) occurred twice as often as alterations in well-differentiated carcinomas (grade 1).

Thus, in the present study no association between the frequency of *PTEN* mutations and the grade of clinical progression and stage of the endometrial cancer were found ($P>0.05$; χ^2-test). To validate these observations, however, a larger sample size representative of all the grades and stages of endometrial carcinoma needs to be analyzed.

CHAPTER 5: CONCLUSION

Endometrial carcinoma affects women worldwide, being rated as the fifth most common cancer in women (Burke *et al.*, 1996; Rose *et al.*, 1996). In South Africa cancer of the uterus is ranked third in Asian, fourth in Black and Coloured and seventh in Caucasian women (Sitas *et al.*, 1998). Various genetic alterations appear to be early events in the pathogenesis of endometrial cancer with the *PTEN* gene most commonly mutated in endometrioid adenocarcinoma (in all three grades) with a reported incidence of 26 % to 52 % (Risinger *et al.*, 1997; Kong *et al.*, 1997; Tashiro *et al.*, 1997; Lin *et al.*, 1998; Simpkins *et al.*, 1998; Risinger *et al.*, 1998; Bussaglia *et al.*, 2000; Sun *et al.*, 2001; Konopka *et al.*, 2002). Carcinogenesis of the endometrium, however, is preceded by pre-malignant hyperplasia. It has been found that *PTEN* mutations occur in the pre-cancerous endometrial tissues in 18 % to 27 % of mainly atypical hyperplasias (Maxwell *et al.*, 1998a; Levine *et al.*, 1998; Sun *et al.*, 2001).

Two independent studies of patients diagnosed with mainly advanced endometrioid adenocarcinoma revealed a disparity between black and white Americans, where the mutation frequency was 22 % to 34 % in white Americans compared to 0 % to 5 % in black Americans (Risinger *et al.*, 1998; Maxwell *et al.*, 2000). Caucasian Americans therefore, have a 4-fold more frequent incidence of *PTEN* alterations relative to African Americans (Maxwell *et al.*, 2000). An association of *PTEN* mutation with clinical outcome has also been shown – patients with *PTEN* mutation-positive endometrial carcinoma had a better prognosis than those without *PTEN* mutations (Risinger *et al.*, 1998). Other complementary prognostic features that correlate with mutated *PTEN* are endometrioid histology, early stage and lower grade. African Americans have a higher frequency of poor prognostic factors such as non-endometrioid, poorly differentiated, advanced staged cancer that is deeply invasive. Their disease-related mortality is significantly higher than white Americans. It is hypothesized that the molecular pathogenesis of endometrial carcinoma within these two distinct ethnic groups may be different (Maxwell *et al.*, 2000). This may also

explain the 2 to 2.5-fold difference in ASIR between the African and Caucasian populations in America as well as South Africa.

The current study aimed to investigate the PTEN gene in Caucasians and Black South African women with endometrial hyperplasia and carcinoma. The frequency and type of mutations as well as the pathological features of the cancers (stage and grade) were also assessed.

Pathogenic mutations were detected in 54 % (20/37) of the endometrioid adenocarcinomas, whereas none of the serous papillary carcinomas and weak differentiated tumours harboured any mutations. Ten % (1/10) of the hyperplasias were found to have a pathogenic mutation. These findings are similar ($P>0.05$; χ^2-test) to those of other studies (Bonneau & Longy, 2000; Yaginuma *et al.*, 2000; Bussaglia *et al.*, 2000; Sun *et al.*, 2001; Konopka *et al.*, 2002). Disease-causing mutations occurred in the tumours of 52 % (13/25) Black and 55 % (6/11) Caucasian South Africans. The tumour of the one Indian patient was also found to carry a pathogenic mutation. Five of the tumours were compound heterozygotes.

In the present study it was shown that *PTEN* gene mutations occurred in all three grades of endometrial adenocarcinomas, however, the majority of the tumours were low grade. When comparing the African and Caucasian groups, there were no significant differences with regards to *PTEN* mutation frequency ($P>0.05$; χ^2-test). Mutations also occurred in early and advanced stage endometrial carcinomas, although the majority of the samples were stage I endometrioid adenocarcinoma. No association between the frequency of *PTEN* mutations and the grade of clinical progression and stage of the endometrial cancer were found ($P>0.05$; χ^2-test). To validate these observations a larger sample size representative of all the grades and stages of endometrial carcinoma needs to be analyzed.

CHAPTER 6: REFERENCES

Ahuja, N. Mohan, A.L., Li, Q., Stolker, J.M., Herman, J.G., Hamilton, S.R., Baylin, S.B. & Issa, J.J. Association between CpG island methylation and microsatellite instability in colorectal cancer. *Cancer Research*, 1997; **57**:3370-3374.

Ambros, R.A., Sherman, ME., Zahn, C.M., Bitterman, P. & Kurman, R.J. Endometrial intra-epithelial carcinoma: A distinctive lesion specifically associated with tumours displaying serous differentiation, *Human Pathology*, 1995; **26**:1260-1267.

Ambros, R.A., Vigna, P.A., Figg, J., Kallakury, B.V.S., Mastrangelo, A., Eastman, A.Y., Malfetano, J., Figge, H.L. & Ross, J.S. Observations on tumour and metastatic tumour suppressor gene status in endometrial carcinoma with particular emphasis on *p53*. *Cancer*, 1994; **73**:1686-1692.

Antonarakis, S.E. & the Nomenclature Working Group. Recommendations for a nomenclature system for human gene mutations. *Human Mutation*, 1998; **11**:1-3.

Arch, E.M., Goodman, B.K., Van Wesep, R.A., Liaw, D., Clarke, K., Parsons, R., McKusick, V.A. & Geraghty, M.T. Deletion of *PTEN* in a patient with Bannayan-Riley-Ruvalcaba syndrome suggests allelism with Cowden disease. *American Journal of Medical Genetics*, 1997; **71**:489-483.

Auger, K.R., Serunian, L.A., Soltoff, S.P., Libby, P. & Cantley, L.C. PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. *Cell*, 1988; **57**:167-175.

Bai, M.K., Costopoulos, J.S., Christoforidou, B.P. & Papadimitriou, C.S. Immunohistochemical detection of the *c-myc* oncogene product in normal, hyperplastic and carcinomatous endometrium. *Oncology*, 1994; **51**:314-319.

Barford, D., Flint, A.J. & Tonks, N.K. Crystal structure of human protein tyrosine phosphatase 1B. *Science*, 1994; **263**:1397-1404.

Berchuck, A., Rodriques, G., Kinney, R.B., Soper, J.T., Dodge, R.K., Clarke-Pearson, D.L. & Bast, R.C. Overexpression of HER-2/*neu* in endometrial cancer is associated with advanced stage disease. *American Journal of Obstetrics and Gynecology*, 1991;**164**:15-21.

Besson, A., Robbins, S.M. & Yong, V.W. *PTEN/MMAC1/TEP1* in signal transduction and tumourigenesis. *European Journal of Biochemistry*, 1999; **263**:605-611.

Biesecker, L.G., Happle, R., Mulliken, J.B., Weksberg, R., Graham, J.M., Viljoen, D.L. & Cohen, M.M. Proteus syndrome: Diagnostic criteria, differential diagnosis and patient evaluation. *American Journal of Medical Genetics*, 1999; **84**:389-395.

Bishop, J.M. Molecular themes in oncogenesis. *Cell*, 1991; **64**:235-248.

Bonneau, B. & Longy, M. Mutations of the human *PTEN* gene. *Human Mutation*, 2000; **16**:109 – 122.

Boström, J., Cobbers, J.M.J.L., Wolter, M., Tabatabai, G., Weber, R.G., Lichter, P., Collins, V.P. & Reifenberger. Mutation of the *PTEN* (*MMAC1*) tumour suppressor gene in a subset of glioblastomas but not in meningiomas with loss of chromosome arm 10q. *Cancer Research*, 1998; **58**:29-33.s

Bruni, P., Boccia, A., Baldassarre, G., Trapasso, F., Santoro, M., Chiappetta, G., Fusco, A. & Viglietto, G. PTEN expression is reduced in a subset of sporadic thyroid carcinomas: Evidence that PTEN-growth suppressing activity in thyroid cancer cells is mediated by $p27^{kip1}$. *Oncogene*, 2000; **19**:3146-3155.

Burke, T.W.; Tortolero-Luna, G.; Malpica, A.; Baker, V.; Whittaker, L.; Johnson, E. & Mitchell, M.F. Endometrial hyperplasia and endometrial cancer. *Gynecologic cancer prevention*, 1996; **23**(2):411-456.

Burks, R.T., Kessis, T.D., Cho, K.R. & Hedrick, L. Microsatellite instability in endometrial carcinoma. *Oncogene*, 1994; **9**(4):1163-1166.

Bussaglia, E., Del Rio, E., Matias-Guiu, X. & Prat, J. *PTEN* mutations in endometrial carcinomas: A molecular and clinicopathologic analysis of 38 cases. *Human Pathology*, 2000; **31**:312-317.

Caduff, R.F., Johnston, C.M., Svoboda-Newman, S.M., Poy, E.L., Merajver, S.D. & Frank, T.S. Clinical and pathological significance of microsatellite instability in sporadic endometrial carcinoma. *American Journal of Pathology*, 1996; **148**:1671-1678.

Cairns, P., Okami, K., Halachmi, S., Halachmi, N., Esteller, M., Herman, J.G., Jin, J., Isaacs, W.B., Bova, G.S. & Sidransky, D. Frequent inactivation of *PTEN/MMAC1* in primary prostate cancer. *Cancer Research*, 1997, **57**:4997-5000.

Cantley, L.C. & Neel, B.G. New insights into tumour suppression: PTEN suppressor tumour formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proceedings of the Natural Academy of Science of the USA*, 1999; **96**:4240-4245.

Cardone, M.H., Roy, N., Stennicke, H.R., Salvesen, G.S., Franke, T.F., Stanbridge, E., Frisch, S. & Reed, J.C. Regulation of cell death protease caspase-9 by phosphorylation. *Science*, 1998; **282**:1318-1321.

Carpenter, C.L., Duchworth, B.C., Auger, K.R., Cohen, B., Schaffhausen, B.S. & Cantley, L.C. Purification and characterization of phosphoinositide 3-kinase from rat liver. *Journal of Biological Chemistry*, 1990; **265**:19704-19711.

Cartegni, L., Chew, S.L. & Krainer, A.R. Listening to silence and understanding nonsense: exonic mutations that affect splicing. *Nature Reviews Genetics*, 2002; **3**: 285-298.

Cary, L.A., Han, D.C., Polte, T.R., Hanks, S.K. & Guan, J. Identification of $p130^{Cas}$ as a mediator of focal adhesion kinase-promoted cell migration. *Journal of Cell Biology*, 1998; **140**:211-221.

Catasus, L.L., Machin, P., Matias-Guiu, X. & Prat, J. Microsatellite instability in endometrial carcinomas clinicopathologic correlations in a series of 42 cases. *Human Pathology*, 1998a; **29**:1160-1164.

Catasus, L.L., Matia-Guiu, X., Machin, P. Munoz, J. & Prat, J. *BAX* somatic frameshift mutations in endometrioid adenocarcinomas of the endometrium: Evidence for a tumour progression role in endometrioid carcinomas with microsatellite instability. *Laboratory Investigations*, 1998b; **78**:1439-1444.

Catasus, L.L., Matia-Guiu, X., Machin, P., Zannoni, G.F., Scambia, G., Benedetti-Panici., P. & Prat, J. Frameshift mutations at coding mononucleotide repeat microsatellites in endometrial carcinomas with microsatellite instability. *Cancer*, 2000; **88**:2290-2297.

Cavenee, W.K. & White, R.L. The genetic basis of cancer. *Scientific American*, 1995; **272**(3):50-57.

Celebi, J.T., Wanner, M., Ping, X.L., Zhang, H & Peacocke, M. Association of splicing defects in *PTEN* leading to exon skipping or partial intron retention in Cowden syndrome. *Human Genetics*, 2000; **107**:234-238.

Charbonneau, H. & Tonks, N.K. 1002 protein phosphatases? *Annual Review of Cell Biology*, 1992; **8**:463-493.

Chen, J., Lindblom, P. & Lindblom, A. A study of the *PTEN/MMAC1* gene in 136 breast cancer families. *Human Genetics*, 1998; **102**:124-125.

Chen, H. & Guan, J. Association of focal adhesion kinase with its potential substrate phosphatidylinositol 3-kinase. *Proceedings of the National Academy of Science of the USA*, 1994; **91**:10148-10152.

Chen, H., Appeddu, P.A., Isoda, H. & Guan, J. Phosphorylation of tyrosine 397 in focal adhesion kinase is required for binding phosphatidylinosinositol 3-kinase. *Journal of Biological Chemistry*, 1996; **271**:26329-26334.

Chung, J., Grammer, T.C., Lemon, K.P., Kazlaukas, A. & Blenis, J. PDGF- and insulin-dependant $p70^{S6k}$ activation mediated by phospatidylinositol-3-OH kinase. *Nature*, 1994; **370**:71-75.

Clark, E.A. & Brugge, J.S. Integrins and signal transduction pathways: The road taken. *Science*, 1995; **268**:233-239.

Clifford, S.L., Kaminetski, C.P., Cirisano, F.D., Dodge, R., Soper, J.T., Clarke-Pearson, D.L., Daniel, L. & Berchuck, A. Racial disparity in overexpression of the *p53* tumour suppressor gene in stage I endometrial cancer. *American Journal of Obstetrics and Gynecology*, 1997; **176**:S229-232.

Coffer, P.J., Jin, J. & Woodgett, J.R. Protein kinase B (c-Akt): A multifunctional mediator of phosphatidylinositol 3-kinase activation. *Biochemical Journal*, 1998; **335**:1-13.

Cooper, D.N. & Krawczak, M. eds. *Human Gene Mutation*, 1st ed. Bios Scientific Publishers, 1993: 240-245, 287-289.

Cotton, R.G.H. & Scriver, C.R. Proof of "disease causing" mutation. *Human Mutation*, 1998; **12**:1-3.

Craven, S.E & Bredt, D.S. PDZ proteins organize synaptic signaling pathways. *Cell*, 1998; **93**:495-498.

Cross, D.A.E., Alessi, D.R., Cohen, P., Andjelkovich, M. & Hemmings, B.A. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature*, 1995; **378**:785-789.

Dahia, P.L.M., Aguiar, R.C., Alberta, J., Kum, J.B., Caron, S., Sill, H., Marsh, D.J., Ritz, J., Freedman, A., Stiles, C. & Eng, C. PTEN is inversely correlated with the cell survival factor Akt/PKB and is inactivated via multiple mechanisms in haematological malignancies. *Human Molecular Genetics*, 1999; **8**:185-193.

Dahia, P.L.M., Fitzgerald, M.G., Zhang, X., Marsh, D.J., Zheng, Z., Pietsch, T., Von Deimling, A., Haluska, F.G., Haber, D.A. & Eng, C. A highly conserved processed *PTEN* pseudogene is located on chromosome band 9p21. *Oncogene*, 1998; **16**:2403-2406.

Dahia, P.L.M., Marsh, D.J., Zheng, Z., Zedenius, J., Komminoth, P., Frisk, T., Wallin, G., Parsons, R., Longy, M., Larsson, C. & Eng, C. Somatic deletions and mutations in the Cowden disease gene, *PTEN*, in sporadic thyroid tumours. *Cancer Research*, 1997; **57**:4710-4713.

Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y. & Greenberg, M.E. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell*, 1997; **91**;231-241.

Davies, M.P.A., Gibbs, F.E.M., Halliwell, N., Joyce, K.A., Roebuck, M.M., Rossi, M.L., Salisbury, J., Sibson, D.R., Tacconi, L. & Walker, C. Mutations in the *PTEN/MMAC1* gene in archival low grade and high grade gliomas. *British Journal of Cancer*, 1999; **79**:1542-1548.

Davies, M.A., Koul, D., Haninder, D., Berman, R., McDonnell, T.J., McConkey, D., Yung, W.K.A. & Steck, P.A. Regulation of Akt/PKB activity, cellular growth and apoptosis in prostate carcinoma cells by MMAC/PTEN. *Cancer Research*, 1997; **59**:2551-2556.

Davies, M.A., Lu, Y., Sano, T., Fang, X., Tang, P., LaPushin, R., Koul, D., Bookstein, R., Stokoe, D., Yung, A., Mills, G.B. & Steck, P.A. Adenoviral transgene expression of *MMAC1/PTEN* in human glioma cells inhibits Akt activation and induces anoikis. *Cancer Research*, 1998; **58**:5285-5290.

Denu, J.M. & Dixon, J.E. Protein tyrosine phosphatases: Mechanisms of catalysis and regulation. *Current Opinion in Chemical Biology*, 1998; **2**:633-641.

Denu, J.M., Stuckey, J.A., Saper, M.A. & Dixon, J.E. Form and function in protein dephosphorylation. *Cell*, 1996; **87**:361-364.

Di Cristofano, A., Pesce, B., Cordon-Cardo, C. & Pandolfi, P.P. *PTEN* is essential for embryonic development and tumour suppression. *Nature Genetics*, 1998; **19**:348-355.

Diehl, J.A., Cheng, M., Roussel, M.F. & Sherr, C.J. Glycogen synthase kinase-3β regulates cyclin D1 proteolysis and subcellular localization. *Genes and Development*, 1998; **12**:3499-3511.

Downward, J. Ras signaling and apoptosis. *Current Opinion in Genetic Development*, 1998; **8**:49-54.

Duerr, E., Rollbrocker, B., Hayashi, Y., Peters, N., Meyer-Puttlitz, B., Louis, D.N., Schramm, J., Wiestler, O.D., Parsons, R., Eng, C. & Von Deimling, A. *PTEN* mutations in gliomas and glioneuronal tumours. *Oncogene*, 1998; **16**:2259-2264.

Duggan, B., Felix, J., Muderspach, L.I. Tsoa, J. & Shibata, D.K. Early mutational activation of the c-Ki-*ras* oncogene in endometrial carcinoma. *Cancer Research*, 1994; **54**:1604-1607.

Easton, D.F. The inherited component of cancer. *British Medical Bulletin*, 1994; **50**(3):527-535.

Eeles, R.A. & Stamps, A.C. Managing the method. In: Eeles, R.A. & Stamps, A.C., eds. *Polymerase chain reaction (PCR): The technique and its applications.* 1 st ed. R.G. Landes Company, Austin, 1993:13.

Eng, C., Murday, V., Seal, S., Mohammed, S., Hodgson, S.V., Chaudary, M.A., Fentiman, I.S., Ponder, B.A.J. & Eeles, R.A. Cowden syndrome and Lhermitte-Duclos disease in a family: A single genetic syndrome with pleiotropy? *Journal of Medical Genetics*, 1994; **31**:458-461.

Enomoto, T., Inoue, M., Perantoni, A.O., Terakawa, N., Tanizawa, O. & Rice, J.M. K-*ras* activation in neoplasms of the human female reproductive tract. *Cancer Research*, 1990; **50**:6139-3145.

Esteller, M., Catasus, L.L., Matias-Guiu, X., Mutter, G.L., Prat, J., Baylin, S.B. & Herman, J.G. *hMLH1* promoter hypermethylation is an early event in human endometrial tumourigenesis. *American Journal of Pathology*, 1999; **155**:1767-1772.

Esteller, M., Levine, R., Baylin, S. B., Ellenson, L.H. & Herman, J.G. *hMLH1* promoter hypermethylation is associated with the microsatellite instability phenotype in sporadic endometrial carcinoms. *Oncogene*, 1998; **17**:2413-2417.

Ewertz, M., Schou, G. & Boice, J.D. The joint effect of risk factors on endometrial cancer. *European Journal for Cancer and Clinical Oncology*, 1988; **24**:189-194.

Fanning, A.S. & Anderson, J.M. Protein molecules as organizers of membrane structure. *Current Opinion in Cell Biology*, 1999;**11**:432-439.

Fauman, E.B. & Saper, M.A. Structure and function of the protein tyrosine phosphatases. *Trends in Biochemical Science*, 1996; **21**:413-417.

Fearon, E.R. & Vogelstein, B.A. A genetic model for colorectal tumourigenesis. *Cell*, 1990: **61**(5):759-767.

Fei, G., Ebert, M.P., Mawrin, C., Leodolter, A., Schmidt, N., Dietzmann, K. & Malfertheiner, P. Reduced PTEN expression in gastric cancer and in the gastric mucosa of gastric cancer relatives. *European Journal of Gastroenterology and Hepatology*, 2002; **14**:297-303.

Fox, H. The female reproductive tract and breast. In: MacSween, R.N.M. & Whaley, K., eds. *Muir's Textbook of Pathology*, 13th ed. Edward Arnold, 1992:1014-1025.

Franke, T.F. & Kaplan, D.R. PI3K: Downstream AKTion blocks apoptosis. *Cell*, 1997; **88**:435-437.

Fujii, G.H., Morimoto, A.M., Berson, A.E. & Bolen, J.B. Transcriptional analysis of *PTEN/MMAC1* pseudogene, Ψ*PTEN*. *Oncogene*, 1999; **18**:1765-1769.

Fukuchi, T., Sakamoto, M., Btsuda, H., Maruyama, K., Nozawa, S. & Hirohashi, S. Beta-catenin mutations in carcinoma of the uterine endometrium. *Cancer Research*, 1998; **58**:3526-3528.

Gallup, D.G. & Stock, R.J. Adenocarcinoma of the endometrium in women 40 years of age and younger. *Obstetrics and Gynecology*, 1984; **64**:417-420.

Gelehrter, T.D., Collins, F.S. & Ginsburg, D. Molecular Genetics: Gene organization, regulation and manipulation. In: Gelehrter, T.D., Collins, F.S. & Ginsburg, D. eds. *Principles of Medical Genetics*, 2nd ed. Williams & Wilkins, 1998: 61-89.

Georgescu, M., Kirsch, K.H., Akagi, T. Shishido, T & Hanafusa, H. The tumour-suppressor activity of PTEN is regulated by its carboxy-terminal region. *Proceedings of the National Academy of Science of the USA*, 1999; **96**:10182-10187.

Giancotti, F.G. & Ruoslahti, E. Integrin signaling. *Science*, 1999; **285**:1028-1032.

Gray, I.C., Stewart, L.M.D., Phillips, S.M.A., Hamilton, J.A., Gray, N.E. Watson, G.J., Spurr, N.K. & Snary, D. Mutation and expression analysis of the putative prostate tumour-suppressor gene *PTEN*. *British Journal of Cancer*, 1998; **78**:1296-1300.

Gu, J., Tamura, M. & Yamada, K.M. Tumour suppressor PTEN inhibits integrin- and growth factor-mediated mitogen-activated protein (MAP) kinase signaling pathway. *Journal of Cell Biology*, 1998; **143**:1375-1383.

Guldberg, P., Thor Straten, P., Birck, A., Ahrenkiel, V., Kirkin, A. F. & Zeuthen, J. Disruption of the *MMAC1/PTEN* gene by deletion or mutation is a frequent event in malignant melanoma. *Cancer Research*, 1997; **57**:3660-3663.

Gurin, C.C., Federici, M.G., Kang, L. & Boyd, J. Causes and consequences of microsatellite instability in endometrial carcinoma. *Cancer Research*, 1999; **59**:462-466.

Han, S., Kato, H., Kato, S., Suzuki, T., Shibata, H., Ishii, S., Shiiba, K., Matsuno, S., Kanamaru, R. & Ishioka, C. Functional evaluation of *PTEN* missense mutations using *in vitro* phosphoinositide phosphatase assay. *Cancer Research*, 2000; **60**:3147-3151.

Hanks, S.K. & Polte, T.R. Signaling through focal adhesion kinase. *Bioessays*, 1997; **19**:137-145.

Hayashi, K. PCR-SSCP: A simple and sensitive method for detection of mutations in the genomic DNA. *PCR Methods and Application*, 1991; 1:34-38.

Hayashi, K. & Yandell, D.W. How sensitive is PCR-SSCP? *Human Mutation*, 1993; **2**:338-346.

Ignar-Trowbridge, D. Risinger, J.I., Dent, G.A., Kohler, M., Berchuck, A., McLachlan, J.A. & Boyd, J. Mutations of the Ki-*ras* oncogene in endometrial carcinoma. *American Journal of Obstetrics and Gynecology*, 1992; **167**:227-232.

Ilyas, M. & Tomlinson, I.P.M. The interactions of APC, E-cadherin and beta-catenin in tumour development and progression. *Journal of Pathology*, 1997; **182**:128-137.

Jones, P.A. & Laird, P.W. Cancer epigenetics comes of age. *Nature Genetics*, 1999; **21**:163-167.

Kapeller, R. & Cantley, L.C. Phosphoinositol 3-kinase. *Bioessays*, 1994; **16**:565-576.

Kaplan, K.B., Bibbins, K.B., Swedlow, J.R., Arnaud, M., Morgan, D.O. & Varmus, H.E. Association of the amino-terminal half of c-Src with focal adhesions alters their properties and is regulated by phophorylation of tyrosine 527. *The EMBO Journal*, 1994; **13**:4745-4756.

Kinzler, K.W. & Vogelstein, B. Lessons from hereditary colorectal cancer. *Cell*, 1996; **87**(2):159-170.

Kohler, M.F., Berchuck, A., Davidoff, A.M., Humphrey, P.A., Dodge, R.K., Iglehart, D., Soper, J.T., Clarke-Pearson, D.L., Bast, R.C. & Marks, J.R. Overexpression and mutation of *p53* in endometrial carcinoma. *Cancer Research*, 1992, **52**:1622-1627.

Kohler, M.F., Carney, P., Dodge, R., Soper, J.T., Clarke-Pearson, D.L., Marks, J.R. & Berchuck, A. *p53* overexpression in advanced-stage endometrial adenocarcinoma. *American Journal of Obstetrics and Gynecology*, 1996; 175:1246-1252.

Kong, D., Suzuki, A., Zou, T., Sakurada, A., Kemp, L.W., Wakatsuki, S., Yokoyama, T., Yamakawa, H., Furukawa, T., Sato, M., Ohuchi, N., Sato, S., Yin, J., Wang, S., Abraham, J.M., Souza, R.f., Smolinski, K.N., Meltzer, S.J. & Horli, A. *PTEN1* is frequently mutated in primary endometrial carcinomas. *Nature Genetics*, 1997; **17**:143-144.

Konopka, B., Paszko, Z., Janiec-Jankowska, A. & Goluda, M. Assessment of the quality and frequency of mutations occurrence in *PTEN* gene in endometrial carcinomas and hyperplasias. *Cancer Letters*, 2002; **178**:43-51.

Kurman, R.J., Kaminski, P.F. & Norris, H.J. The behaviour of endometrial hyperplasia: A long-term study of "untreated" hyperplasia in 170 patients. *Cancer*, 1985; **56**:403-412.

Kurman, R.J. & Norris, H.J. Evaluation of criteria for distinguishing atypical endometrial hyperplasia from well differentiated carcinoma. *Cancer*, 1982; **49**:2547-2559.

Lagarda, H., Catasus, L., Arguelles, R., Matia-Guiu, X. & Prat, J. K-*ras* mutations in endometrial carcinoma with microsatellite instability. *Journal of Pathology*, 2001; **193**:193-199.

Lax, S.F., Kendall, B., Tashiro, H., Slebos, R.J.C. & Ellenson, L.H. The frequency of *p53*, K-*ras* mutations and microsatellite instability differs in uterine endometrioid and serous carcinoma: Evidence of distinct molecular genetic pathways. *Cancer*, 2000; **88**:814-824.

Lee, J., Yang, H., Georgescu, M., Di Cristofano, A., Maehama, T., Shi, Y., Dixon, J.E., Pandolfi, P. & Pavletich, N.P. Crystal structure of the *PTEN* tumour suppressor: Implications for its phosphoinositide phosphatase activity and membrane association. *Cell*, 1999; **99**:323-334.

Levine, A.J. Tumour suppressor genes. *Scientific American*, 1995; **272**(1): 28-37.

Levine, R.L., Cargile, C.B., Blazes, C.B., Van Rees, B., Kurman, R.J. & Ellenson, L.H. *PTEN* mutations and microsatellite instability in complex atypical hyperplasia, a precursor lesion to uterine endometrioid carcinoma. *Cancer Research*, 1998; **58**:3254-3258.

Li, D. & Sun, H. *TEP1*, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor β. *Cancer Research*, 1997; **57**:2124-2129.

Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S.I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S.H., Giovanella, B.C., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M.H. & Parsons, R. *PTEN*, a putative protein tyrosine phosphatase gene mutated in human brain, breast and prostate cancer. *Science*, 1997; **275**:1943-1947.

Liaw, D., Marsh, D.J., Li, J., Dahia, P.L.M., Wang, S.I., Zheng, Z., Bose, S., Call, K., Tsou, H.C., Peacocke, M., Eng, C. & Parsons, R. Germline mutations of the *PTEN* gene in Cowden disease, an inherited breast and thyroid cancer syndrome. *Nature Genetics*, 1997; **16**:64-67.

Lin, W.M., Forgacs, E., Warshal, I., Martin, J.S., Ashfaq, R. & Muller, C.Y. Loss of heterozygosity and mutational analysis of the *PTEN/MMAC1* gene in synchronous endometrial and ovarian carcinomas. *Clinical Cancer Research*, 1998; **4**:2577-2583.

Liu, W., James, C.D., Frederick, L., Alderete, B.E. & Jenkins, R.B. *PTEN/MMAC1* mutations and EGFR amplification in glioblastomas. *Cancer Research*, 1997; **57**:5254-5257.

Loeb, L.A. Mutator phenotype may be required for multistage carcinogenesis. *Cancer Research*, 1991; **51**:3075-3079.

Longy, M., Coulon, V., Duboué, B., David, A., Larrègue, M., Eng, C., Amati, P., Kraimps, J., Bottani, A., Lacombe, D. & Bonneau, D. Mutations of *PTEN* in patients with Bannayan-Riley-Ruvalcaba phenotype. *Journal of Medical Genetics*, 1998; **35**:886-889.

Lukes, A.S., Kohler, M.F., Pieper, C.F., Kerns, B.J., Bentley, R., Rodriquez, G.C., Soper, J.T., Clarke-Pearson, D.L., Bast, R.C. & Berchuck, A. Multivariable analysis of DNA ploidy, p53 and HER-2/neu as prognostic factors in endometrial cancer. *Cancer*, 1994; **73**:2380-2385.

Lynch, H.T., Kimberling, W., Albano, W.A., Lynch, J.F., Biscone, K., Schuelke, G.S., Sandberg, A.A., Lipkin, M., Deschner, E.E., Mikol, Y.B., Elston, R.C., Bailey-Wilson, J.E. & Danes, B.S. Hereditary nonpolyposis colorectal cancer (Lynch syndromes I and II). *Cancer*, 1985; **56**:934-938.

Lynch, E.D., Ostermeyer, E.A., Lee, M.K., Arena, J.F., Ji, H., Dann, J., Swisshelm, K., Suchard, D., MacLeod, P.M., Kvinnsland, S., Gjertsen, B.T., Heimdal, K., Lubs, H., Moller, P., King, M. Inherited mutations in *PTEN* that are associated with breast cancer, Cowden disease and juvenile polyposis. *American Journal of Human Genetics*, 1997; **61**:1254-1260.

MacDonald, F. & Ford, C.H.J. Oncogenes. Tumour suppressor genes. Cell cycle control and mismatch repair genes In: MacDonald, F. & Ford, C.H.J., eds. *Molecular Biology of Cancer,* 1st ed. Bios Scientific Publishers, 1997:13-72.

Maehama, T. & Dixon, J.E. The tumour suppressor, *PTEN/MMAC1*, dephosphorylates the lipid second messenger phosphoinositol 3,4,5-triphosphate. *Journal of Biological Chemistry*, 1998; **273**:13375-13378.

Maehama, T. & Dixon, J.E. PTEN: A tumour suppressor that functions as a phospholipid phosphatase. *Trends in Cell Biology*, 1999; **9**:125-128.

Marsh, D.J., Coulon, V., Lunetta, K.L., Rocca-Serra, P., Dahia, P.L.M., Zheng, Z., Liaw, D., Caron, S., Duboué, B., Lin, A.Y., Richardson, A., Bonnetblanc, J., Bressieux, J., Cabarrot-Moreau, A., Chompret, A., Demange, L., Eeles, R.A., Hodgson, S.V., Huson, S., Lacombe, D., LePrat, F., Odent, S., Toulouse, C., Olopade, O.I., Sobol, H., Tishler, S., Woods, C.G., Robinson, B.G., Weber, H.C., Parsons, R., Peacocke, M., Longy, M. Eng, C. Mutation spectrum and genotype-phenotype analyses in Cowden disease and Bannayan-Zonana syndrome, two hamartoma syndromes with germline *PTEN* mutations. *Human Molecular Genetics*, 1998; **7**(3):507-515.

Marsh, D.J., Kum, J.B., Lunetta, K.L., Bennett, M.J., Gorlin, R.J., Ahmen, S.F., Bodurtha, J., Crowe, C., Curtis, M.A., Dasouki, M., Dunn, T., Feit, H., Geraghty, M.T., Graham, J.M., Hodgson, S.V., Hunter, A., Korf, B.R., Manchester, D., Meisfeldt, S., Murday, V.A., Nathanson, K.L., Parisi, M., Pober, B., Romano, C., Tolmie, J.L., Trembath, R., Winter, R.M., Zackai, E.H., Zori, R.T., Weng, L., Dahia, P.L.M. & Eng, C. *PTEN* mutation spectrum and genotype-phenotype correlations in Bannayan-Riley-Ruvalcaba syndrome suggest a single entity with Cowden syndrome. *Human Molecular Genetics*, 1999; **8**:1461-1472.

Marsh, D.J., Dahia, P.L.M., Zheng, Z., Liaw, D., Parsons, R., Gorlin, R.J. & Eng, C. Germline mutations in *PTEN* are present in Bannayan-Zonana syndrome. *Nature Genetics*, 1997; **16**:333-334.

Marshall, C.J. Tumour suppressor genes. *Cell*, 1991; **64**:313-326.

Matias-Guiu, X., Catasus, L., Bussaglia, E., Lagarda, H., Garcia, A., Pons, A., Muñoz, J., Argüelles, R., Machin, P & Prat, J. Molecular pathology of endometrial hyperplasia and carcinoma. *Human Pathology*, 2001; **32**:569-577.

Maxwell, G.L., Risinger, J.I., Gumbs, C., Shaw, H., Bentley, R.C., Barrett, J.C., Berchuck, A. & Futreal P.A. Mutation of the *PTEN* tumor suppressor gene in endometrial hyperplasias. *Cancer Research*, 1998a; **58**:2500-2503.

Maxwell, G.L., Risinger, J.I., Hayes, K.A., Alvarez, A.A., Dodge, R.K., Barrett, J.C. & Berchuck, A. Racial disparity in the frequency of *PTEN* mutations, but not microsatellite instability, in advanced endometrial cancers. *Clinical Cancer Research*, 2000; **6**:2999-3005.

Maxwell, G.L., Risinger, J.I., Tong, B., Shaw, H., Barrett, J.C., Berchuck, A. & Futreal, P.A. Mutation of the *PTEN* tumour suppressor gene is not a feature of ovarian cancers. *Gynecologic Oncology*, 1998b; **70**:13-16.

Mayo, L.D. & Donner, D.B. A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. *Proceedings of the National Academy of Science of the USA*, 2001, **98**:11598-11603.

Mayo, L.D. & Donner, D.B. The PTEN, Mdm2, p53 tumour suppressor-oncoprotein network. *Trends in Biochemical Sciences*, 2002; **27**(9):462-467.

Mayo, L.D., Dixon, J.E., Durden, D.L., Tonks, N.K. & Donner, D.B. PTEN protects p53 from Mdm2 and sensitizes cancer cells to chemotherapy. *Journal of Biological Chemistry*, 2002; **277**:5484-5489.

Mazure, N.M., Chen, E.Y., Laderoute, K.R. & Giaccia, A.J. Induction of vascular endothelial growth factor by hypoxia is modulated by a phosphatidylinositol 3-kinase/Akt signaling pathway in Ha-*ras*-transformed cells through a hypoxia inducible factor-1 transcriptional element. *Blood*, 1997; **90**:3322-3331.

Mirabelli-Primdahl, L., Gryfe, R., Kim, H., Millar, A. Luceri, C., Dale, D., Holowaty, E., Bapat, B., Gallinger, S. & Redston, M. Beta-catenin mutations are specific for colorectal carcinomas with microsatellite instability but occur in endometrial carcinomas irrespective of mutator pathways. *Cancer Research*, 1999; **59**:3346-3351.

Morin, P.J., Sparks, A.B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B. & Kinzler, K.W. Activation of β-cadherin-Tcf signaling in colon cancer by mutations in β-cadherin or *APC*. *Science*, 1997; **275**:1787-1790.

Mutter, G.L., Lin, M., Fitzgerald, J.T., Kum, J.B., Baak, J.P.A., Lees, J.A., Weng, L. & Eng, C. Altered PTEN expression as a diagnostic marker for the earliest endometrial precancers. *Journal of the National Cancer Institute*, 2000a; **92**(11):924-931.

Mutter, G.L., Lin, M., Fitzgerald, J.T., Kum, J.B. & Eng, C. Changes in endometrial PTEN expression throughout the human menstrual cycle. *The Journal of Clinical Endocrinology and Metabolism*, 2000b; **85**(6):2334-2338.

Myers, M.P., Pass, I., Batty, I.H., Van der Kaay, J., Stolarov, J.P., Hemmings, B.A., Wigler, M.H., Downes, C.P. & Tonks, N.K. The lipid phosphatase activity of PTEN is critical for its tumour suppressor function. *Proceedings of the National Academy of Science of the USA*, 1998; **95**:13513-13518.

Myers, M.P., Stolarov, J.P, Eng, C., Li, J., Wang, S.I., Wigler, M.H., Parsons, R. Tonks, N.K. PTEN, the tumour suppressor from human chromosome 10q23, is a dual specific phosphatase. *Proceedings of the National Academy of Science of the USA*, 1997; **94**:9052-9057.

Nakai, K. & Sakamoto, H. Construction of a novel database containing aberrant splicing mutations of mammalian genes. *Gene*, 1994; **141**:171-177.

Navari, J.R., Roland, P.H.Y., Keh, P. Salvesen, H.B., Akslen, L.A., Iversen, O.E., Das, S., Kothari, R., Howey, S. & Phillips, B. Loss of estrogen receptor expression in endometrial tumours is not associated with the *de novo* methylation of the 5' end of the ER gene. *Clinical Cancer Research*, 2000; **6**:4026-4032.

Neel, B.G. & Tonks, N.K. Protein tyrosine phosphatases in signal transduction. *Current Opinion in Cell Biology*, 1997; **9**:193-204.

Nelen, M.R., Padberg, G.W., Peeters, E.A.J., Lin, A.Y., Van den Helm, B., Frants, R.R., Coulon, V., Goldstein, A.M., Van Reen, M.M.M., Easton, D.F., Eeles, R.A., Hodgson, S., Mulvihill, J.J., Murday, V.A., Tucker, M.A., Tucker, M.A., Mariman, E.C.M., Starink, T.M., Ponder, B.A.J., Ropers, H.H., Kremer, H., Longy, M. & Eng, C. Localization of the gene for Cowden disease to 10q22-23. *Nature Genetics*, 1996; **13**:114-116.

Obata, H. , Morland, S.J., Watson, R.H., Hitchcock, A., Chenevix-Trench, G., Thomas, E.J. & Campbell, I.G. Frequent *PTEN/MMAC1* mutations in endometrioid but not serous or mucinous epithelial ovarian tumours. *Cancer Research*, 1998; **58**:2095-2097.

Okamoto, A., Sameshima, Y., Yamada, S., Terashima, Y., Teada, M. & Yokota, J. Allelic loss on chromosome *17p* and *p53* mutations in human endometrial carcinoma of the uterus. *Cancer Research*, 1991; **51**:5632-5636.

Okami, K., Wu, L., Riggins, G., Cairns, P., Goggins, M., Evron, E., Halachmi, N., Ahrendt, S.A., Reed, A.L., Hilgers, W., Kern, S.E., Koch, W.M., Sidransky, D. & Jen, J. Analysis of *PTEN/MMAC1* alterations in aerodigestive tract tumours. *Cancer Research*, 1998; **58**:509-511.

Ong, S.H., Hadari, Y.R., Gotoh, N., Guy, G.R., Schlessinger, J. & Lax, I. Stimulation of phosphatidylinositol 3-kinase by fibroblast growth factor receptors is mediated by coordinated recruitment of multiple docking proteins. *Proceedings of the National Academy of Science of the USA*, 2001; **98**:6074-6079.

Ouyang, H., Shiwaku, H.O., Hagiwara, H., Miura, K., Abe, T., Kato, Y., Ohtani, H., Shiiba, K., Souza, R.F., Meltzer, S.J. & Horii, A. The insulin-like growth factor II receptor gene is mutated in genetically unstable cancers of the endometrium, stomach and colorectum. *Cancer Research*, 1997; **57**:1851-1854.

Ouyang, H., Furukawa, T., Abe, T., Kato, Y. & Horii, A. The *BAX* gene, the promoter of apoptosis is mutated in genetically unstable cancers of the colorectum, stomach and endometrium, *Clinical Cancer Research*, 1998; **4**:1071-1074.

Parazzini F., Negri, E., La Vecchia, C., Bruzzi, P. & Decarli, A. Population attributable risk for endometrial cancer in northern Italy. *European Journal for Cancer Clinical Oncology*, 1989; **25**:1451-1456.

Parazzini, F., La Vecchia, C., Bocciolone, L. & Franceschi, S. The epidemiology of endometrial cancer. *Gynecologic Oncology*, 1991; **41**:1-16.

Parazzini, F., Franceschi, S., La Vecchia, C., Chatenoud, L. & Di Cintio, E. The epidemiology of female genital tract cancers. *International Journal of Gynecologic Cancer*, 1997; **7**:169-181.

Parkin, D.M., Läärä, E. & Muir, C.S. Estimates of the worldwide frequency of sixteen major cancers in 1980. *International Journal of Cancer*, 1989; **41**:184-197.

Parsons, J.P. Integrin-mediated signalling: Regulation by protein tyrosine kinases and small GTP-binding proteins. *Current Opinion of Cell Biology*, 1996; **8**:146-152.

Parsons, R. Phosphatases and tumourigenesis. *Current Opinion in Oncology*, 1998; **10**:88-91.

Peiffer, S.L. Herzog , T.J., Tribune, D.J. Mutch, D.J., Gersell, D.J. & Goodfellow, P.J. Allelic loss of sequences from the long arm of chromosome 10 and replication errors in endometrial cancers. *Cancer Research*, 1995; **55**:1922-1926.

Perren, A., Weng, L.P., Boag, A.H., Ziebold, U., Thakore, K., Dahia, P.L., Komminoth, P., Lees, J.A., Mulligan, L.M., Mutter, L.M. & Eng, C. Immunohistochemical evidence of loss of PTEN expression in primary ductal adenocarcinoma of the breast. *American Journal of Pathology*, 1999; **155**:1253-1260.

Peterson, E.P. Endometrial cancer in younger women: A clinical profile. *Obstetrics and Gynecology*, 1968; **31**:702-707.

Podsypanina, K., Ellenson, L.H., Nemes, A., Gu, J., Tamura, M., Yamada, K.M., Cordon-Cardo, C., Catoretti, G., Fisher, P.E. & Parsons, R. Mutation of *PTEN/MMAC1* in mice causes neoplasia in multiple organ systems. *Proceedings of the National Academy of Science of the USA*, 1999; **96**:1563-1568.

Prat, J. Female reproductive system. In: Damjanov, I. & Linder, J. eds. *Anderson's Pathology*, 10th ed. Mosby, 1996(2): 2261 – 2271.

Prives, C. Signaling to p53: Breaking the Mdm2-p53 circuit. *Cell*, 1998; **95**:5-8.

Rasheed, B.K.A., Stenzel, T.T., McLendon, R.E., Parsons, R., Friedman, A.H., Friedman, H.S., Bigner, D.D. & Bigner, S.H. *PTEN* gene mutations are seen in high-grade but not in low-grade gliomas. *Cancer Research*, 1997; **57**:4187-4190.

Rhei, E., Kang, L., Bogomolniy, F., Federici, M.G., Borgen, P.I. & Boyd, J. Mutation analysis of the putative tumour suppressor gene *PTEN/MMAC1* in primary breast carcinomas. *Cancer Research*, 1997; **57**:3657-3659.

Risinger, J.I., Berchuck, A., Kohler, M.F., Watson, P., Lynch, H.T. & Boyd, J. Genetic instability of microsatellites in endometrial carcinoma. *Cancer Research*, 1993; **53**:5100-5103.

Risinger, J.I., Hayes, A.K., Berchuck,A. & Barrett, J.C. *PTEN/MMAC1* mutations in endometrial cancers. *Cancer Research*, 1997; **57**:4736-4738.

Risinger, J.I., Hayes, K., Maxwell, L., Carney, M.E., Dodge, R.K., Barrett, C. & Berchuck, A. *PTEN* mutations in endometrial cancers is associated with favourable clinical and pathologic characteristics. *Clinical Cancer Research*, 1998; **4**:3005-3010.

Rizo, J. & Südhof, T.C. C2 domains, structure and function of a universal Ca^{2+}-binding domain. *Journal of Biological Chemistry*, 1998; **273**:15879-15882.

Ronnet, B.M., Zaino, R.J., Ellenson, L.H. & Kurman, R.J. Endometrial carcinoma In: Kurman R.J. ed. *Blaustein's pathology of the female genital tract*, 5th ed. Springer-Verlag, 2002:501-558.

Rose, P.G. Endometrial carcinoma. *The New England Journal of Medicine*, 1996; **335**:640-649.

Sato, N., Tsunoda, H., Nishida, M., Morishita, Y., Takimoto, Y., Kubo, T. & Noguchi, M. Loss of heterozygosity on 10q23.3 and mutation of the tumour suppressor gene *PTEN* in benign endometrial cyst of the ovary: Possible sequence progression from benign endometrial cyst to endometrial carcinoma and clear cell carcinoma of the ovary. *Cancer Research*, 2000; **60**:7052-7056.

Saegusa, M., Hashimura, M. & Okayasu, I. Beta-catenin mutations and aberrant expression in endometrial tumourigenesis. *British Journal of Cancer*, 2001; **84**(2): 209-217.

Sakai, A., Thieblemont, C., Wellman, A., Jaffe, E.S. & Raffeld, M. *PTEN* gene alterations in lymphoid neoplasms. *Blood*, 1998; **92**:3410-3415.

Salvesen, H. B., Das, S. & Akslen, L.A. Loss of nuclear p16 protein expression is not associated with promotor methylation but defines a subgroup of aggressive endometrial carcinomas with poor prognosis. *Clinical Cancer Research*, 2000; **6**: 153-159.

Sasaki, H., Nishii, H., Tada, A., Furusato, M., Terahima, Y., Siegal, G.P., Parker, S.L., Kohler, M.F., Berchuck, A. & Boyd, J. Mutation of the Ki-*ras* proto-oncogene in human endometrial hyperplasia and carcinoma. *Cancer Research*, 1993; **53**:1906-1910.

Schaller, M.D., Otey, C.A., Hildebrand, J.D. & Parsons, T. Focal adhesion kinase and paxillin bind to peptides mimicking β integrin cytoplasmic domains. *Journal of Cell Biology*, 1995; **130**:1181-1187.

Schlaepfer, D.D., Hanks, S.K., Hunter, T & Van der Geer, P. Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature*, 1994; **372**:786-791.

Schlaepfer, D.D., Broome, M.A. & Hunter, T. Fibronectin-stimulated signaling from a focal adhesion kinase-c-Src complex: Involvement of the Grb2, $p130^{Cas}$ and Nck adaptor proteins. *Molecular and Cellular Biology*, 1997:**17**:1702-1713.

Schlaepfer, D.D. & Hunter T. Focal adhesion kinase overexpression enhances ras-dependent integrin signaling to ERK2/mitogen-activated protein kinase through interactions with and activation of c-Src. *Journal of Biological Chemistry*, 1997; **272**:13189-13195.

Schottenfeld, D. Determinants of black/white survival differences among women with endometrial cancer. *Gynecologic Oncology*, 1995; **56**:151-153.

Schwartz, M.A. Integrins, oncogenes and anchorage independence. *Journal of Cell Biology*, 1997; **139**:575-578.

Scully, R.E., Bonfiglio, T.A., Kurman, R.J., Silveberg, S.G. & Wilkinson, E.J. In: Scully, R.E., Poulsen, H.E., Sobin, L.H., eds. *Histological typing of the female genital tract tumours.* 2nd ed. Springer-Verlag, 1994.

Serunian, L.A., Auger, K.R., Roberts, T.M. & Cantley, L.C. Production of novel polyphosphoinositides *in vivo* is linked to cell transformation by polyomavirus middle T antigen. *Journal of Virology*, 1990; **64**:4718-4725.

Simpkins, S.B., Peiffer-Schneider, S., Mutch, D.G., Gersell, D. & Goodfellow, P.J. *PTEN* mutation in endometrial cancers with 10q LOH: Additional evidence for the involvement of multiple tumour suppressors. *Gynecologic Oncology*, 1998; **71**:391-395.

Sitas, F., Madhoo, J. & Wessie, J. Incidence of histologically diagnosed cancer in South Africa, 1993 - 1995. *National Cancer Registry of South Africa*, South African Institute of Medical Research, Johannesburg, 1998.

Smith, J.M., Kirk, E.P., Theodosopoulos, G., Marshall, G.M., Walker, J., Rogers, M., Field, M., Brereton, J.J. & Marsh, D.J. Germline mutation of the tumour suppressor *PTEN* in Proteus syndrome. *Journal of Medical Genetics*, 2002, **39**:937-940.

Stambolic, V., Suzuki, A., De la Pompa, J.L., Brothers, G.M., Mirtsos, C., Sasaki, T., Ruland, J., Penninger, J.M., Siderovski, D.P. & Mak, T.W. Negative regulation of PKB/Akt-dependant cell survival by the tumour suppressor PTEN. *Cell*, 1998; **95**:29-39.

Stambolic, V., Tsao, M., Macpherson, D., Suzuki, A., Chapman, W.B. & Maj, T.W. High incidence of breast and endometrial neoplasia resembling human Cowden syndrome in *PTEN* +/- mice. *Cancer Research*, 2000; **60**:3605-3611.

Starink, T.M., Van der Veen, J.P.W., Arwert, F., De Waal, L.P., De Lange, G.G., Gille, J.J.P. & Eriksson, A.W. The Cowden syndrome: A clinical and genetic study in 21 patients. *Clinical Genetics*, 1986; **29**:222-233.

Steck, P.A., Pershouse, M.A., Jasser, S.A., Yung, W.K.A., Lin, H., Ligon, A.H., Langford, L.A., Baumgard, M.L. Hattier, T., Davis, T., Frye, C., Hu, R., Swedlund, B., Teng, D.H.F. & Tavtigian, S.V. Identification of a candidate tumour suppressor gene, *MMAC1*, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nature Genetics*, 1997; **15**:356-362.

Stuckey, J.A., Schubert, H.L., Fauman, E.B., Zhang, Z., Dixon, J.E. & Saper, M.A. Crystal structure of *Yersinia* protein tyrosine phosphatase at 2.5Ă and the complex with tungstate. *Nature*, 1994; **370**:571-575.

Sun, H., Enomoto, T., Fujita, M., Wada, H., Yoshino, K., Ozaki, K., Nakamura, T. & Murata, Y. Mutational analasis of the *PTEN* gene in endometrial carcinoma and hyperplasia. *American Journal of Clinical Pathology*, 2001; **115**:32-38.

Suzuki, A., De la Pompa, J.L., Stambolic, V., Elia, A.J., Sasaki, T., Barrantes, I.D., Ho., A., Wakeham, A., Itie, A., Khoo, W., Fukumoto, M. & Mak, T.W. High cancer susceptibility and embryonic lethality associated with mutation of the *PTEN* tumour suppressor gene in mice. *Current Biology*, 1998a; **8**:1169-1178.

Suzuki, H., Freije, D., Nusskern, D.R., Okami, K., Cairns, P., Sidransky, D., Isaacs, W.B. & Bova, G.S. Interfocal heterogeneity of *PTEM/MMAC1* gene alterations in multiple metastatic prostate cancer tissues. *Cancer Research*, 1998b; **58**:204-209.

Takahashi-Tezuka, M., Yoshida, Y., Fukada, T., Ohtani, T., Yamanaka, Y., Nishida, K., Nakajima, K., Hibi, M. & Hirano, T. Gab1 acts as an adapter molecule linking the cytokine receptor gp130 to ERK mitogen-activated protein kinase. *Molecular and Cellular Biology*, 1998; **18**:4109-4117.

Tamura, M., Gu, J., Matsumoto, K., Aota, S., Parsons, R. & Yamada, K.M. Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. *Science*, 1998; **280**:1614-1617.

Tamura, M., Gu, J., Takino, T. & Yamada, K.M. Tumour suppressor PTEN inhibition of cell invasion, migration and growth: Differential involvement of focal adhesion kinase and $p130^{Cas}$. *Cancer Research*, 1999a; **59**:442-449.

Tamura, M., Gu, J., Tran, H. & Yamada, K.M. *PTEN* gene and integrin signaling in cancer. *Journal of the Cancer Institute*, 1999b; **91**(21):1820-1828.

Taniyama, K., Goodison, S., Ito, R., Bookstein, R., Miyoshi, N., Tahara, E., Tarin, D. & Urquidi, V. PTEN expression is maintained in sporadic colorectal tumours. *Journal of Pathology*, 2001; **194**:341-348.

Tashiro, H., Blazes, M.S., Wu, R., Bose, S., Wang, S.I., Li, J., Parsons, R. & Ellenson, L.H. Mutations in *PTEN* are frequent in endometrial carcinoma but rare in other common gynecological malignancies. *Cancer Research*, 1997; **57**:3935-3940.

Teng, D.H., Hu, R., Lin, H., Davis, T., Iliev, D., Frye, C., Swedlund, B., Hansen, K.L., Vinson, V.L., Gumpper, K.L., Ellis, L., El-Naggar, A., Frazier, M., Jasser, S., Langford, L.A., Lee, J., Mills, G.B., Pershouse, M.A., Pollack, R.E., Tornos, C., Troncoso, P., Yung, W.K.A., Fujii, G., Berson, A., Bookstein, R., Bolen, J.B., Tavtigian, S.V. & Steck, P.A. *MMAC/PTEN* mutations in primary tumour specimens and tumour cell lines. *Cancer Research*, 1997; **57**:5221-5225.

Tonks, N.K. & Neel, B.G. From form to function: Signalling by protein tyrosine phosphatases. *Cell*, 1996; **87**:365-368.

Torres, J. & Pulido, R. The tumour suppressor PTEN is phosphorylated by the protein kinase CK2 at its C terminus. Implications for the PTEN stability to proteasome-mediated degradation. *Journal of Biological Chemistry*, 2001; **276**:993-998.

Toyota, M., Ahuja, N., Suzuki, H. Itoh, F., Ohe-Touota, M., Imai, K., Baylin, S.B. & Issa, J.J. Aberrant methylation in gastric cancer associated with the CpG island methylator phenotype. *Cancer Research*, 1999a; **59**:5438-5442.

Toyota, M., Ahuja, N. Ohe-Toyota, M., Herman, J.G., Baylin, S.B. & Issa, J.J. CpG island methylator phenotype in colorectal cancer. *Proceedings of the National Academy of Science of the USA*, 1999b; **96**:8681-8686.

Tsou, H.C., Teng, D.H., Ping, X.L., Brancolini, V., Davis, T., Hu, R., Xie, X.X., Gruener, A.C., Schrager, C.A., Christiano, A.M., Eng, C., Steck, P., Ott, J., Tavtigian, S.V. & Peacocke, M. The role of *MMAC1* mutations in early onset breast: Causative in association with Cowden syndrome and excluded in *BRCA1*-negative cases. *American Journal of Human Genetics*, 1997; **61**:1036-1043.

Tsou, H.C., Ping, X.L., Xie, X.X., Gruener, A.C., Zhang, H., Nini, R., Swisshelm, K., Sybert, V., Diamond, T.M., Sutphen, R. & Peacocke, M. The genetic basis of Cowden's syndrome: Three novel mutations in *PTEN/MMAC1/TEP1*. *Human Genetics*, 1998; **102**:467-473.

Tsuchiya, K.D., Wiesner, G., Cassidy, S.B., Limwongse, C., Boyle, J.T. & Schwartz, S. Deletion 10q23.2-q23.33 in a patient with gastrointestinal juvenile polyposis and other features of a Cowden-like syndrome. *Genes, Chromosomes and Cancer*, 1998; **21**:113-118.

Van Rensburg, J.E. The genetic basis of cancer. *Specialist Medicine*, 1997; **19**(5):46-53.

Vanhaesebroeck, B., Leevers, S.J., Panayotou, G. & Waterfield, M.D. Phosphoinositide 3-kinases: A conserved family of signal transducers. *Trends in Biochemical Science*, 1997; **22**:267-272.

Varras, M.N., Koffa, M., Koumantakis, E., Ergazaki, M., Protopapa, E., Michalas, S. & Spandidos, D. *ras* Gene mutations in human endometrial carcinoma. *Oncology*, 1996; **53**:505-510.

Vazquez, F., Ramaswamy, S., Nakamura, N. Sellers, W.R. Phosphorylation of the PTEN tail regulates protein stability and function. *Molecular and Cellular Biology*, 2000; **20**:5010-5018.

Versteeg, R. Aberrant methylation in cancer. *American Journal of Human Genetics*, 1997; **60**:751-754.

Vuori, K. & Ruoslahti, E. Association of insulin receptor substrate-1 with integrins. *Science*, 1994; **266**:1576-1578.

Waite, K.A. & Eng, C. Protean PTEN: Form and Function. *American Journal of Human Genetics*, 2002; **70**:829-844.

Wang, D., Huang, H.S., Kazlauskas, A. & Cavenee, W.K. Induction of vascular endothelial growth factor expression in endothelial cells by platelet-derived growth factor through the activation of phospatidylinositol 3-kinase. *Cancer Research*, 1999; **59**: 1464-1472.

Wang, L., Ignat, A & Axoitis, C.A. Differential expression of the PTEN tumour suppressor protein in fetal and adult neuroendocrine tissues and tumours: Progressive loss of PTEN expression in poorly differentiated neuroendocrine neoplasms. *Applied Immunohistochemistry and Molecular Morphology*, 2002; **10**:139-146.

Wang, Z., Taylor, F., Churchman, M., Norbury, G. & Tomlinson, I. Genetic pathways of colorectal carcinogenesis rarely involve the *PTEN* and *LKB1* genes outside the inherited hamartoma syndromes. *American Journal of Pathology*, 1998; **153**(2):363-366.

Wary, K.K., Mariotti, A., Zurzolo, C. & Giancotti, F.G. A requirement for caveolin-1 and associated kinase Fyn in integrin and achorage-dependant cell growth. *Cell*, 1998; **94**:625-634.

Weinberg, R.A. How cancer arises. *Scientific American*, 1996; **275**(3):62-70.

Weng, P.A., Boag, A.H., Ziebold, U., Thakore, K., Dahia, P.L., Kommonoth, P., Lees, J.A., Mulligan, L.M., Mutter, G.L. & Eng, C. Immunohistochemocal evidence of loss of PTEN expression in primary ductal adenocarcinomas of the breast. *American Journal of Pathology*, 1999a; **155**:1253-1260.

Weng, L., Smith, W.M., Brown, J.L. & Eng, C. PTEN inhibits insulin-stimulated MEK/MAPK activation and cell growth by blocking IRS-1 phosphorylation and IRS-1/Grb-2/Sos complex formation in a breast cancer model. *Human Molecular Genetics*, 2001; **10**(6):605-616.

Weng, L., Smith, W.M., Dahia, P.L.M., Ziebold, U., Gil, E., Lees, J.A. & Eng, C. PTEN suppresses breast cancer cell growth by phosphatase function-dependent G1 arrest followed by apoptosis. *Cancer Research*, 1999b; **59**:5808-5814.

Whang, Y.E., Wu, X. & Sawyers, C.L. Identification of a pseudogene that can masquerade as a mutant allele of the *PTEN/MMAC1* tumour suppressor gene. *Journal of the National Cancer Institute*, 1998; **90**(11):859-861.

Wu, X., Hepner, K., Castelino-Prabhu, S., Do, D., Kaye, M.B., Yuan, X., Wood, J., Ross, C., Sawyers, C.L. & Whang, Y.E. Evidence for regulation of PTEN tumour suppressor by a membrane-localized multi-PDZ domain containing scaffold protein MAGI-2. *Proceedings of the National Academy of Science of the USA*, 2000; **97**:4233-4238.

Yaginuma, Y., Yamashita, T., Ishiya, T., Morizaki, A., Katoh, Y., Takahashi, T., Hayashi, H. & Ishikawa, M. Abnormal structure and expression of *PTEN/MMAC1* gene in human uterine cancers. *Molecular Carcinogenesis*, 2000; **27**:110-116.

Yamada, K.M. & Geiger, B. Molecular interactions in cell adhesion complexes. *Current Opinion in Cell Biology*, 1997; **9**:76-85.

Yart, A., Laffargue, M., Mayeux, P., Chretien, S., Peres, C., Tonks, N., Roche, S., Payrastre, B., Chap., H. & Raynal, P. A critical role for phosphoinositide 3-kinase upstream of Gab1 and SHP2 in the activation of *ras* and mitogen-activated protein kinases by epidermal growth factor. *The Journal of Biological Chemistry*, 2001; **276**:8856-8864.

Yokoyama, Y., Wan, X., Shinohara, A., Takahashi, S., Takahashi, Y., Niwa, K. & Tamaya, T. Expression of *PTEN* and *PTEN* pseudogene in endometrial carcinoma. *International Journal of Molecular Medicine*, 2000; **6**:47-50.

Yuvaniyama, J., Denu, J.M., Dixon, J.E. & Saper, M.A. Crystal structure of the dual specificity protein phosphatase VHR. *Science*, 1996; **272**:1328-1331.

Zhou, X., Marsh, D.J., Hampel, H. Mulliken, J.B., Gimm, O. & Eng, C. Germline and germline mosaic mutations associated with Proteus-like syndrome of hemihypertrophy, lower limb asymmetry, arterio-venous malformations and lipomatosis. *Human Molecular Genetics*, 2000; **9**:765-768.

Zhou, X., Li, Y., Hoang-Xuan, K., Laurent-Puig, P., Mokhtari, K., Longy, M., Sanson, M., Delattre, J., Thomas, G & Hamelin, R. Mutational analysis of the *PTEN* gene in gliomas: Molecular and pathological correlations. *International Journal of Cancer*, 1999; **84**:150-154.

Zundel., W., Schindler, C., Haas-Kogan, D., Koong, A., Kaper, F., Chen, E., Gottschalk, A.R., Ryan, H.E., Johnson, R.S., Jefferson, A.B., Stokoe, D. & Giaccia, A.J. Loss of *PTEN* facilitates HIF-1α-mediated gene expression. *Genes and Development*, 2000; **14**:391-396.

APPENDIX A

ENDOMETRIAL CANCER:

Patient	Ethnic group	Age at diagnosis	Histological type	*Grading	*Staging
END1	African	56	Endometrioid adenocarcinoma	II	III
END2	Caucasian	82	Endometrioid adenocarcinoma	II	Ib
END3	African	75	Papillary serous carcinoma	III	
END4	Caucasian	58	Endometrioid adenocarcinoma	II	IIb
END5	African	45	Endometrioid adenocarcinoma	II	Ia
END6	African	73	Endometrioid adenocarcinoma	II	Ic
END7	Caucasian	74	Endometrioid adenocarcinoma	II	Ia
END8	Indian	47	Endometrioid adenocarcinoma	II	III
END9	African	53	Endometrioid adenocarcinoma	II	III
END10	Caucasian	49	Endometrioid adenocarcinoma	II	Ia
END11	African	65	Endometrioid adenocarcinoma	II	I
END12	Caucasian	72	Endometrioid adenocarcinoma	I	Ic
END13	African	53	Endometrioid adenocarcinoma	II	Ib
END14	Caucasian	47	Endometrioid adenocarcinoma	II	Ib
END15	African	73	Endometrioid adenocarcinoma	II	III
END16	African	71	Endometrioid adenocarcinoma	II	IIb
END17	Caucasian	51	Endometrioid adenocarcinoma	II	Ib
END19	African	53	Endometrioid adenocarcinoma	I	Ic
END20	Caucasian	42	Endometrioid adenocarcinoma	II	Ia
END21	African	60	Endometrioid adenocarcinoma	II	II
END22	African	56	Endometrioid adenocarcinoma	II – III	Ib
END23	African	68	Endometrioid adenocarcinoma	II	III
END24	Caucasian	71	Endometrioid adenocarcinoma	II	III
END25	Caucasian	59	Endometrioid adenocarcinoma	III	III
END26	African	92	Endometrioid adenocarcinoma	II	Ic
END27	Caucasian	70	Endometrioid adenocarcinoma	II	Ic
END28	African	71	Endometrioid adenocarcinoma	II	Ic
END29	African	87	Papillary serous carcinoma	III	
END30	African	61	Endometrioid adenocarcinoma	II	IIIc
END31	African	84	Endometrioid adenocarcinoma	II	Ia
END32	African	43	Endometrioid adenocarcinoma	II	IVa
END33	Caucasian	79	Poorly differentiated carcinoma		
END34	African	57	Endometrioid adenocarcinoma	II	III
END35	African	66	Endometrioid adenocarcinoma	II – III	Ic
END36	African	70	Endometrioid adenocarcinoma	II – III	Ib
END37	African	53	Endometrioid adenocarcinoma	I	Ib
END38	African	64	Endometrioid adenocarcinoma	II	Ib
END39	African	68	Endometrioid adenocarcinoma	II	Ib
END40	African	66	Endometrioid adenocarcinoma	II	Ib
END41	African	62	Endometrioid adenocarcinoma	II	Ia
END42	African	58	Papillary serous carcinoma	III	
END43	African	54	Endometrioid adenocarcinoma	II	III
END44	African	68	Endometrioid adenocarcinoma	II	IIb
END45	African	81	Endometrioid adenocarcinoma	III	IV
END46	Caucasian	76	Endometrioid adenocarcinoma	II	Ib
END47	African	61	Papillary serous carcinoma	II	
END48	Caucasian	78	Endometrioid adenocarcinoma	II	Ic

* Criteria of architectural grading and FIGO staging of endometrioid adenocarcinoma (Appendix B).

ENDOMETRIAL HYPERPLASIA :

Patient	Ethnic group	Age at diagnosis	Histological type
EHYP1	Caucasian	Unknown	Atypical hyperplasia
EHYP2	Caucasian	Unknown	Atypical hyperplasia
EHYP3	Caucasian	Unknown	Atypical hyperplasia
EHYP4A EHYP4C	Caucasian	Unknown	Atypical hyperplasia Complex atypical hyperplasia
EHYP5	Caucasian	Unknown	Atypical hyperplasia
EHYP6	Caucasian	Unknown	Atypical hyperplasia
EHYP7	Caucasian	Unknown	Atypical hyperplasia
EHYP8A EHYP8C	Caucasian	Unknown	Atypical hyperplasia Complex atypical hyperplasia
EHYP9	Caucasian	Unknown	Atypical hyperplasia
EHYP10	Caucasian	Unknown	Atypical hyperplasia