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The role of the tumour suppressor gene PTEN in the etiology of cancers of the female genital tract

by

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Abstrak

Die rol van die tumoronderdrukker geen PTEN in die etiologie van kankers van die vroulike genitale traktus

Fosforilering en defosforilering van die tirosien aminosure in proteine speel ‘n belangrike rol in die regulering van sellulêre prosesse in alle eukariotiese organismes. Dit sluit die regulering van selsikluskontrole, groeikontrole, sellulêre differensiasie sowel as genetiese en sinaptiese oordrag in. Dit word lank reeds gespekuleer dat die fosfatase-gene betrokke is in menslike karsinogenese, maar die PTEN geen is die eerste fosfatase geen wat bewys word om ‘n ware tumoronderdrukker geen te wees. As basiese funksie defosforileer normale PTEN die kinases en inhibeer dit die kinase sinjaal kontrolepaaie wat deur integrien en groeifaktor beheer word.

Die sentrale hipotese van hierdie studie is dat PTEN ‘n belangrike rol speel in tumore van die boonste genitale traktus. Die frekwensie van abnormaliteite in die koderingsareas van hierdie geen is bestudeer in spesifieke ginekologiese tumore en weefsels met die gebruik van polimerase kettingreaksie gebaseerde mutasie-analise. Die maligne tumore sowel as die mees verwante pre-maligne of benigne weefsel- of tumortipes wat beskikbaar was, is gebruik as navorsingsmateriaal om sodoende die verskillende vlakke van PTEN betrokkenheid in die ontwikkeling van neoplasie te demonstreer.

Intieme betrokkenheid van die PTEN geen is gevind in endometriële karsinogenese. PTEN mutasies is in hiperplasie gevind en dit was algemeen in endometroiede karsinoom (54%). Patogene mutasies was baie meer algemeen in kanker as in hiperplasie (10%). Veelvuldige mutasies is in sommige laat stadium tumore aangetoon, wat suggereer dat reeds maligne selle meer genetiese mutasies oor tyd verkry. Alle tumore waar meer as een patogeniese mutasie gevind is het voorgekom by swart pasiënte. Die laaste twee bevindinge is uniek tot hierdie studie.

Selektiewe betrokkenheid van die PTEN geen is gevind in die ontwikkeling van sagte weefsel tumore van die uterus. PTEN mutasies is nie in benigne sagte weefsel tumore gevind nie en geen betekenisvolle betrokkenheid is in leiomiosarkome of endometriële stromale sarkome aangetoon nie. PTEN was egter betekenisvol betrokke in karsinosarkome van die uterus (13%) en veral in tumore met ‘n endometrioiede epiteelkomponent waar mutasies in 17% gevind is. Hierdie bevinding is ‘n hoogs betekenisvolle en unieke navorsingsbevinding wat die hipotese ondersteun dat hierdie tumore uit die endometrium ontstaan. Dit onderskryf ook die indruk dat ‘n sterk band bestaan tussen hierdie geen en endometroiede differensiasie, met morfologie sterk gekoppel aan sellulêre genetika.

Mutasie in die PTEN geen is aangetoon in ovariële endometroiede karsinoom in ~29% van gevalle wat ondersoek is. Die bevinding bevestig PTEN betrokkenheid in karsinogenese in hierdie tumortipe. Weereens toon die resultaat dat PTEN betrokkenheid gekoppel is aan endometroiede morfologie. Die ondersoek van benign of pre-maligne letsels in hierdie orgaan was nie voldoende om kommentaar oor die tydsberekening van mutasie te kan lewer nie.

Met alle tumortipes in ag genome, is daar ‘n tendens aangetoon van minder PTEN mutasies in swart vroue. PTEN mutasies korreleer met endometroiede histologie. In kombinasie bevestig hierdie resultaat ‘n rasse-diskrepansie in die distribusie van tumourtipe of morfologie.

In opsomming is die bevinding van hierdie studie dat daar betekenisvolle dog hoogs selektiewe PTEN geen betrokkenheid in boonste genitale traktus tumore is. ‘n Sterk en interessante verband is bevestig tussen genotipe en histologiese fenotipe. Hierdie resultate verbeter die begrip van karsinogenese en behoort ‘n bydrae te lewer in die soeke na nuwe anti-neoplastiese middels.

Abstract

The role of the tumour suppressor gene PTEN in the etiology of cancers of the female genital tract

The phosphorylation and dephosphorylation of the tyrosine amino-acids in proteins play an important role in the regulation of many cellular processes in all eukariotic organisms, including the regulation of cell cycle control, growth control, cellular differentiation and gene and synaptic transmission. The involvement of the phosphatase genes in human carcinogenesis was long-suspected, but PTEN is the first important phosphatase gene proven to be a true tumour suppressor. The basic function of normal PTEN is the dephosphorylation of the kinases and inhibition of the integrin and growth factor mediated kinase signalling pathways.

The central hypothesis of this study is that PTEN plays an important role in tumours of the upper female genital tract. The involvement of aberrations in the coding regions of this gene was studied in specific gynaecologic tumours and tissues using polymerase chain reaction based mutation analysis. The research model was to study both the malignant tumour and the closest available pre-malignant or benign counterpart to demonstrate different levels of involvement of PTEN in the evolving steps.

The PTEN gene was found to be intimately involved in endometrial carcinogenesis. Involvement was demonstrated in hyperplasia and was common in endometroid carcinoma (54%). Pathogenic PTEN mutations were much more common in cancer than in hyperplasia (10%). Multiple mutations were found in some late stage tumours, suggesting that the already malignant tumour cells accumulate more genetic mutations over time. All tumours with more than one pathogenic mutation occurred in African patients. The latter two findings are unique to the current study.

Selective involvement of the PTEN gene was demonstrated in uterine soft tissue tumours. PTEN involvement was neither found in benign soft tissue tumours nor significantly in leiomyosarcoma or endometrial stromal sarcoma. However, PTEN plays a significant role in uterine carcinosarcoma (13%) and specifically in tumours with an endometroid epithelial component, where mutations were found in 17%. This finding is a highly significant and unique research result which supports the hypothesis of the endometrial origin of these tumours. It also supports the observation of a strong link between this gene and endometroid differentiation, with morphology strongly linked to cellular genetics.

PTEN gene mutation was demonstrated in ovarian endometroid carcinoma in ~29% of cases investigated. This finding confirms PTEN involvement in carcinogenesis in this tumour type. The finding suggests that PTEN involvement is linked to endometroid epithelial morphology. We could not sufficiently test the involvement of the gene in benign or pre-malignant ovarian endometroid lesions and thus cannot comment on the chronology of mutations in this tissue type.

When all tumour types were included, there was a tendency towards a lower frequency of PTEN mutations in African women. PTEN mutations correlated with endometroid histology. In combination, these results confirm the racial disparity in tumour type distribution or morphology.

In summary this study demonstrated significant though highly selective PTEN gene involvement and a strong and interesting association between genotype and histological phenotype was confirmed. The findings enhance our understanding of carcinogenesis and should lead to translational research into new anti-neoplastic drugs.

Short summary

The role of PTEN gene mutation in the evolution of gynaecologic malignancies was analysed using polymerase chain reaction based mutation analysis.

Benign, pre-malignant and malignant tumours of the upper female genital tract were examined. The accumulation of cellular genetic damage during carcinogenesis were studied and compared in the different tissue and tumour types.

The study demonstrated significant though highly selective PTEN gene involvement and a strong and interesting association between genotype and histological phenotype was confirmed.

The findings enhance our understanding of carcinogenesis and should lead to translational research into new therapies.

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1 Introduction to the study

This first chapter serves as an introductory chapter and aims to describe the background to and purpose of the research, the research questions and the objectives. The study will be justified by a discussion of the significance of the research topic.

The literature review will provide an overview of the current knowledge, provide a background to the rest of the work and focus on the intricate role and function of the tumour suppressor PTEN.

Existing knowledge of genetic changes as part of the carcinogenetic process in general, the role and cellular function of kinases and phosphatases and the role and function of the PTEN gene and pten protein will be described.

The outlay of the thesis consisting of three separate studies and the conclusive chapter will be described.

1.1 Background

Carcinogenesis is now widely accepted to be a multistep process where the combination of a few mutational genetic events and subtle changes of the transcribed proteins lead to a widespread disruption of cellular functions. The disruption of proto-oncogenes leading to activation and overexpression of the oncogene protein product as well as inactivation of tumour suppressor genes plays a central role in the pathogenesis of neoplastic disease. In some cancer types the detailed characterization of these abnormalities played an important role in the classification of the disease. In leukemias and lymphomas it led to treatment individualization and defining the prognosis.

The identification of the targets of genetic disruption has greatly advanced our understanding of tumorigenesis. Unravelling the specific functions and the interactions of these different genes will play an important role in furthering our knowledge of anti-tumoral immunity and will in future play an important role in the treatment of neoplastic disease.

Protein tyrosine phosphorylation has long been known to be hugely important in cell cycle and growth control and abnormality of tyrosine phosphorylation has been shown to occur in many human cancer types. Several protein kinases have been implicated as oncogenes and phosphatases have long been known to antagonise the action of the kinases.

PTEN (phosphatase and tensin homologue) is a new and unique tumour suppressor gene found on chromosome 10q23.3. In 1997 three independent research groups published their findings of a candidate tumour suppressor gene located in this region on chromosome ten. The name PTEN (phosphatase and tensin homologue, situated on chromosome ten) was given by J. Li and co-authors (Li et al 1997a) and is now used almost universally. This tumour suppressor has subsequently been found to be intimately involved in a multitude of tumour types and in various parts of the carcinogenetic pathway. Inevitably it was also found to be involved in gynaecological cancers.

The protein product (called pten) of PTEN plays an important role in cytoskeletal organization, cell growth regulation, and apoptosis and has been shown to be frequently mutated in multiple human cancers. PTEN encodes a protein-tyrosine phosphatase and the first protein plays its role as a phosphatase opposing the kinase pathways.

Kinases bring about phosphorylation while removal of the phosphate is controlled by phosphatase-enzymes. More than 95 protein-tyrosine kinases have been identified in humans and more than 55 genes encoding for protein-tyrosine phosphatases are currently known. Although the involvement of the phosphatase genes in human carcinogenesis has long been suspected, PTEN is the first important protein-tyrosine phosphatase gene to be proven to be a true tumour suppressor.

The research road leading to the identification of the PTEN gene started when loss of heterozygosity was found frequently in a variety of human tumours at chromosome ten, region 10q23-25. This alteration was particularly common in brain tumours (Rasheed et al 1995) and prostate cancer (Fults et al 1990).

This novel tumour suppressor gene was mapped to 10q23.3 and it was found to encode a 403 amino acid polypeptide chain. The gene has a coding region of 1 212 nt and consists of nine exons. The protein sequence is similar to that of the cytoskeletal proteins tensin and auxilin. The N-terminal domain of the PTEN-protein shows extensive homology to the cytoskeletal protein tensin, implicating roles for PTEN in the maintenance of cellular structure and in signal transduction (Tamura et al 1999).

1.2 Research questions

The involvement of the PTEN gene and its protein product in gynaecologic cancer is the topic of this study. The current knowledge about the place of PTEN in the carcinogenetic pathway in general and the role of the protein product, pten, will be discussed. The existing knowledge about the different tumour types will be discussed in the relevant chapters.

1.2.1 Endometrial hyperplasia and carcinoma

The most important current deficiencies in knowledge about the involvement of PTEN in endometrial cancer include differences among population groups and the involvement of the gene in early or late carcinogenesis or in cancer progression.

By studying PTEN mutations in endometrial hyperplasia and endometroid adenocarcinoma of different stages and histological grades in two population groups, information on these aspects will be collected.

The research questions for this study will be:

1. What role do PTEN gene mutation and pten protein inactivation play in the etiology of endometrial carcinoma?

2. What is the frequency of PTEN mutations in endometrial cancers and pre-cancers?

3. When in the carcinogenetic process do these mutations occur?

4. How does PTEN mutations correlate with disease stage and grade?

5. How does the involvement of the PTEN gene differ between the different population groups in South Africa?

6. How does the involvement of the gene differ between South African and European patients?

1.2.2 Uterine soft tissue tumours

Little was known about the involvement of PTEN in uterine tumours other than endometroid endometrial cancer before this study was done. The etiopathogenesis of uterine soft tissue tumours in general is also poorly understood. The role of somatic mutations in the PTEN gene in uterine sarcomas and the benign counterparts will be studied.

The research questions for this study will be:

1. What role do PTEN gene mutation and pten protein inactivation play in the etiology of uterine leiomyomas and uterine sarcomas?

2. What is the frequency of PTEN mutations in uterine soft tissue tumours?

3. Can these mutations be predicted by the finding of micro-satellite instability?

4. Can these mutations also be shown in uterine leiomyomas?

5. How does PTEN mutations correlate with histological type, disease stage and grade?

6. How does the involvement of the PTEN gene differ between the different population groups in South Africa?

1.2.3 Endometroid ovarian cancer

The role of the PTEN gene in the etiology of ovarian cancer and in progression of the disease has not been sufficiently investigated. The involvement of this gene in malignant transformation of (ovarian) endometriosis was not studied before 2000 and the role of PTEN in carcinogenesis in the ovary was not evaluated sufficiently.

The research questions for this study will be:

1. What role do PTEN gene mutation and pten protein inactivation play in the etiology of ovarian endometroid adenocarcinoma?
2. What is the frequency of PTEN mutations in these tumours?
3. Can (ovarian) endometriosis be regarded as the benign counterpart or pre-malignant lesion of ovarian endometroid adenocarcinoma?
4. Do PTEN mutations also occur in ovarian endometriosis ?
5. Do PTEN mutations correlate with histological type, disease stage and grade?

1.3 Objectives

The role of this new tumour suppressor gene in gynaecological neoplasms will be studied by analysing the role of PTEN gene mutation in the chain of events leading to a clone of invasive malignant cells in specific gynaecologic tumours and tissues.

2 Justification of the study

2.1 Motivation

PTEN was recently identified as a tumour suppressor gene. Mutations in both germline and somatic cells were linked very convincingly to malignant neoplastic disease.

The function and interaction of this gene in the growth control pathway is so intricate and varied that this tumour suppressor gene is involved in a lot of cross-talk between different pathways.

More knowledge about the function of this important intracellular role player will undoubtedly contribute largely to the understanding of these pathways and carcinogenesis in general.

Understanding carcinogenesis is the gateway to cancer prevention. With its intricate involvement in the inhibition of cellular growth, the understanding of PTEN involvement will also enhance our ability to fight tumour progression using targeted therapies.

Adding new knowledge to the large pool of molecular knowledge on cancer cell growth control will contribute to improve cancer management in future.

2.2 Significance

The findings of the individual studies that will form this thesis will be highly relevant in the South African context. This is the only research study on the role of the PTEN gene in gynaecologic tumours done in South Africa, in Africa or on patients from the continent. Research findings from the rest of the world have thus never been confirmed in this context. It was shown previously that racial and population differences predict important differences in genetic carcinogenesis.

It is of importance for South Africa to build on existing knowledge and skills and to develop the potential of molecular laboratories and of young researchers. All molecular work in the thesis was done at the molecular laboratory of the Cancer Genetics section of the Department of Human Genetics (later a section of Department of Genetics), University of Pretoria. The molecular work done at the Genetics Department of the University of Utrecht was done by the author and was later confirmed at the Cancer Genetics laboratory in Pretoria.

The findings of molecular research studies will usually not be immediately applicable to clinical management. These findings are expected to enhance our understanding of carcinogenetic processes as well as the relations between different benign, pre-malignant and malignant neoplasms. Without doubt the current results will in future become the topic of translational research.

3 Theory base and general literature overview

3.1. Genetic changes as part of the carcinogenetic process

Three groups of genes are known to influence and determine tumorigenesis, namely the DNA repair genes, proto-oncogenes (and oncogenes) and tumour

suppressor genes. These groups, with the most important examples from each group, will be discussed.

3.1.1 DNA repair genes

Recently, micro-satellite instability (MSI) was demonstrated in colon cancer and endometrial cancer as a genetic alteration that occurs in many familial and in some sporadic tumours (Ionov et al 1993; Risinger et al 1993). DNA repair genes have the function of maintaining the integrity and stability of the genome. Abnormality of these genes manifests through the alteration of DNA repeats, called micro-satellites. Because of the repeating nature, these repeated sequences are more prone to **R**eplication **Er**rors, named RER+, which is then recognised by the instability of micro-satellites. In hereditary non-polyposis colorectal carcinoma (HNPCC) these genetic abnormalities are inherited in a dominant fashion, leading to a germline mutation causing the syndrome (Aaltonen et al 1993).

It is now known that inherited mutations in the DNA mismatch repair genes, eg. hMLH1, hMSH2, hMSH6, hPMS2, cause HNPCC. Endometrial cancer is the commonest non-colonic cancer occurring in females born with these mutations, resulting in a lifetime risk of between 22% and 43% (Watson & Lynch 1993; Aarnio et al 1995). Lynch syndrome or HNPCC is the only known familial cancer syndrome that often causes endometrial cancer. Endometrial cancers occurring in females with mutations in these genes will almost invariably demonstrate MSI when tested. Mutations in MSH2 explain 60% of cases and in MLH1 another 30% (Salvesen 2000).

Although MSI is frequently present (15% to 34%) also in sporadic endometrial cancer, somatic mutations in the DNA repair genes are not frequent in these sporadic cases. Instead, it has been shown that the finding of MSI in sporadic cancer correlates strongly with methylation of the hMLH1 promoter region (Simpkins et al 1999), which is an epigenetic finding, causing inactivation of the hMLH1 gene (Salvesen et al 2000). The result on cellular level is the same as a mutation in the gene causing defects in the DNA repair system (Peiffer et al 1995).

3.1.2 Proto-oncogenes and oncogenes

Varmus and Bishop originally described the oncogene hypothesis in 1976 (Maxwell & Carlson 1996). The concept is now well recognised as a common part of carcinogenesis and a huge number of potential oncogenes have since been described. Oncogenes are derived from normal cellular genes normally involved in cell survival and cellular growth. Proto-oncogenes normally encode proteins that stimulate the growth-signalling pathway at cellular level, often via growth factors. Genetic changes can cause over-expression or hyperactivity of the proteins leading to a name-change to 'oncogene'. Gene activation can be caused by amplification, translocation, mutation (point mutations, deletions, etc), and chromosomal re-arrangements or even by the integration of viral DNA.

Proto-oncogenes shown to play a role in gynaecological cancer include the *ras* family, the *myc* family, C-*fms*, C-erb B (HER-2/neu) and bcl-2 and these will be discussed.

3.1.2.1 Extra-cellular peptide growth factors with their cell membrane receptors

The extra-cellular peptide growth factors bind to cell membrane receptors that consist of a binding domain situated extra-cellularly, a membrane spanning domain and a tyrosine kinase domain that is cytoplasmic. Activation of the latter form the basis of activation of the peptide-receptor complex, leading to secondary signals transferred to the nucleus. Several growth factor-receptor complexes have been identified that can potentially have an oncogenetic effect when actified (Morsi et al 2000).

3.1.2.1.1 HER- 2/*neu* or C-*erb* B-2

The HER-2/*neu* gene or c-*erb* B-2 encodes a transmembrane receptor tyrosinase that is structurally similar to a receptor for human epidermal growth factor. The receptor is named p185neu and it controls tyrosine kinase activation that starts the cell proliferation pathway. Protein binding causes internalisation of the receptor complex and increased cellular growth (Maxwell & Carlson 1996). Amplification or over-expression of the protein product has been linked rather consistently to a poor outcome in many tumours and in endometrial carcinoma to non-endometroid histology (Reinatz 1994; Lukes 1994; Rolitzky et al 1999; Silverman 2000). The

importance of the HER-2/*neu* gene for this study is that it forms an integral part of the kinase / phosphatase pathway and thus is an integral part of the PTEN-pathway.

3.1.2.1.2 Proto-oncogene *bcl*-2

The proto-oncogene *bcl-2*, encoding for the Bcl-2 protein, inhibits programmed cell death and so prolongs cell survival. It also interacts with p53, which is an inducer of apoptosis, and there seems to be an inverse correlation with the *bcl-2* counteracting p53 and *vice versa*. P53 induced apoptosis can be completely blocked by the Bcl-2 protein (Burton & Wells 1998).

The level of the Bcl-2 protein has been found to fluctuate during the menstrual cycle in normal human endometrium and is lower in hyperplastic and lowest in malignant endometrial tissue (Morsi et al 2000; Zheng et al 1996; Geisler et al 1998). Immunohistochemical detection of the protein levels is used in research studies and results are conflicting and difficult to interpret (Seagusa & Okayasu 1997). Nuclear location of the protein also correlated with neoplastic behaviour, while location in the cytoplasm is the norm (Ioffe et al 1998).

3.1.2.1.3 Epidermal growth factor (EGF)

The epidermal growth factor-receptor complex was one of the first to be identified and characterised on the molecular level. It is a growth factor- receptor tyrosine kinase complex with expression in normal and atrophic endometrium. Although amplification has not been demonstrated in endometrial cancer, the EGF-receptor expression may be decreased in neoplastic endometrium. EGF-receptor expression does not, however, correlate with clinical and pathologic features in most of the reports (Khalifa et al 1994) and its importance in gynaecologic oncogenesis remains to be clarified.

EGF activates protein kinase C (PKC) through increased breakdown of phosphatidyl-inositol (PI) (Connor et al 1997). It seems that the effects of EGF on growth stimulation acts via the PI signal transduction cascade.

3.1.2.1.4 C-*fms*

The C-*fms* proto-oncogene encodes a transmembrane tyrosine kinase receptor for the M-CSF 1 (macrophage colony stimulating factor), which regulates growth of

phagocytes (Kimura et al 1991). M-CSF serum levels seem to increase in patients with some malignancies, but it is not known whether it plays a role in carcinogenesis or whether this is in response to an existing malignancy (Scambria et al 1994).

3.1.2.2 Cell membrane proteins (The ras oncogenes)

The *ras* gene family consists of three genes located on different chromosomes, but encoding for a similar transmembrane protein, named p21. This protein is similar to the other G-proteins involved in adenylate cyclase activation. The *ras* family of G-proteins are located on the inner aspect of the cell membrane and are probably involved in the transfer of external stimuli to the cell via second messenger activation. These proto-oncogenes are thought to play a critical role in the control of cell proliferation and have inherent GTPase activity. After the binding of several different growth factors to the receptor kinases, the activation of *ras* is the final common pathway (Berchuck & Boyd 1995).

The H-*ras* (Harvey-*ras*) is located on chromosome 11, K-*ras* (Kirsten-*ras*) on chromosome 12 and N-*ras* (neuroblastoma-*ras*) on chromosome 1.

Activation of the *ras* proto-oncogene family has been detected in a number of malignancies at frequencies depending on the type of tumour. Activation occurs mostly by point mutation and was found most frequently in codon 12, 13 and 61 and most frequently involving the K-*ras*. Pancreatic carcinoma has a mutation rate of about 90% in the *ras*-family, while mutations occur infrequently in gynaecological cancers (Koffa & Spandidos 1997). In endometrial cancer mutations occur mostly in the K-*ras* (14%-30%), sometimes in the H-*ras* (7%) and several groups have shown racial difference, with tumours from Japanese women harbouring more mutations than American counterparts (Boyd & Risinger 1991; Enomoto et al 1990). Two groups have correlated activation of these genes with a poor outcome (Mizuuchi et al 1992; Fujimoto et al 1993). Another group have found the opposite (Sasaki et al 1993), while *ras* activation has also been demonstrated in pre-cursor lesions (Duggan et al 1994), suggesting involvement also at the early stages of carcinogenesis.

3.1.2.3 Nuclear transcription factors (Proto-oncogene c-*myc)*

Signals from the cell membrane are transferred to the nucleus by so called nuclear transcription factors, situated in the cytoplasm. Overexpression of these peptides can potentially result in oncogenesis. Among the described proto-oncogenic nuclear transcription factors, the *myc*-family has been implicated most in human cancer. Little is known about involvement of this gene in female genital cancer, but it has been implicated in endometriosis, normal endometrium and to some extent in endometrial cancer (Monk et al 1994; Niederacher et al 1999).

3.1.3 Tumour suppressor genes

In 1969 Harris and co-workers published the findings of their experiments with the fusion of normal and tumour-forming cells (Harris et al 1969). Some, but not all the hybrid cells were still able to produce tumours and when analysed, these cell lines were shown to lack one or more chromosomes from the normal parent. Their results suggested that the normal set of chromosomes contained genetic material which could inhibit tumour growth and that the introduction of even a single normal allele can restore this function. The concept of a tumour suppressor gene was born, and the retinoblastoma (RB) gene was the first tumour suppressor gene to be identified.

Loss of heterozygosity

When one copy of a gene is lost via loss of a chromosome, deletion of a genetic locus or genetic conversion, the genetic area is reduced to homozygosity. A common term for this genetic finding is ‘loss of heterozygosity’ or LOH. Finding LOH in cancer cells is indicative of the involvement of a tumour suppressor gene and therefore plays an important role in the discovery of new candidate tumour suppressor genes.

The ‘two hit’ genetic model for tumorigenesis

Knudson (1971) was the first to propose the two-mutation model which describes that tumorigenesis only follows when two mutations occur in the same cell. His model suggests that this is the pathway of both genetic and sporadic tumorigenesis.

In the genetic form, a germline mutation occurs which is followed by an essentially recessive loss of function at cellular level in all cells. In a single cell

this is then followed by a somatic mutation in the same gene, which paralyses the genetic function followed by uninhibited growth of the cell and its offspring. The likelihood of this happening in any single cell when all germline cells have a mutation or the so-called 'first hit' is very high, leading to the autosomal dominant pattern of inheritance in genetic cancer syndromes.

In sporadic cancer both mutations are somatic. It is most likely that the second mutation follows the first one in one of the population of descendants of the cell in which the first mutation occured, rather than at the same time in a single cell. It follows that the natural mutation rate determines the occurrence of double mutations and the tumour incidence.

Knudson described that both mutations have to occur in the same chromosomal location and found that his model was applicable to a major portion of human cancers in the revision of his model, published in 1984 (Knudson 1984).

Tumour suppressor genes and the 'two-hit' model

The model descibed above fit tumour suppressor genes perfectly. Because loss of function only occurs when both alleles are mutated, a large group of descendant cells of a single proliferating cell with a single genetic mutation will survive as normal cells. This will greatly increase the chance of a second mutation and tumour formation.

Most tumour suppressor genes have been shown to play a major role only in a number of sites. Examples are many and include the APC gene on chromosome 5q21 causing familial adenomatous polyposis, the NF1 and NF2 genes located on 17q11.2 and 22q12.2 causing neurofibromatosis and the BRCA1 and 2 genes on 17q21 and 13q12 involved in familial breast cancer. The specificity of tumour suppressors vary and the incidence of different cancers differs from site to site. Some genes seem to be more specific than others and the type and site of the mutation within the gene might even influence the tumorigenic potential. The tissue specificity determines the risk of tumorigenesis.

Tumour suppressor genes most often implicated in gynaecologic cancer include tp53, the DCC gene, the RB gene and the topic of this study, namely

PTEN. These tumour suppressor genes will be discussed in short and PTEN extensively in the next section.

3.1.3.1 The *tp53*-gene

The *tp53* tumour suppressor gene is located on chromosome 17p13.1 and encodes a 53kDa phosphoprotein, p53, situated in the nucleus. Point mutations of this gene leads to overexpression of the mutant protein product which has a much longer half-life than the wild type. With mutations occurring mostly in exons 5-8, this is the commonest molecular alteration described in human cancer to date. Normally the p53-protein inhibits progression of the cell cycle via activation of cell cycle regulators. An important example is the kinase inhibitor p21, the product of the WAF-1 gene. This protein mediates p53 –induced G1 arrest, but also maintains growth arrest in some cells.

During cell cycle arrest in G1, time is allowed for the repair of DNA damage and to allow for the initiation of apoptosis or natural cell death of damaged cells. It is therefore thought that normal p53 acts as a tumour suppressor by preventing the replication of damaged cells and thus discouraging malignant clones (Ioffe et al 1998).

The p53 protein can be demonstrated well with immunohistochemistry. It is well known that the mutated gene encodes a protein product with a much longer half-life than the wild type. Abnormal proteins are therefore overexpressed and many researchers have demonstrated that there is a strong correlation between a mutated gene and overexpression. Immunochemistry results can be difficult to interpret and the correlation is also not absolute because some mutations will result in total arrest of protein production. Results therefore always have to be interpreted carefully. P53 mutation generally correlates with aggressive histological subgroups, high nuclear grading, stage and several poor prognostic features.

3.1.3.2 The DCC gene

The DCC gene (deleted in colorectal carcinoma) is frequently found to be deleted in colorectal cancers and is associated with tumour progression. In endometrial

cancer loss of expression seems to correlate with poor differentiation grade (Enomoto et al 1995).

3.1.3.3 The retinoblastoma gene

The retinoblastoma gene was the first tumour suppressor gene to be described and was initially shown to be mutated in inherited retinoblastomas. Alterations have now also been described in other human neoplasms, but researchers have failed to demonstrate significant involvement in neoplasms of the female genital tract (Niemann et al 1997).

3.1.3.4 The PTEN gene

PTEN (phosphatase and tensin homologue) is a new and unique tumour suppressor gene found on chromosome 10q23.3. The protein product plays an important role in cytoskeletal organization, cell growth regulation, and apoptosis. The gene has been shown to be frequently mutated in multiple human cancers and in some tumour types it correlates with a poor outcome and with tumour progression.

PTEN encodes a protein-tyrosine phosphatase and is the first gene of this type to be proven to be a true tumour suppressor. The protein plays its role as a phosphatase opposing the kinase pathways. The function of the protein and its role in the tumour suppressor pathways in as far as it is currently understood and unravelled will now be extensively discussed.

3.2. The function of the phosphatases and kinases

3.2.1 Protein phosphatases and protein kinases

The phosphorylation and dephosphorylation of the tyrosine amino-acids in proteins play a very important role in the regulation of many cellular processes in mammalians and in fact in all eukariotic organisms. Examples include the regulation of cell cycle control, growth control, cellular differentiation and gene and synaptic transmission. Phosphorylated tyrosines activate their proteins because they are recognised by specialized binding domains on other proteins that will then initiate intracellular signalling pathways, often via integrins (Hunter 1997).

Because of the importance of this process in the cell cycle and growth control, abnormality of tyrosine phosphorylation has been shown to occur in many human cancer types. Several protein kinases have been implicated as oncogenes and phosphatases have long been known to antagonise the action of the kinases.

Kinases bring about phosphorylation while removal of the phosphate is controlled by **phosphatase**-enzymes. More than 95 protein-tyrosine kinases have been identified in humans and more than 55 genes encoding for protein-tyrosine phosphatases are currently known. Although the involvement of the phosphatase genes in human carcinogenesis has long been suspected, PTEN is the first important protein-tyrosine phosphatase gene to be proven to be a true tumour suppressor (Li et al 1998b).

Protein kinases attach phosphate groups to proteins, and several oncogenes have been shown to be kinases acting on various proteins. The addition of the phosphate groups activates cells by accelerating cell growth and division via signalling pathways and a wide variation of growth factors (Parsons 1998).

Protein kinase B/Akt

Protein kinase B was identified by three research groups in 1991 as a kinase similar to protein kinases A and C. This kinase was also identified as the product of the retrovirus AKT8 found in a T-cell lymphoma. The concerned research group named the viral oncogene *v-akt* and the protein product Akt (Downward 1998). Both the names PKB and Akt are still widely used, but the name RAC1 or RAC-PK (related to A and C protein kinases) is now discouraged to avoid confusion. The names PKB, Akt and PKB/Akt will be used here. Recently Akt has been shown to interact with PTEN and therefore this kinase or oncogene will be discussed in some detail (Kulik et al 1997)..

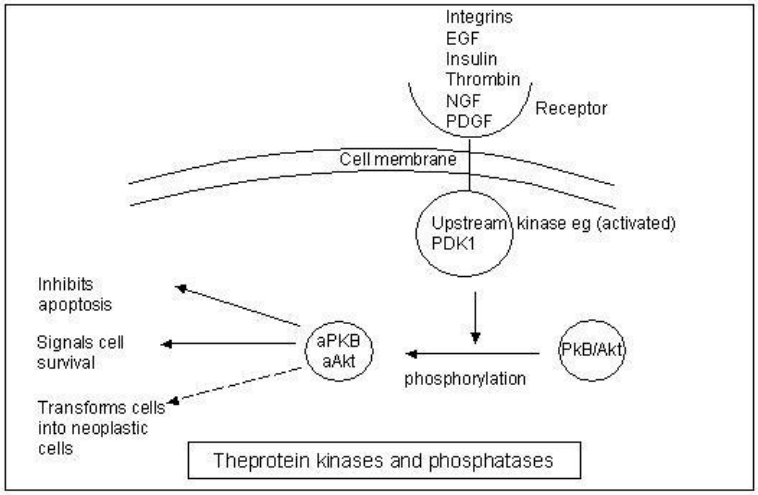
Protein kinase B/Akt (PKB/Akt), which is a serine/ treonine kinase, has been shown to be an extremely important physiological mediator of the effects of insulin, several growth stimuli and growth factors and it protects cells against natural cell death. Activated PKB/Akt will provide a cell survival signal that will inhibit stress induced apoptosis and therefore Akt is a known cell survival

promotor (Kulik et al 1997). This important anti-apoptotic role has been demonstrated in neuronal cells by Kennedy, Wagner and Conzen (1999).

PKB/Akt is also considered to be a proto-oncogene and when activated, Akt has been shown to induce cellular transformation (Wu et al 1998) to neoplastic cells. The activation of this kinase is a complex process induced by various stimuli and mediated by many growth factors and an intricate signalling process. The activation starts via translocation of the kinase to the plasma membrane, where it is activated by phosphorylation of upstream kinases like phosphoinoside-dependent kinase 1 (PDK1). The activation can be in response to growth stimuli, including platelet derived growth factor, insulin, thrombin, epidermal growth factor and nerve growth factor.

Cellular stress, like heat shock and hyperosmolarity, has also been shown by Konishi, Matsuzaki and colleagues (1996) to cause activation of this pathway. This activation may well be through the p38/HOG1 kinase cascade as suggested by Dudek and colleagues (1997). Dahia has shown that activation occurs especially when the protein is phosphorylated in particular at the Thr308 and Ser473 residues (Dahia et al 1999).

Figure 1.1 demonstrates and summarises the actions of the protein kinases and the phosphatases as discussed in the text above. Integrin signalling and the function of the other protein kinases will be discussed below and is summarised in figure 1.2.



EGF = Epidermal growth factor

NGF = Nerve growth factor

PDGF = Platelet derived growth factor

PDK = Phosphoinoside-dependent kinase

PKB = Protein Kinase B

Akt = protein product of viral oncogene *v-akt*

Figure 1.1: The function of the protein kinases and phosphatases.

Other protein kinases and integrin signaling

A number of other protein kinases have been implicated in the pathways closely related and linked to PTEN activity and to PKB/Akt, often in response to integrin signaling.

Integrins consist of pairs of α and β transmembrane subunits, forming highly selective receptors on the cell surface. Ligand occupancy by extracellular proteins or antibodies can cause direct activation of intracellular signalling. Integrins are known to be remarkably multifunctional and are involved in, among others, the cytoskeletal organization, control of the cell cycle and growth, cell adhesion and migration and electrolyte exchange (Tamura et al 1999b).

Important intracellular signal pathways include the activation of different kinases by phosphorylation, the formation of large complexes of signalling molecules and interaction (stimulation, co-operation) with growth factors.

Focal adhesion kinase (FAK) is one important integrin signalling protein. This kinase is activated by phosphorylation and seems to be a major mediator of integrin-dependent cell survival. FAK is important in cell proliferation and cell

cycle control, cell survival and to help prevent anoikis, or cell detachment-induced apoptosis. FAK is also an important upstream mediator of the PKB/Akt pathway, regulated among others by PIP3 (Tamura et al 1999a).

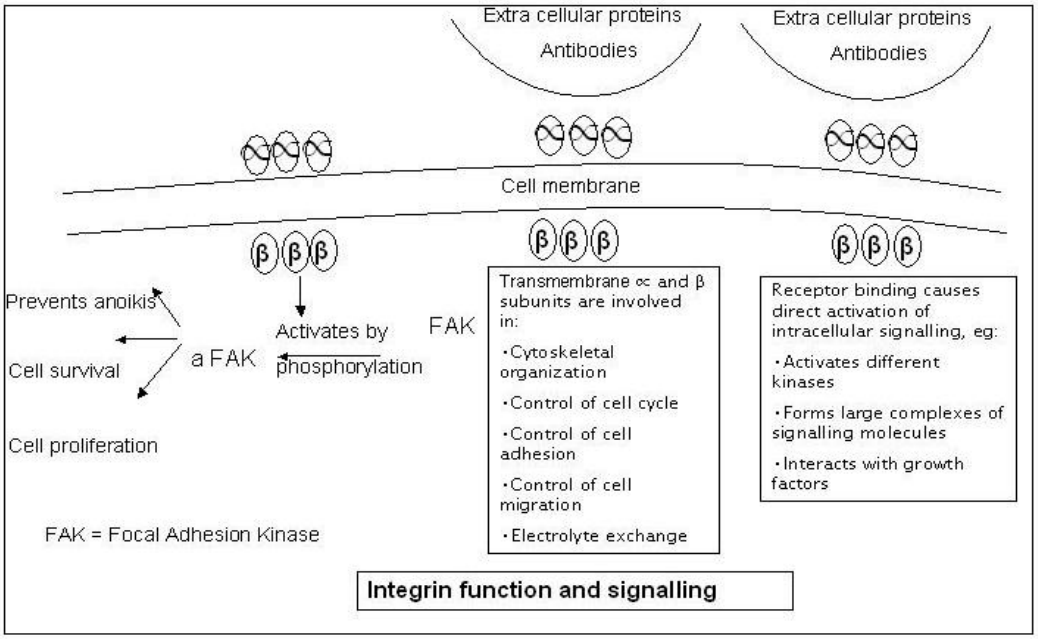


Figure 1.2: Integrin function and signalling.

Examples of molecules involved in the large cytoplasmic complexes formed as the intracellular tail of integrins, include the Src family of kinases, cytoskeletal proteins and the mitogen activated protein kinase (MAP kinase/MAPK) pathway. MAPK is activated by signal transduction and by extracellular signal regulated kinase (ERK) and Ras and Raf are regulated downstream (Wu et al 1998). This pathway can also be activated by other growth factors, like EGF and PDGF (Gu et al 1998).

3.2.2 The lipid kinases and phosphatases in the phospholipid pathway

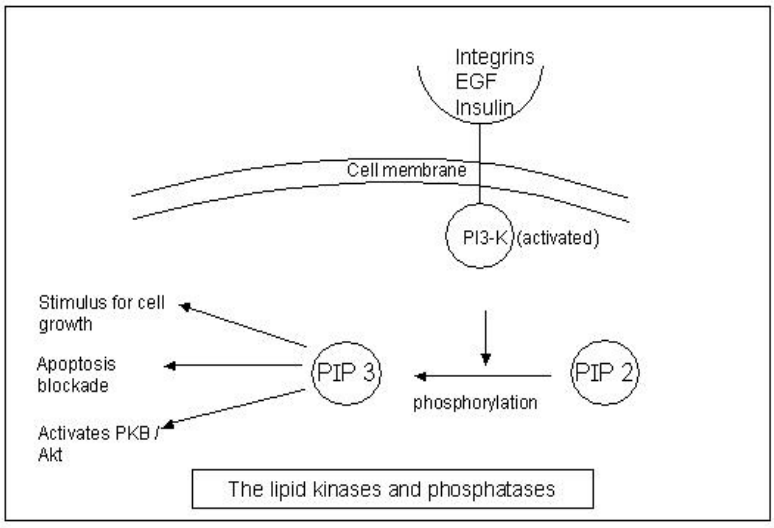
In the phospholipid metabolism pathway, phosphatidylinositol 3,4-biphosphate (PI(3,4)P2 or PIP2) is phosphorylated to phosphatidylinositol 3,4,5-triphosphate (PIP3 or PI-P3) and this reaction is catalysed by the phosphatidylinositol 3-kinase (PI3-K) enzyme. The reaction is stimulated, as in the case of the protein kinases,

by integrins and growth factors like insulin and epidermal growth factor (Hopkins 1998).

PIP3 is the key component of this control pathway, controlling cell growth by blocking apoptosis and by direct stimulation of cell growth. These interactions are shown in figure 1.3 below.

PIP3 is also the upstream stimulus for the activation of PKB/Akt and some

Figure 1.3: The function of the lipid kinases and phosphatases.



EGF = epidermal growth factor
PI3-K = phosphatidylinositol 3-kinase
PIP-3 = phosphatidylinositol 3,4,5 – triphosphate
PIP-2 = phosphatidylmositol 3,4-6: biphosphate
PKB = protcinkinase B

other kinases. These enzymes encourage cell division and inhibits apoptosis.

3.3. PTEN: The tumour suppressor gene and its protein product

3.3.1 The isolation of PTEN as a novel tumour suppressor gene

The research road leading to the identification of the PTEN gene started when loss of heterozygosity was found frequently in a variety of human tumours at chromosome ten, region 10q23-25. This alteration was particularly common in

brain tumours (Rasheed et al 1995; Fults et al 1990) and prostate cancer (Gray et al 1995).

In 1997 three independent research groups published their findings of a candidate tumour suppressor gene located in this region on chromosome ten. The name PTEN (phosphatase and tensin homologue, situated on chromosome ten) was given by J. Li and co-authors (Li J et al 1997b), MMAC1 (mutated in multiple advanced cancers-1) by the group of Steck (Steck et al 1997) and TEP1 (transforming growth factor β-regulated, epithelial cell enriched phosphatase-1) (Li DM et al 1997a) by D. M. Li and his group. Currently the name PTEN is used almost universally and will be used in this study.

This novel tumour suppressor gene was mapped to 10q23.3 and it was found to encode a 403 amino acid polypeptide chain. The gene has a coding region of 1 212 nt and consists of nine exons. The protein sequence is similar to that of the cytoskeletal proteins tensin and auxilin. The N-terminal domain of the PTEN-protein shows extensive homology to the cytoskeletal protein tensin, implicating roles for PTEN in the maintenance of cellular structure and in signal transduction (Tamura et al 1999a).

The protein was initially identified as a phosphatase as it was found to contain a protein tyrosine phosphatase (PTP-ase) catalytic domain. The phosphatase function has now been shown to be dual protein specific and to play an important role in the phospholipid pathways as well. The protein is active in the cytoplasm, which is unique for a tumour suppressor gene and plays its role on cytogenetic level in the carcinogenetic process.

The protein product of the PTEN gene was subsequently shown to play an important role in human malignancy with somatic mutations observed in multiple cell lines and in primary tumours such as endometrial cancer, melanoma, thyroid carcinoma, glioblastoma and other neuro-malignancies.

The PTEN gene was also linked to families with Cowden syndrome (Nelen et al 1997) where germline mutations in this gene lead to a syndrome consisting of multiple hamartomas and an elevated risk for tumours of the breast, thyroid and

skin (Liaw et al 1997; Lynch et al 1997; Marsh et al 1997; Tsou et al 1998). Germline mutations are also linked to the related familial hamartomatous polyposis syndrome, called Bannayan-Zonana syndrome (Marsh et al 1997) and to a juvenile polyposis coli syndrome (Olschwang et al 1998).

3.3.2 PTEN as a protein phosphatase and lipid phosphatase

Initially called a 'dual specificity phosphatase', it has been demonstrated by numerous authors that the tumour suppressor function of PTEN indeed relies strongly on its phosphatase activity. From the amino-acid sequence, the PTEN protein resembles two different types of enzyme, namely a protein phosphatase and a lipid phosphatase.

Early reports suggested that PTEN was a protein tyrosine phosphatase (PTP-ase), i.e. mainly a protein phosphatase, which is an enzyme that removes phosphate groups from the tyrosine groups of other proteins. Many protein kinases (enzymes with the opposite effect) have been proven in the past to have oncogenic effects, so this finding was welcomed by the scientific world. Interestingly, though, it was subsequently shown that the most important target of this phosphatase is probably not tyrosine, but rather the phospholipids.

lt is thus currently known that PTEN can act as a dual specificity phosphatase and can thus remove phosphate *in vitro* as a lipid phosphatase from among others phosphatidylinositol phosphate and inositol phosphate and as a protein phosphatase from phosphoserine, phosphotreonine and phosphotyrosine (Li & Sun 1997; Myers et al 1997; Maehama & Dixon 1998). The relevance of these actions on cellular level is still incompletely understood.

Studying cellular function in the offspring of cells known to harbour a disease causing mutation can shed light on the role of a gene and its protein product. Patients with germline mutations in PTEN as well as DNA isolated from tumours known to have PTEN mutations have enabled further research to understand the function of PTEN. It is now known that disease-causing mutations virtually always involve the phosphatase region of the gene and it has been shown that such mutations always cause diminished phosphatase activity in the protein product.

These findings support *in vitro* data that the tumour suppressor function relies on phosphatase activity.

The groups of Tonks (1997) and Myers (1997) showed that PTEN preferentially strips phosphate from negatively charged amino acid residues. As such sequences do not occur naturally in proteins known to be involved in the phosphorylation by kinases, many authors investigated phospholipids as the alternative negatively charged substrate available. Dixon and his group (Dixon 1999) found that pure PTEN-protein can remove a phosphate group from PIP3. They also demonstrated that overproduction of the PTEN protein leads to lowered intracellular PIP3 concentrations.

This discovery demonstrated an elegant possible tumour suppressor function for PTEN. Phosphorylation of PIP2 results in PIP3, which is the active molecule leading to the activation of other kinases in the pathway, like PKB/Akt. These kinases block apoptosis and causes cell survival and cell division. Removing the phosphate from PIP3 will revert it to the inactive PIP2, working against cell survival and growth.

Additionally, Tonks and co-workers have demonstrated that tumour inhibition activity relies heavily on maintenance of the lipid phosphatase function of the gene and not really on the tyrosine or protein phosphatase activity (Tonks 2003). They introduced mutant PTEN and normal (wild type) PTEN into tumour cells, showing that mutant PTEN lacking specifically lipid phosphatase activity were unable to halt cell growth, while normal PTEN inhibited it effectively.

3.3.3 The interaction of PTEN with known kinases, phosphatases and growth factors

As discussed above, PTEN is a unique tumour suppressor with both lipid phosphatase and protein tyrosine phosphatase activity (Tamura et al 1998).

Phosphatases have been implicated in several critical pathways responsible for cell growth, differentiation, cytoskeletal organization and even B cell activation after antigen activation. Phosphatases have also long been known to antagonise the action of the kinases. Although the kinases are well known

stimulators of cell growth and proliferation, involvement of the phosphatases in human tumorigenesis have not been directly demonstrated before PTEN studies.

The basic function of normal PTEN seems to be the dephosphorylation of the kinases and the inhibition of integrin and growth factor mediated kinase signaling pathways. In the growth regulatory pathway, the most important currently known interactions are with PIP3 and PKB/Akt.

PTEN dephosphorilates phosphatidylinositol 3,4,5-triphosphate and inositol phosphate in the same way as the other dual specificity phosphatases (Fauman & Saper 1996). Ramaswamy and co-workers (1999) also showed that PIP3 dephosphorylation (or inactivation) is a function of PTEN and that this ability inhibits Akt-kinase or PKB activation. PTEN is therefore also called the upstream regulator of PKB/Akt.

Increased activated PKB/Akt levels have been demonstrated to be strongly inversely related to pten-protein levels (Dahia et al 1999). PTEN deactivation leads to excessive levels of activated PKB/Akt, demonstrating that PTEN is needed as a negative regulator of the Akt / PI 3-kinase pathway (Wu et al 1998). As a regulator of the PKB pathway, PTEN opposes not only the level, but also the phosphorylation function of PKB/Akt directly. The downstream target of PKB/Akt, namely 4E-BP1, is thus also inhibited because phosphorylation by PKB (and therefore activation) is inhibited. 4E-BP1 is a translation repressor.

The interactions of the PTEN-protein with many of the other protein kinase pathways are intricate and as yet incompletely understood. It has been shown that wild type PTEN will inhibit the activation of MAP kinase, focal adhesion kinase (FAK) and ERK. It is also known that PTEN inhibits the activity of the Ras oncogene, which functions in this same pathway.

Huang and Kontos (2002) also showed that PTEN modulates the transmembrane vascular epithelial growth factor (VEG-F) mediated signalling process. This implicates the intimate involvement of the PTEN gene in angiogenesis. This is yet another way in which PTEN is not only involved in malignant transformation of cells but also in proliferation of the malignant clone.

Many other growth factors are involved in the integrin and phosphatase transmembrane and intracellular signalling pathways which interact with the PTEN regulated PKB and PIP3 growth regulatory pathways. This implicates indirect involvement of the growth factors and even of the hormone driven growth regulation paths with the PTEN gene and pten protein. Direct involvement of these growth factors is plausible, possible but not yet proven.

3.3.4 PTEN and the control of cellular growth

3.3.4.1 Arresting cells in G1

PTEN regulates the G1 growth cycle progression by arresting cells in G1 (Furnari et al 1998a). It is known that this arrest is mediated by the ability of intact pten-protein to dephosphorylate a lipid substrate, (PIP3 inactivation) as demonstrated by Ramaswamy and co-workers (1999). PIP3 inactivation correlates strongly with inactivation of the Akt signalling pathway as discussed and demonstrated above.

It has been shown that cell growth arrest in G1 as caused by intact PTEN can be overrided by adding previously activated Akt. This proves that preventing the activation of Akt is one of the main mechanisms of cell growth control utilized by PTEN (Ramaswamy et al 1999). It also shows that overamplification of PKB/Akt can cause this kinase to act as an oncogene, overcoming the normal cell growth control mechanisms.

3.3.4.2 Induction of apoptosis

Li, Simpson, Takahashi and colleagues (Li et al 1998a) showed that PTEN can also be involved directly in induction of cell death by apoptosis. This involvement is not fully understood but is exciting for anti-tumour drug development. It appears that induction of apoptosis is again reliant on a functioning phosphatase domain and that activity is also via interaction with the kinase pathway and signalling proteins. PTEN interferes with the PIP3 signalling to activate Akt. It appears that the induction of apoptosis happens via the Akt-dependent apoptosis pathway, also because these investigators could show that PTEN expressing cells could be rescued from apoptosis by excess Akt (see also section 3.3.4.1).

Induction of apoptosis has not been demonstrated for many tumour suppressor genes. P53 specifically induces apoptosis in genetically damaged cells, while the

mechanisms of involvement for BRCA1 (Shao, Chai et al 1996) and APC (Clarke et al 1993) are not well understood but theoretically includes the induction of apoptosis. Apoptosis induction by PTEN was confirmed by other investigators (Xu et al 2002) who demonstrated this in a T cell model again via the PI3K/ Akt pathway.

Normal PTEN function and thus apoptosis was shown to be overcome by excess Akt, when researchers showed that lethal levels of PIP3 could be tolerated by cells with a mutation in the Akt/PKB pathway (Stocker et al 2002). These high levels of PIP3 would stimulate PTEN induced Akt/PKB inactivation when no such mutation exists.

Bcl-2 was another molecule that was shown to rescue cells from PTEN induced apoptosis and bcl-2 is also induced by Akt, thus using the same pathway.

3.3.4.3 Effect on cell and soma size

Interestingly it was shown that the PTEN gene also regulates neuronal soma size. This extra cellular growth modulation effect of the gene was confirmed when Crackower and colleagues (2002) showed that PI3K-PTEN pathways regulate myocardial cell size and even contractility.

Other investigators documented that PTEN is a very important factor in regulating mammalian cell size (Backman et al 2002).

3.3.5 Biological activities of PTEN not directly related to growth

3.3.5.1 Cell adhesion, the intracellular matrix and PTEN

The structural homology of PTEN with tensin and auxillin, which are important cell adhesion molecules, pointed towards a role for PTEN in cell adhesion, cell motility, the cytoskeleton and in intracellular signalling (Steck et al 1997; Li et al 1997b). Tensin is intimately involved in integrin-signalling, which is a complex system activated via receptor binding or stimulation by extracellular proteins.

PTEN tumour suppressor function may be mediated by its down-regulating of cellular interaction with the extracellular matrix (Tamura et al 1998). While many cells depend upon adhesion to the extra-cellular matrix to survive and escape apoptosis, tumour cells often lack this requirement.

PTEN inhibits cell migration, spreading and focal adhesions. It was shown that PTEN disrupts the architecture of focal adhesions and the cytoskeleton when introduced into fibroblasts and glial tumour cell lines. It interacts with Focal Adhesion Kinase, which is thought to be an important molecule in integrin signalling pathways. It seems that PTEN reduces FAK function by inhibiting its tyrosine phosphorylation and thereby inhibiting its activation. FAK is thought to play an important role in protecting adhesion-independent neoplastic cells from apoptosis (Berchuck & Boyd 1995).

FAK is seen as an upstream mediator of the PI3-K/Akt pathway, which acts pro-cell-survival and will inhibit apoptosis. Activated FAK binds to integrin receptors to activate cell survival signalling, ending with PI 3-K activation of Akt, which is a serine-threonine protein kinase (Gilmore & Romer 1996).

3.3.5.2 Cell migration and cell proliferation

Tamura and co-workers (Berchuck & Boyd 1995) demonstrated that PTEN has a direct inhibitory effect on cell invasion and migration and that this function relies on its phosphatase domain. They showed that this function is mediated by the effect of the tumour suppressor gene on FAK and on p130 Cas. Overexpression of these two proteins can antagonize PTEN inhibition. Fumari and co-workers (1997) also showed that the growth inhibition function of PTEN relies on intact phosphatase function.

It was showed in various tumour cell lines, that when PTEN wildtype was re-introduced into a tumour lacking normal PTEN-protein, cell migration was halted and growth inhibited (Cheney et al 1998). This re-establishes PTEN as a true tumour suppressor gene.

In neuronal neoplasms PTEN mutation is indeed not associated with neoplastic transformation but rather with increased aggressiveness and invasive potential of the tumour cells. An important effect of intact PTEN to inhibit cell migration and proliferation while supporting apoptosis in neuronal cells was recently suggested and partly proven (Li et al 2002). Thus PTEN plays an important role not only to limit normal cellular growth and migration, but also to induce natural cell death.

3.3.6 Interaction of PTEN with other tumour suppressor genes, oncogenes and chemotherapeutic agents

PTEN interacts largely with other genes as most of these also influence the same growth stimulatory pathways. It is also known and feasible that multiple genetic defects in the cell causes neoplastic behaviour and will usually increase the aggressiveness of the tumour. This also supports the two-hit model of Hudson (see 3.1.3) albeit in a way a little different from the classical model as described.

The biology of cancer cells are predicted largely by the genetic mutation patterns. In this way chemosensitivity should also be dependent upon these patterns. Indeed various researchers have looked at chemo-response in relation to PTEN status as measured on immunohistochemistry and mutation analysis.

PTEN protects p53 from Mdm2 and seems to sensitize cancer cells to chemotherapy as shown by Mayo and co-workers (2001). It has also been shown that PTEN can induce chemosensitivity by the reduction of Bcl-2 protein suppression (Huang et al 1997).

PTEN sensitization of cancer cells to drug induced apoptosis was demonstrated in a prostate cancer cell line by Yuan, Whang and co-workers (2004), while the group of Saga (2002) showed that PTEN overexpression increases the sensitivity of ovarian cancer cells to the chemotherapeutic agent irinotecan.

Many more interactions have been described and will still be unmasked. It is clear that all these genes involved in growth control have contact via the intracellular pathways and they also dictate malignant behaviour and chemo- and radiation response.

3.3.7 Interaction of PTEN with steroid hormone receptors

The sex steroid hormone receptors are also situated on the cell membrane and receptor binding has a domino but ill-understood effect on intracellular signalling. Recently many researchers have suggested interaction between the steroid hormone driven growth control pathway and the growth factor controlled path. The suggestion is that one of the pathways will be dominant for a time, but that

malignant cells have the ability, over time, to switch their dominant pathways to ensure uninhibited growth. Cross-talk between these intricate signalling paths are extremely difficult but also important to investigate further.

Involvement of the PTEN / PI3K pathway has been demonstrated in the regulation of steroid receptors by two groups of authors. Li, Nicosia and Bai (2001) demonstrated that PTEN can antagonize the androgen receptor and thus cause apoptosis of prostate cancer cells. Campbell, Bhat-Nakshatri and Patel (2001) showed that Akt/ PI3K can activate estrogen receptor alpha, thereby inducing anti-estrogen resistance.

3.4 The involvement of PTEN in non-gynaecologic neoplasms

3.4.1 Brain tumours

Shortly after the detection of PTEN, various research groups examined neural tumours for PTEN involvement. Interestingly most groups found involvement in higher grade but not in lower grade tumours. This suggested an influence on aggressiveness and not in initial oncogenesis. Duerr and co-workers (1998) reported mutations in glioblastomas and subtypes of gliomas but not in astrocytomas and its subtypes.

The finding that primary but not secondary glioblastomas (Tohma et al 1998) contain PTEN mutations, supports the notion that this is a genetic alteration that occurs late in the process. Davies and colleagues (1999) also found that mutations occurred more frequently in high grade than in low grade gliomas.

The study of neuronal tumours has since contributed significant to the understanding of the function of PTEN.

3.4.2 Breast cancer

Initial reports found that LOH in the region of the PTEN gene was frequent in sporadic breast cancer. Mutation analysis, however, could not confirm that PTEN plays an important role in etiopathogenesis of sporadic breast cancer (Feilotter et al 1999). Many researchers still suspect that another tumour suppressor is still

undetected in the same region as PTEN, which may play an important role in breast cancer.

Similar to the findings in neural tumours, LOH studies also suggested more involvement of this area associated with disease progression (Bose et al 1998). Again mutation analysis could not confirm that the involved gene was indeed PTEN (Rhei et al 1997).

3.4.3 Other malignancies

Because PTEN is the causative gene in Cowden syndrome, characterised by hamartomas in the gastro-intestinal tract, researchers investigated the role of the gene in sporadic bowel neoplasms. Similar to the findings with BRCA, however, the gene was found not to be involved in sporadic cases (Wang et al 1998).

The gene and especially the protein were confirmed to play a role in a percentage of melanomas. The early reports were by Tsao et al (1998), Guldberg et al (1997) and Shao et al (1998).

Involvement in haematological malignancies include non-Hodgkin's leukemia (Dahia et al 1998; Nakahara et al 1998).

In prostate cancer it was shown that not only genetic mutation (in one chromosome), but also subsequent pten protein levels play a role in tumour progression. Pandolfi first reported the measurement of pten levels (2003).

Numerous other reports have been published regarding the involvement of this gene in the etiology of different cancers. However, so far no other tumour type has matched the frequency of PTEN mutations found consistently in endometrial cancers.

3.5 The involvement of PTEN in the etiology of cancers of the female genital tract

The involvement of PTEN in especially early stages of tumorigenesis in the upper female genital tract will be the focus of the rest of this report. Detail of current knowledge about its role in the different tumour types will be discussed in the

relevant chapters. This section contains a short summary which will serve as an introduction to the further discussion.

3.5.1 Neoplasms of the uterine epithelium

Molecular work on endometrial cancer played an important role in the discovery of the PTEN gene. Microsatellite instability is frequent in endometrial cancer, and abnormality in DNA repair mechanisms plays an important role in these tumours. Loss of heterozygosity was found to occur frequently on the 10q chromosome in endometrial carcinomas (Peiffer et al 1995; Kong et al 1997). This led to the search for a tumour suppressor at this locus and to the discovery of PTEN and its protein pten.

The role of the PTEN tumour suppressor gene in endometrial carcinogenesis has been studied extensively. The incidence of somatic mutations in endometrial cancer is the highest of any primary malignancy analysed so far. The frequencies reported vary from approximately 40% to even 76% (Nagase et al 1997; Peiffer et al 1995; Risinger et al 1997; Tashiro et al 1997). It is not completely understood what role these mutations play in tumorigenesis and in proliferation of existing cancers.

3.5.2 Neoplasms of the uterine soft tissue

Loss of heterozygosity (LOH) for chromosome 10q has recently been shown by Quade and colleagues (1999) to be frequent in leiomyosarcomas. This finding supported the hypothesis that the PTEN gene plays a role in neoplastic transformation in these tumours.

The frequency and role of microsatellite instability in various subtypes of uterine sarcoma and leiomyoma is not known and very little was known about the involvement of PTEN in uterine soft tissue tumours before this study. It is also unknown how the findings in these two tumour types will correlate.

3.5.3 Neoplasms of the ovarian epithelium

In endometrial cancer, MSI was found almost exclusively in the endometroid adenocarcinoma histological subtype. Many other molecular studies have found correlation or strong correlation with histological subtype and even across

different tissues of origin. It was therefore logical to expect that the involvement of the PTEN gene will also be more impressive in endometroid ovarian tumours.

Microsatellite instability was found in both ovarian endometroid carcinoma and endometriosis although it was less frequent than in endometrial tumours. Results of analysis on endometriosis samples have been inconsistent.

Martini and co-workers (2002) were able to demonstrate hypermethylation of both hMLH1 and PTEN with inactivation of protein expression in atypical endometriosis cases as well as in endometroid cancer specimens.

4 Delineation of the research

4.1 Tumours of the female genital tract

4.1.1 Etiology of tumours of the upper vs lower female genital tract

The involvement of PTEN in gynaecological cancers seems to be limited to tumours of the upper genital tract and this thesis will therefore focus only on tumour types of the upper genital tract. It is well known and proven that various types of human papilloma virus are almost solely responsible for the carcinogenesis of epithelial tumours of the vulva, vagina and cervix. No further attention will be paid to these tumour types in this thesis.

In the upper genital tract classical risk factors include endogenous and exogenous hormonal factors, factors related to growth factors (including insulin levels) and inherited defects in tumour suppressor gene function, including HNPCC (endometrial cancer), family history and BRCA mutations (ovarian cancer). It is logical and to some extent proven that the other tumour suppressor genes active in these pathways, including then PTEN, will be involved in tumorigenesis in these cancers.

4.1.2 Models of neoplastic transformation

Other proliferative conditions and benign neoplasms share many of these known risk factors for upper genital tract cancer. These conditions include endometrial hyperplasia and polyps (hormonal, hyperstimulation and inherited factors as risk

factors), leiomyomas, adenomyosis and endometriosis (family history, race and related risk factors).

Benign proliferative diseases and neoplasms have often been used as a model in the study of carcinogenesis. Some proliferative conditions are not considered to be risk factors for the development of cancer and many others have only a weak association with risk for malignancy. No uniform definition for a pre-malignant condition exists, but to be considered a true pre-malignancy, most authorities agree that the risk for subsequent or co-existing cancer must be much elevated from the background or population risk.

Although precursor lesions or true pre-malignant lesions are not available for all the tumour types studied, the model of comparison between the malignant tumour and either the pre-malignant lesion or the benign counterpart was selected for this study. This model provides us with a theoretical model of progression and with a model to compare gene involvement early in carcinogenesis with involvement later in the evolution of the malignant cell.

4.1.2.1 Neoplasms of the uterine epithelium

Endometrial hyperplasia is currently only considered to be pre-malignant if it is atypical in histological appearance. Atypical endometrial hyperplasia is probably a true precursor lesion of endometroid adenocarcinoma. All other hyperplasias as well as endometrial polyps are considered benign proliferative disorders, although they share many risk factors with endometrial cancer. Endometrial intra-epithelial dysplasia or neoplasia is considered the pre-invasive counterpart of poorly differentiated endometrial cancer, often of a non-endometroid subtype. Although some endometrial cancers originate from endometrial polyps, the latter is not considered the pre-cursor lesion of endometrial cancer.

Endometroid carcinoma of the uterus will be compared with hyperplasia and atypical hyperplasia of the endometrium. This is considered a good model using a true precursor lesion. This model is often used in studies of this nature and the results will be compared with the outcome of similar studies.

4.1.2.2 Neoplasms of the uterine soft tissue

Different types of uterine sarcoma probably develop along different histological pathways.

Leiomyosarcoma is a truely malignant tumour developing in a clonal fashion from a smooth muscle cell of the uterine muscle wall. No precursor lesion exists for this tumour, but the degrees of malignancy are graded. Related tumours range from truely benign (leiomyoma), to fast growing benign (cellular leiomyoma), to malignant (leiomyosarcoma with low mitotic index) and highly malignant (high mitotic index).

Carcinosarcoma is probably also a monoclonal tumour but is from endometrial or archimetrial (the earliest undifferentiated uterine cell) origin. This tumour is considered to be most related to endometrial cancer of all the sarcomas and is considered by many to represent the least differentiated carcinoma of all. As such the precursor lesion for this tumour could be atypical hyperplasia or endometrial intra-epithelial dysplasia.

Endometrial stromal sarcoma is a malignant soft tissue tumour of the stroma of the endometrium and thus has a lot in common with endometrial polyps. These tumours seem to be highly hormone sensitive and degrees of malignancy also varies.

Leiomyoma and cellular leiomyoma of the uterus will be compared with uterine sarcomas. These tumours are not considered pre-malignant, but rather as benign neoplastic counterparts. This is considered a relatively good model as long as it is interpreted with caution.

Endometrial hyperplasia and endometrial carcinoma results will be compared with results of carcinosarcoma. This model will be further explained in the relevant chapters.

4.1.2.3 Neoplasms of the ovarian epithelium

The origin of endometroid ovarian cancer is not totally clear. It is, however known that the incidence of this disease as well as that of clear cell cancer of the ovary is definitely raised in women with a former diagnosis of endometriosis. This

association becomes important with advanced age (over 50 years) and in women with ovarian endometriosis. It is probably harsh to call ovarian endometriosis a pre-malignant condition, but it is known that these tumours arise from the endometriotic lesions and that they often co-exist, thereby fulfilling the criteria.

Ovarian and pelvic endometriosis will be compared with ovarian endometroid carcinoma. Theoretically this model is very good but practical problems have been reported by other investigators.

The models used to study malignant transformation will be further explained and fully referenced in the relevant sections.

4.2 Methods to study the role of PTEN

4.2.1 Detecting somatic genetic mutation

Frequent LOH at 10q led to the narrowing of the critical region on chromosome 10q and the subsequent identification of a gene with nine exons, located on BAC46b12 (Steck et al 1997). The protein encoded is a polypeptide of 403 amino acids with various functions in the control of cell growth and others as discussed above.

After the initial work to identify the gene and develop primers for the various exons, most researchers focused on mutation analysis. Mutation analysis on blood enabled researchers to link germ-line PTEN mutations with inherited disorders as discussed above. DNA can also be isolated from tissue, including tumour and subsections of neoplasms. PTEN-coding sequences of the gene is then amplified with the polymerase chain reaction (PCR) method using target-specific oligo-deoxynucleotide primers. Mutation analysis is used to detect abnormalities in the different exons of the gene.

This method of gene sub-section amplification will be used throughout this study to determine not pten protein expression, but rather true somatic mutations typically happening early in a cell destined to be cloned into a benign or malignant lesion.

4.2.2 Detecting the aberrant protein product

The group of Perren (1999) developed a monoclonal antibody (6H2.1) against an amino acid oligopeptide identical to the C-terminal end of the human pten-protein and demonstrated specificity by western blot analysis of known wild-type and PTEN-null cell lines.

PTEN immunohistochemistry requires freshly cut paraffin sections from recently embedded tissue. Stained tissue samples are usually scored from 0 to 3 to give a semi-quantitative result. This technique has enabled researchers to study the expression of the pten protein in normal tissues better and has enabled differentiation of involvement in subsections of tissue, for instance in endometrial glands but not in stroma or abnormal expression in certain glands but not in others (Mutter et al 2000).

This study was mostly done on stored tumour samples, and DNA was extracted from paraffin embedded tissue. Such samples are not suitable for protein detection. Secondly it is much easier to compare findings with those of other researchers if the same method is used. Most researchers have reported on mutation analysis. Thirdly the correlation with protein expression and immunohistochemistry is not at all absolute with cross-binding with both protean pten and aberrant pten possible. The semi-quantitative method used to interpret results of immunohistochemistry sometimes makes results difficult to interpret and to compare.

5 Conclusion

Most of the initial work on PTEN that was performed in the late 1990s, were discussed in detail in this chapter. Additionally the importance of this gene and its position in the tumour suppressor pathways were considered, including the intricate interactions between the different pathways and genes.

The tumour types chosen for the rest of this study and the motivation was also described and discussed.

In the following chapters the known role and importance of PTEN in these different neoplasms will be discussed. In addition the findings of our own research work will be described and put into perspective.

The concluding chapter will try to answer the research questions set out here.

Chapter 2

The role of the tumour suppressor gene PTEN in the etiology of endometrial cancer and hyperplasia

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1. Introduction

1.1 Background

Endometrial cancer is the most frequently diagnosed female genital cancer in the western world and it is second only to cervical cancer in the developing world. The incidence worldwide is steeply increasing and so is the number of deaths from endometrial cancer especially in the developing world, as it is frequently not diagnosed early in less developed parts of the world. Various epidemiological studies have also suggested a poorer prognosis for non-European racial groups.

The stage distribution of endometrial cancer is generally very favourable, as it tends to be a symptomatic cancer early in the disease process. The prognosis for the disease is therefore also relatively good although the prognosis per stage is slightly worse than that for cervical cancer. Large differences exist for the prognosis between histological types, differentiation grades and population groups even when corrected for stage.

The relative advantages of different (new) treatment strategies are still hotly debated. Examples of treatments that may offer advantage to some subsets of patients include lymph node dissection, adjuvant radiation, adjuvant chemotherapy and upper abdominal staging.

The classical epidemiological risk factors are listed below in table 2.1. Numerous studies suggest that both endogenous and exogenous estrogen and Tamoxifen play important roles, as does the balance of stimulating versus stabilizing sex hormone (progestogen). Uterine carcinomas are histologically diverse and it should be expected that the pathogenesis of tumour types be different.

Table 2.1: Classical risk factors for endometrial carcinoma

Endogenous hyperestrogenism
Obesity
Polycystic ovary syndrome
Chronic anovulation
Functional ovarian tumours

Exogenous hyperestrogenism
Unopposed HRT
Tamoxifen
Hereditary
Lynch II, hereditary non-polyposis colon cancer
Family history colorectal / endometrial cancer
Other

Endometroid adenocarcinoma is the most common type, with clear cell and serous papillary types much less common and also more aggressive. Endometrial hyperplasia is the ideal benign counterpart for endometroid adenocarcinoma with atypical hyperplasia a proven precursor lesion. These benign and pre-malignant lesions share the etiological factors of endometrial cancer.

Carcinogenesis is a multistep process where genetic lesions are accumulated. These lesions occur mainly in oncogenes and tumour suppressor genes, resulting in the initiation of uninhibited growth and eventually in tumour progression and metastatic potential. Renan (1993) estimated that six genetic mutations are needed for tumorigenesis in the endometrium, based on the age-specific incidence of these cancers.

After some years of study, endometrial carcinoma still displays the highest percentage of PTEN mutations of all tumour types. Additionally, PTEN is the most frequently mutated tumour suppressor gene in endometrial carcinoma (Mutter et al, see also 2.3.4.1.2). It is not known whether this is also true for the South African population as previous reports pointed towards differences between population groups. Some differences between the different local population groups are also expected.

It is not very clear when in the carcinogenetic process these mutations occur and what influence the mutations have on tumour biology and growth patterns. Hormone receptor positivity is another molecular cellular change that correlates strongly with tumour biology and behaviour. There also exists a strong inverse correlation with differentiation grade. It is unknown how PTEN mutation rate correlates with differentiation grade.

In a study comparing 15 Afro-American with 115 European-American endometrium carcinoma patients, Hicks found poorer survival in the first group, which could best be explained by the increased number of poor prognostic factors in the group of Afro-American women (Hicks et al 1997).

Matthews and co-workers conducted a retrospective study on 401 patients to examine the influence of race and histology on outcome. They also found tumour type and stage to be the important predictors of outcome. Again a strong association was found between aggressive histological types and Black race (Matthews et al 1997).

In an important population based study, Plaxe and Saltzstein (1997) reported that Black women develop significantly less low risk tumours and in fact have the same incidence of high risk endometrial cancer. This leads to the bias towards high-risk tumours and poor outcome in Black women. The incidence of these tumours and the outcome in different South African race groups has not been studied.

Bokhman described two different clinical pictures of endometrial cancer and related it to histological features. This classification is discussed below under histology. These two types also display important molecular differences and although these features are not absolute the differences are usable in the clinic and correlates with outcome. These features are summarized in table 2.2.

Table 2.2: Typical clinical and molecular features of type I and type II endometrial cancer

Feature	Type I	Type II
Clinical features		
Etiology	estrogen related	age related
Race distribution	more in European	equal
Histological type	endometroid	other
Differentiation grade	grade 1	grade 3
stage	early	late
prognosis	good	poor

Molecular features		
MSI	positive	negative
PTEN	positive	negative
Her 2/neu	negative	positive
K-ras	positive	positive
P53	negative	positive

1.2 Research quest

In this chapter existing evidence of involvement of the PTEN gene in endometrial carcinogenesis will be explored and interpreted using the knowledge of endometrial carcinogenesis in histology, genetics and molecular medicine. Additionally, involvement of the tumour suppressor gene in subsets of patients with endometrial cancer of different grades and endometrial hyperplasia specimens will be tested.

The hypothesis is that the PTEN gene is intimately involved in endometrial carcinogenesis and may be involved in endometrial hyperplasia.

The research questions as listed in chapter 1 are:

1. What role does PTEN gene mutation and pten protein inactivation play in the etiology of endometrial carcinoma?
2. What is the frequency of PTEN mutations in endometrial cancers and pre-cancers?
3. When in the carcinogenetic process do these mutations occur?
4. How does PTEN mutations correlate with disease stage and grade?
5. How does the involvement of the PTEN gene differ between the different population groups in South Africa?
6. How does the involvement of the gene differ between South African patients and other reported populations?

2. Literature overview

2.1 Genetic changes in endometrial proliferative disorders

2.1.1 DNA repair genes and micro-satellite instability

Micro-satellite instability (MSI) was first demonstrated in colon cancer and endometrial cancer as another genetic alteration that occur in many familial and in some sporadic tumours (Ionov et al 1993; Risinger et al 1993). This anomaly manifests through the alteration of DNA repeats, called micro-satellites. Due to the repeating nature, these repeated sequences are more prone to replication errors, named RER+, which is then recognised by the instability of the microsatellites.

In hereditary non-polyposis colorectal carcinoma, HNPCC, these genetic abnormalities are inherited in a dominant fashion, leading to a germline mutation causing the syndrome (Aaltonen et al 1993). It is now known that inherited mutations in the DNA mismatch repair genes, namely hMLH1, hMSH2, hMSH6, hPMS2, cause HNPCC. Endometrial cancer is the commonest non-colonic cancer in females born with these mutations, causing a lifetime risk of between 22% and 43% (Watson & Lynch 1993; Aarnio et al 1995). Lynch syndrome or HNPCC is the only known familial cancer syndrome that often causes endometrial cancer.

Although MSI is frequent (15% to 34%) also in sporadic endometrial cancer, somatic mutations in the DNA repair genes are not frequent in sporadic endometrial cancers. Instead, it has been shown that the finding of MSI correlates strongly with methylation of the hMLH1 promoter region (Simpkins et al 1999), which is an epigenetic finding, causing inactivation of the hMLH1 gene (Salvesen et al 2000). The result on cellular level is the same as a mutation in the gene causing defects in the DNA repair system (Peiffer et al 1995). Impaired DNA repair may be an important reason for mutations in the tumour suppressor genes to go unchecked and be a pre-cursor to further pre-malignant genetic change.

In endometrial cancer MSI has been found almost exclusively in the endometroid adenocarcinoma histological subtype. No association was found with either differentiation grade or tumour stage by Smid-Koopman in 2002, but others

have shown association with poor differentiation grade and other markers of poor prognosis (An et al 2007).

Some authors have investigated the correlation between PTEN mutations and MSI, but results are not easy to interpret and study size tend to be small (Kanaya et al 2005).

2.1.2 Proto-oncogenes and oncogenes

2.1.2.1 K-ras

Activation of the Ras proto-oncogene family has been detected in a number of malignancies at frequencies depending on the type of tumour. Activation occurs mostly by point mutation and was found most frequently in pancreas carcinoma (~90%) but relatively infrequently in gynaecological cancers (Kofa & Spandidos 1997). In endometrial cancer mutations occur mostly in the K-ras (14%-30%) and sometimes in the H-ras (7%) gene.

Two groups correlated activation of these genes with a poor outcome (Mizuuchi et al 1992; Fujimoto et al 1993), while another group found the opposite (Sasaki et al 1993). K-ras mutations is now frequently quoted to be associated with type I cancers although the evidence is somewhat unconvincing (Cerezo et al 2006). Ras activation was also demonstrated in precursor lesions, suggesting involvement at the early stages of carcinogenesis (Mutter et al 1999).

Turbiner and co-authors found more K-ras mutations in Tamoxifen exposed endometrial cancers and fewer PTEN mutations (2008).

2.1.2.2 HER *2/neu* or c-erbB-2

Since 1991 several groups have studied the involvement of this oncogene in endometrial cancer, by studying over-expression, mutation and amplification. Many attempts have been made to find an association with histological grade, stage and prognosis with varying results. An important study by Macwhinnie and Monaghan (2004) could not show a difference between serous papillary and endometoid histological subtypes.

The majority of published results suggest higher grade, and some also higher stage at diagnosis or worst prognosis in patients with over-expression (Saffari et al

1999; Rolitsky et al 1999; Riben et al 1997). Konopka and co-workers (2004) could not demonstrate any erbB-2 amplification in 43 endometroid adenocarcinomas.

2.1.2.3 Bcl-2

The proto-oncogene Bcl-2 is an inhibitor of programmed cell death. It counteracts the action of p53, which induces apoptosis. Morsi and colleagues studied bcl-2 protein expression in normal endometrium (2000). This group observed cyclical changes of bcl-2 expression in normal endometrium and decreased expression levels in hyperplasia and carcinoma. Several groups have since showed that expression levels correlate negatively with differentiation grade (Geisler et al 1998; Zheng et al 1996).

The correlation of the bcl-2 activity with carcinogenesis is still incompletely expression seems to be important and not the quantity. The function and inhibition of this proto-oncogene remains difficult to study and the various measures of activity difficult to interpret. Levels of expression may correlate with some other measures of aggressive tumour growth but is not an independent prognostic indicator (Peiro et al 2003).

2.1.2.4 C-fms

The proto-oncogene c-fms encodes a transmembrane tyrosine kinase receptor for the growth factor CSF-1 or colony stimulating factor-1. CSF-1 was found to inhibit growth and induce cellular differentiation. Altered expression of this factor and thus of the CSF-1 receptor is found in 50-60% of endometrial carcinomas and has been correlated with high grade tumors (Leiserowitz et al 1993; Smit et al 1995; Kimura et al 1991).

2.1.2.5 C-myc

Proto-oncogene c-myc is an early response gene and is essential in controlling cell proliferation. Mutations in this gene seem to be rare in endometrial carcinomas (Monk et al 1994; Niederacher et al 1999).

2.1.3 Tumour-suppressor genes and onco-suppressor genes

2.1.3.1 P 53

Mutations of the tumour-suppressor gene P53 occur very commonly in human tumours. Wildtype P53 functions as a G1 arrest and such arrest at the G1-S checkpoint creates extra time for DNA repair meganisms. P53 may initiate cell death via apoptosis (Lain 1992) if DNA repair fails. Mutations in the P53 gene can result in a protein with increased stability, leading to a longer half life of the mutant protein. Functionally inactive P53 protein can thus become over-expressed in the tumour cell (Findley 1988). Total loss of P53 expression is a relatively uncommon event, which can result from complete deletion of the P53 gene.

Several groups studied the expression level of the p53 protein in endometrial cancer using immuno-histochemical staining. In endometroid adenocarcinomas, p53 overexpression occurs in 15-55%, but in the more aggressive papillary serous adenocarcinomas 52-95% of tumours show over-expression. Various authors found a significant correlation with high stage tumours (Kohler et al 1992; Ito et al 1994; Lax et al 2000). In serous adenocarcinomas, p53 overexpression seem to occur as an early event with overexpression shown in high and low stages (Geisler et al 1999). This group and others (Kohler et al 1992) demonstrated that p53 overexpression is an independent prognostic marker, using multivariate analysis.

Recently, however, many authors challenged immuno-histochemical staining as a method to evaluate p53-activity. Methods to quantify staining results, cut off levels for defining overexpression and the antibodies used, differ widely between the studies. Importantly, Ito et al (1994) also found that about 15% of the P53 mutations show no immunoreactivity.

Overexpression of p53 seems to be a rare event in pre-malignant hyperplastic endometrium and even in stage 1 endometroid adenocarcinomas. However, in serous papillary adenocarcinomas the precursor lesion, EIC (endometrial intraepithelial carcinoma) is always associated with p53 overexpression if the tumour is p53 positive (Sherman et al 1995), demonstrating involvement early in carcinogenesis.

2.1.3.2 DCC

The tumor suppressor gene DCC (Deleted in Colorectal Carcinoma) plays a roll both in cell growth and cellular differentiation. Three groups have investigated DCC-expression in endometrial carcinoma (Seagusa et al 1999; Enomoto et al 1995; Gima et al 1994). These groups reported mutations and loss of protein expression in 30-50% of endometrial carcinomas without association with stage or grade.

2.1.3.3 Rb-gene

The retinoblastoma gene (Rb) was the very first tumour suppressor gene to be described and it was found to be responsible for hereditary retinoblastoma syndrome (Friend et al 1986; Lee et al 1987). In endometrial cancer alterations in this gene seem to be very rare. Loss of heterozygosity (LOH) at the Rb locus have been demonstrated in 10% of endometrial carcinomas and immuno-histochemistry has been used to demonstrate loss of Rb protein expression by Niemann et al (1997) and Semczuk et al (2000).

2.1.3.4 PTEN

The role of the PTEN tumour suppressor gene in endometrial carcinogenesis has been studied extensively and will be critically analysed and discussed below.

2.2 Histology of endometrial proliferative disorders

2.2.1 Endometrial hyperplasia and precursors of endometrial cancer

2.2.1.1 Endometrial hyperplasia

Endometrial hyperplasia essentially implies overgrowth of endometrium, consisting of endometrial glands and stroma. It is almost exclusively associated with a relative excess of endogenous or exogenous estrogen. Various histological subtypes are identified, according to the degree of cellular and structural differentiation and atypia. Simple hyperplasia (SH) resembles the normal endometrial tissue growth pattern, while complex hyperplasia (CH) has a more complex and thus more abnormal architectural growth pattern.

Both simple and complex hyperplasia can be associated with cellular atypia (SAH and CAH), which seems to be the most important predictor of malignant

potential (Scully et al 1994). Complex hyperplasia with atypia is the most dangerous type, with an estimated risk of simultaneous malignancy of about 20% (Prat 1996). Molecular markers that will predict progression to malignancy with accuracy are still outstanding (Orbo et al 2004).

2.2.1.2 Endometrial intra-epithelial neoplasia (EIN) or carcinoma (EIC)

This precursor of malignancy is the non-hyperplastic precursor lesion associated with serous papillary endometrial cancer (Fox 1992). This lesion is not associated with hyper-estrogenism, and will commonly arise in a background atrophic endometrium (Ambros et al 1995). When computerised morphometric analysis is used, the term is sometimes also used for atypical hyperplasias (Mutter 2000). This is probably warranted in a lesion co-existing with carcinoma in 20% of cases.

Precursor lesions have for the most part the genetic aberrations of the malignant lesion that will typically follow them. This correlation is so strong that markers of genetic abnormality are now used to detect the pre-cursor lesions in the background “normal” tissue. Maia and co-workers (2003) used PTEN and bcl-2 markers to help detect EIN in background epithelium of patients with carcinoma.

In the same way endometrial hyperplasia and specifically atypical hyperplasia (CAH) can be used to study the carcinogenesis of endometroid adenocarcinoma and endometrial intra-epithelial carcinoma (EIC) to study serous papillary adenocarcinoma.

2.2.2 Endometrial polyps

Various scientific findings support the idea of a common etiology or at least some shared etiological factors for endometrial thickening, endometrial hyperplasia, endometrial polyps and cancer. While endometrial hyperplasia presents overgrowth of both components of the endometrium, endometrial polyps are formed by stromal overgrowth covered in a normal fashion by glandular epithelium.

The abundance of literature on tamoxifen and its effects on the uterus includes benign, premalignant and malignant changes of both myometrium and endometrium, but with a very definite emphasis on the latter. The most common

lesions associated with its use are endometrial polyps and cystic hyperplasia. This also supports the idea of a shared etiology.

On a tissue level it can be postulated that endometrial polyps are the most probable benign counterpart of endometrial stromal sarcoma. Endometrial polyps and these models of carcinogenesis were not explored further in the current study but would deserve future attention.

2.2.3 Endometrial cancer

2.2.3.1 Pathogenetic subtypes

Since Bokhman described two types of endometrial cancer (Bokhman 1983), many authors have investigated and confirmed that there are two main etiological or pathogenetic pathways. In the majority of young patients the carcinoma is associated with hyperestrogenism and a better prognosis, while older patients typically have endometrial atrophy, low estrogen levels and more aggressive tumours. Molecular findings support this view and have identified various cellular genetic differences between the groups (Kaku et al 1999, Matias-Guiu et al 2001). Some of these findings were summarized by Ryan and colleagues in 2005.

Concomitant endometrial hyperplasia seems to be the most constant histological differentiator between these two groups (Beckner et al 1985, Deligdisch et al 1985). Tumours developing in a background of hyperplasia display a better prognosis and association with the type of genetic aberrations frequently seen in such better prognostic groups and in well-differentiated tumours (Scully et al 1994, Kurman et al 1994).

It is important that the pathogenetic subtypes were described initially within the group of endometroid adenocarcinomas, as a way to differentiate between two prognostic groups with essentially the same histopathologic tumour type. It is widely accepted that the etiopathogenesis of serous papillary adenocarcinoma overlaps mainly with Bokhman type 2 and arises mainly from atrophic endometrium.

Other non-endometroid carcinomas are rare and the pathogenetic differences are poorly understood (Darvishian et al 2004). Some endometrial carcinomas with

tubular growth may indeed represent serous carcinomas on molecular basis. When the treatment strategies for these tumour types diverge molecular tumour typing will become extremely important.

2.2.3.2 Histological subtypes

Endometroid adenocarcinoma is by far the commonest type, accounting for 80% of tumours. These tumours are subdivided in adenocarcinoma, adeno-acanthoma and adenosquamous carcinoma (Pecorelli et al 1999) according to the presence and type of squamous component. The degree of cellular atypia further divides the group into well, moderately and poorly differentiated groups, which has been convincingly shown to have important prognostic implications and has been included in FIGO staging in 1988.

Non-endometroid carcinomas include papillary serous adenocarcinoma (the most common subtype, accounting for about 10% of cases), clear-cell carcinoma, mucinous adenocarcinoma, adenosquamous carcinoma, undifferentiated and mixed carcinoma (Scully et al 1995, Pecorelli et al 1999). The latter four subtypes are extremely rare and are therefore inadequately studied.

With improved techniques more tumours are shown to be of the mixed subtype. As is the case in carcinosarcoma, these tumours are convincingly shown to be monoclonal with molecular techniques. An and co-workers (2004) showed monoclonality by identifying identical mutations in PTEN and P53 genes in different sections of several mixed tumours.

2.3 PTEN gene and endometrial proliferative disorders

2.3.1 PTEN in normal endometrium

Pten expression in the normal endometrium changes in response to hormonal variations (Mutter et al 2000). During the proliferative phase pten-protein is expressed in all tissue types, while expression is increased in the early secretory phase and lowered in the late secretory phase. These changes seem to be confined to the functionally active and hormonally responsive layers of endometrium.

Interestingly, Mutter and co-workers also found a proportion of normal, non-hyperplastic, endometrial lining cells already deficient in pten expression. PTEN

mutations were not studied in these cells, thus abnormal pten expression can be due to many factors. These cells probably do not progress to clonal representation, due to exfoliation. The apoptotic effect of progesterone would also induce natural cell death of genetically abnormal cells.

2.3.2 PTEN in endometrial hyperplasia

Should PTEN mutations be found to occur at about the same rate in pre-cancerous proliferative disorders as in endometrial cancer, this finding would suggest early inactivation of the PTEN-gene and thus an important role in carcinogenesis.

In the very limited reports available, PTEN mutations have indeed been demonstrated in endometrial hyperplasia and specifically in higher grades of hyperplasia like CAH. Sun and co-workers (2001) found different rates of PTEN mutations when analysing different subtypes of endometrial hyperplasia. They report an incidence of only 1 in 40 hyperplasias without atypia, but an incidence of 18% in CAH. The same group reported an incidence of 26% in endometroid endometrial cancer.

Kanaya and co-authors (2005) reported PTEN mutations in 5 of 27 (19%) endometrial hyperplasia specimens, all five were all atypical hyperplasia. Both frameshift mutations were associated with hMLH1 hypermethylation and the authors postulate that the latter is the earlier event leading to the PTEN mutation. PTEN mutation is then probably the important pre-cancerous event.

When computerised morphometry was used to identify EIC, this precursor lesion showed a high frequency of PTEN mutation (55%). This series included mostly complex atypical hyperplasia and not the EIC originally described in the absence of hyperplasia as a pre-cursor of papillary serous carcinoma. This important difference explains the high frequency of mutations as this series can then be seen as including mostly true clonal pre-cursor lesions of endometroid adenocarcinoma. In the same series the highest ever PTEN mutation rate of 83% is reported for endometrial carcinoma. These authors could demonstrate an absence of mutations in normal endometrium (Mutter et al 2000).

The group of Levine examined a series of 29 complex atypical hyperplasias and report an incidence of 27% in patients with synchronous cancer and 22% PTEN mutations without synchronous invasive disease (Levine et al 1998). Maxwell et al (1998) could not demonstrate differences in the incidence of PTEN mutations in hyperplasia with and without cellular atypia, finding 19% versus 21%.

It is considered important to study this topic further in the light of limited information and discrepancies in the literature.

2.3.3 PTEN in progression to endometrial cancer

Although evidence points toward increased incidence of PTEN mutations in precursor lesions of higher order, no direct association has been demonstrated between mutation of the PTEN-gene or loss of protein function and the development of an invasive lesion. On the contrary, evidence seems to support the early development of PTEN mutation as a factor supporting uncontrolled growth and associated also with pre-invasive lesions.

Interestingly, small clusters of PTEN deficient glandular cells have even been demonstrated in hyperstimulated endometrium not yet fitting the diagnostic criteria for hyperplasia (Mutter et al 2000). These results also demonstrate the cellular genetic variance of normal endometrium versus the cellular monoclonality of true pre-invasive hyperplasia.

The results of PTEN gene involvement in normal endometrium, as for most normal tissues, have been obtained using immunohistochemical staining for the pten protein, a technique showing very high rates of altered pten levels in endometrial cancer (up to 83%) and hyperplasia (55%). This technique also demonstrates the involvement of pten protein levels in cellular growth abnormalities by other methods than PTEN gene mutation. Pten protein expression can, for example, also be abnormal due to inhibition of upstream growth regulators.

2.3.4 PTEN in endometrial cancer

2.3.4.1 Frequency of PTEN mutations in endometrial cancer

2.3.4.1.1 Germline mutations

As described in chapter 1, PTEN was initially found as a result of the mapping of the susceptibility gene for Cowden syndrome. Germline mutations in the PTEN gene has subsequently also been linked to the Banayan-Zonana syndrome and to Proteus syndrome. It is thought that the latter can also be the result of mosaic germline mutations.

Mice with pten protein knockout develop complex proliferative endometrial lesions pointing to importance in cellular growth regulation in the female reproductive tract (Podsypanina et al 1999).

Despite the association of malignant epithelial and sometimes endometrial cancer with the Cowden syndrome (a cancer predisposition syndrome) (Mutter et al 2000), germline mutations are very uncommon in sporadic endometrial cancer. Black and co-workers (2004) recently published the results of 240 consecutive patients with endometrial cancer in whom they found only one scarce polymorhism (exon 4) and no disease causing germline mutations. This work confirms the results of other workers in the field.

2.3.4.1.2 Somatic mutations

Loss of heterozygosity was found to occur frequently (in about 40%) on the 10q chromosome (LOH 10q) in endometrial carcinomas (Peiffer et al 1995; Kong et al 1997). This led to the search for a tumour suppressor at this locus and the PTEN or MMAC1 gene, situated on 10q23.3, was found to fit the description. Evidence for the co-involvement of multiple other genes at this site has since emerged and continues to be investigated (Nagase et al 1996; Nagase et al 1997; Simpkins et al 1998).

The incidence of somatic mutations in PTEN in endometrial cancer is the highest of any tumour suppressor gene in any primary malignancy analysed so far. The frequencies reported vary from approximately 40% to even 76% and 83% (Nagase et al 1997; Peiffer et al 1995; Risinger et al 1997; Tashiro et al 1997; Mutter et al 2000).

Risinger and co-workers (1997) examined 70 endometrial cancers and found PTEN mutations of 34%, making it at the time the most commonly mutated known gene in endometrial cancer. Tashiro et al (1997) reported a 50% mutation rate in endometroid endometrial cancer. Kurose first described inactivation of both alleles, reporting an incidence of 33%, and confirmed the pattern that defines tumour suppressors for PTEN mutation in endometrial cancer (Kurose et al 1997).

2.3.4.2 PTEN gene inactivation by other methods

Not only genetic change but also epigenetic change can inactivate this important gene. It is now widely recognised that promotor PTEN methylation plays a role in tumorigenesis in some neoplasms. Some tumours have more than one genetic abnormality or "two hits" in PTEN and this may be two mutations or one mutation plus promoter methylation. This finding correlates with abnormality of DNA repair (Salvesen et al 2004) which is often also methylation of the hMLH1 gene.

The relation of genetic changes to pten protein expression is extremely complex. PTEN activity can be tested by mutation analysis, pten protein immunohistochemistry (Kimura et al 2004), and PTEN-antibody 6H2.1. PTEN antibody abnormality is decreased with exon 8 mutations. The latter two are low in about 20% of endometroid tumours, while about 54% will display PTEN mutations.

2.3.4.3 PTEN mutations in endometrial cancer subtypes

Sun and co-workers found a PTEN gene mutation incidence of 26% in endometroid and only 1 in 7 (14%) in non-endometroid tumours (Sun et al 2001). This confirmed the results of Risinger et al (1998) who found 37% in endometroid and 5% in non-endometroid carcinomas and Tashiro et al (1997) who reported 50% in endometroid and 0% in non-endometroid carcinomas.

Simpkins examined 34 endometroid tumours shown to have loss of 10q sequences and found an incidence of 38% PTEN mutations. The numbers in all these studies are small, but the results compare well, supporting the overall accuracy of the findings. This research has not been repeated in South Africa, in African patients or outside the developed world.

2.3.4.4 Association of PTEN with other genetic anomalies

Loss of **P53** function and overexpression of p53 protein product is strongly associated with serous papillary tumour type and with poorly differentiated tumours lacking hormone receptors (Kovalev et al 1998; Koul et al 1997; Boyd 1996). Tumours with PTEN mutations typically lack p53 overexpression, suggesting that the same biological endpoint, namely inhibition of apoptosis, is reached via either of these pathways but typically not via both.

Microsatellite instability or MI (typically defined as tumours with detectable alterations at two or more different microsatellite loci) is strongly associated with PTEN mutations in both endometrial carcinoma and hyperplasia (Levine et al 1998; Tashiro et al 1997). This strong association suggests that PTEN mutations may arise secondary to loss of the DNA repair system, although PTEN mutations can also precede the development of the MI phenotype (Levine et al 1998).

Loss of heterozygosity (LOH) of chromosome 10q is associated in about 40% of cases with PTEN mutation. Many other genes may be responsible for this finding. LOH of many chromosomes are typically associated with poorly differentiated tumours and serous papillary subtype. This finding has a low predictive value and no current therapeutic potential.

Although **K-ras activation** has been demonstrated frequently in precursor lesions and in endometrial cancer, the association with PTEN is unclear. This association will be discussed in more detail in chapter 3.

2.3.4.5 PTEN mutations and the prognosis of endometrial cancer

PTEN mutation is associated with tumour characteristics that generally have a good outcome. The most important is the close association with endometroid histology. PTEN mutation also correlates with the presence of MSI and absence of p53 overexpression, both predictors of good clinical outcome (Risinger et al 1998).

In many other tumours PTEN mutation has been found to predict a worse outcome and higher metastatic potential. Glioblastoma multiforme is one important example of a tumour where PTEN anomalies are frequently associated with late stage and a poor outcome. In endometrial cancer PTEN mutation

correlates with a better rather than worse outcome. This difference in the role of the tumour suppressor in different cancers is poorly understood. It probably depends on the specific sequence of genetic alterations that would lead to tumorigenesis in the specific organ and histological or morphological tumour type.

2.3.4.6 PTEN in different population groups and races

Little is known about PTEN mutation rates in different races. It is expected that the total incidence of PTEN mutations in Black women would be lower, because of the lower incidence of low risk, endometroid adenocarcinomas. Large differences in tumour types and tumour incidence exists between different population groups, and a difference in tumour type and also in etiopathogenesis on molecular level should be expected.

This study aims to address this as an important research question.

3. Materials and methods

3.1 Materials

3.1.1 Sampling and clinical material

Purposive sampling was used to select cases in a non-randomized way. This case selection is a non-probability type of sampling suited to this kind of research where cases typical of a category are of interest to the researcher. Forty-eight consecutive cases of endometrial carcinoma were identified in a retrospective review of the gynaecologic oncology clinical filing system. All these patients were surgically treated at the Gynaecologic Oncology Unit at the University of Pretoria during the years 1996 to 1998. Clinical data was available from these files on presentation, treatment, stage and outcome on most patients.

While on a study visit in Utrecht, the Netherlands, I was also allowed to include samples from the University Hospital of Utrecht (AZU). The anatomical pathologist (Dr Daisy Sie-Go) identified twenty-three suitable cases diagnosed from 1996 to 1999. These cases were selected for endometroid histology, all occurred in Caucasians and the clinical and histological characteristics will be explored further.

PTEN mutations have been reported in endometrial hyperplasia before, with a higher frequency in premalignant types. These findings have not been repeated locally and have not been correlated with race. In any study of PTEN involvement in endometrial cancer, it would be important to correlate the findings with that of the benign counterpart. Here we wanted to correlate the findings of the different grades of tumour with mutation frequency including hyperplasia of different aggressiveness on histology.

Slides of paraffin embedded endometrial tissue samples of different types of endometrial hyperplasia were kindly presented to us by the department of Anatomical Pathology, University of Leuven in Belgium. Twelve tissue samples with histological diagnosis were received, originating from ten patients.

3.1.2 Histology reports

Histology results from the original reports were available for all patients. These reports were used in the analysis. All tumours were also reviewed, histological diagnoses were confirmed and the tissues containing tumour and normal tissue were outlined.

The pathologist in Utrecht, Dr Daisy Sie-Go, also reviewed all tumours from her subset. Abnormal areas containing tumour material were marked and the material was then analysed as described below.

Two of the twelve tissue samples of endometrial hyperplasia had the histopathological diagnosis of complex atypical hyperplasia and ten had simple atypical hyperplasia. All the patients were Caucasian.

3.1.3 Tissue for DNA analysis

Paraffin embedded tissue was retrieved from the existing stored blocks of tissue. The first section of each block was stained and studied to confirm the diagnosis and presence of tumour and normal tissue. DNA from tumour and normal tissue was obtained from each case. The details of DNA extraction will be outlined below.

The endometrial hyperplasia samples were received and cut in sections. The first slide was marked to show the areas of hyperplasia reported on the histology. Tissue was obtained directly from the glass plates with a sterile sharp scalpel.

3.2 Methods

3.2.1 DNA extraction

Paraffin embedded tissue previously confirmed to contain either tumour or normal tissue was used. After hematoxylin staining, the pathologist reviewed the histological diagnosis and indicated normal (myometrial) and tumour areas on one slide. Using this slide as a guide, normal and tumour tissue was removed with a sterile blade from five to ten consecutive sections per patient.

Tissue samples were carefully transferred to micro-tubes where it was treated with the extraction buffer (10mM Tris-HCL, pH8.0; 0,45% Nonidet P40; 0,45% Tween-20).

Extraction buffer (200 µl) and 0,2mg/ml Proteinase K (Roche) was added to the tissue. After overnight digestion at 55°C, the proteinase K was inactivated by boiling (5 minutes at 95°C). The DNA solutions were quenched on ice and centrifuged. The supernatant, containing the DNA was transferred to new sterile tubes and used or stored at 5°C.

3.2.2 DNA amplification

PTEN-coding sequences were amplified by polymerase chain reaction using the primers described by Guldberg et al (1997) (Utrecht) and Davies et al (1999) (Pretoria). The nine exons were amplified in ten and eleven sections, with exons five in two sections and eight in one (Utrecht) or two (Pretoria) sections. Intron-based primers were used to minimise the risk of amplifying the processed PTEN pseudogene on chromosome 9, as described by Dahia et al (1998).

The primer sequences, the amplification conditions and product lengths are displayed in tables 2.3 and 2.4.

3.2.3 PTEN mutation analysis

Samples in the Pretoria group were analysed using the SSCP-method and exons displaying aberrant bands were directly sequenced. Materials collected in Utrecht were analysed in Utrecht using the DGGE-method. Direct sequencing was not completed there on the aberrant exons and the results were later confirmed by SSCP and sequencing of abnormalities in the cancer genetics laboratory under supervision of professor EJ van Rensburg.

Although the initial screening for mutations were done using two different methods, all material was eventually screened using SSCP and the methods were found to render comparable results.

3.2.3.1 Denaturing gradient gel electrophoresis (DGGE)

The melting temperature of amplicons varies according to nucleotide composition. Using DGGE, the 5% glycerol and 15-50% urea-formamide gradient simulates the temperature gradient and separates the amplicons.

Under the optimised conditions displayed below (table 2.3), the PCR-products move through the gel until the level of the melting temperature of its lower domain. Migration of the product immediately ceases when this point is reached, with the product then visible on the gel at this point. Mutant DNA exhibits changed migration and usually shows up as double bands: wild type from contaminating normal tissue or from the normal allele plus the altered band of mutant PTEN product.

Ten primer sets, as described by Guldberg et al (1997), were obtained from Eurogentec. Exon five was amplified in two parts. DGGE gels were stained with ethidium bromide and read by UV lamp illumination. Results were photographed using Kodac film.

Table 2.3: Primers and optimised conditions used for amplification and mutation detection with DGGE (Utrecht)

Exon	Primers	Primer sequence (5' → 3')	pcr conditions (35 cycles)	DGGE gradient	Volt-hours at 60°C
1	pten 1F pten 1R	ccgtcctcctttttcttcagccac (gc)-gaaaggtaaagaggagcagcc	30" 95°C 30" 57°C	20%–50%	600

			1' 72°C		
2	pten 2F pten 2R	(gc)ttagtttgattgctgcatatttc cggcgacatcaatatttgaaatagaaaagc	30" 95°C 30" 50°C 1' 68°C add 10% DMSO	20%-50%	600
3	pten 3F pten 3R	tgttaatggtggctttttg (gc)gcaagcatacaaataagaaaac	30" 95°C 30"55°C 1' 72°C (= standard)	10%-40%	500
4	pten 4F pten 4R	(gc)ttcctaagtgcaaaagataac tacagtctatcgggtttaagt	standard	20%-50%	600
5-1	pten 5F1 pten 5R1	(gc)ttttttcttattctgaggttatc tcattacaccagttcgtcc	standard	20%-50%	600
5-2	pten 5F2 pten5R2	tcatgttgccgaaattcac (gc)gaagaggaaaggaaaaacatc	standard	20%-50%	600
6	pten 6F pten 6R	(gc)agtgaaataactataatggaaca gaaggatgagaatttcaagc	standard	20%-50%	600
7	pten 7F pten7R	cgcgcccgaatactggtatgtatttaaccat (gc)tctcccaatgaaagtaaagta	standard	20%-50%	600
8	pten 8F pten8R	gcccgtttttaggacaaaatgtttcac (gc)cccacaaaatgtttaatttaac	standard	20%-50%	600
9	pten 9F pten 9R	gttttcattttaaattttctttc (gc)tggtgttttatgggtcttg	standard	20%-50%	600

Table 2.4: Primers and optimised conditions used for amplification and mutation detection by SSCP method - Pretoria

Exon	Primer name	Primer sequence	Product length (bp)	PCR conditions Temp (°C) MgCl2 (mM)	SSCP conditions (hours at 8W)
1	PTEN 1F PTEN 1R	caagtccagagccatttcc cccacgttctaagagagtga	233	58 2.0	16
2	PTEN 2F PTEN 2R	ttcttttagtttgattgctg gtatctttttctgtggcttag	239	50 2.0	16
3	PTEN 3F PTEN 3R	ctgtcttttggtttttctt caagcagataactttcactta	213	50 2.0	15
4	PTEN 4F PTEN 4R	tataaagattcaggcaatgtt cagtctatcgggtttaagtta	190	50 2.0	15
5A	PTEN 5AF PTEN 5AR	ttgttaattaaaaattcaagag gcacatatcattacaccagt	217	48 2.0	15
5B	PTEN 5BF PTEN 5AR	tgaccaatggctaagtgaa aaaagaaacccaaaatctgtt	248	50 2.0	16
6	PTEN 6F PTEN 6R	cccagttaccatagcaat taagaaaactgttccaataca	275	50 2.0	16

7	PTEN 7F PTEN 7R	ttgacagttaaaggcatttc cttattttggatatttctcc	264	50 2.0	16
8A	PTEN 8AF PTEN 8AR	ttcatttctttttctttttctttt ggttggctttgtctttctt	238	53 2.5	15,5
8B	PTEN 8BF PTEN 8BR	ccaggaccagaggaaac cacatacatacaagtcacaa	235	56 1.5	16
9	PTEN 9F PTEN 9R	agtcatatttgtgggtttt ttattttcatggtgttttatc	268	48 3.0	17

3.2.3.2 Single strand conformational polymorphism (SSCP)

The gene was amplified in eleven pieces, with exons five and eight in two parts. The primer sequences described by Davies et al (1999) were used.

PCR products were diluted 1:10 with denaturing buffer, denatured at 95°C (5 minutes), quenced on ice and 3 µl product was loaded on a 0.5X Mutation Detection Enhancement (MDE) gel. The gels were run at 8 Watts, 14-20 h in 0,6X TBE buffer and read after drying using exposure to medical X-ray film (Fuji).

3.2.3.3 Sequence analysis

Direct DNA sequencing was performed on all samples displaying abnormal SSCP or DGGE patterns. Sequenced samples were diluted and heat denatured. Three microliter was loaded on a 6% denaturing polyacrylamide gel. Electrophoresis was performed in 1X TBE buffer at 60 Watts and results read as for SSCP gels.

4. Results

4.1 Clinical data

Of the 48 initial patients, 14 were Caucasian, one was Indian and 33 were African. Mutation analysis was completed for 47 patients who had enough suitable tissue material for DNA analysis. The age distribution of these 47 patients is shown in table 2.5 below. The majority of African patients were in the age category 60 to 69 (11/32), while the Caucasian patients had an almost bimodal pattern with six under the age of sixty and eight (the rest) over the age of seventy. The one Indian patient was 47 years at diagnosis.

The age distribution according to race is displayed in figure 2.1 below. All 23 patients in the Utrecht subset were included in this dataset.

Of the original subset, 8/14 (57%) Caucasian patients were older than 70, while 10/48 (21%) African patients were older than 70. This tendency towards higher age in the Caucasian racial group did not reach significance. The age distribution of Caucasians in the Utrecht group is similar to the African group.

Table 2.5: Age distribution of women at diagnosis per racial group.

	African (%)		Caucasian Pretoria (%)		Caucasian Utrecht (%)		Indian (%)	
< 50	2	(6)	3	(21)	2	(9)	1	(100)
50 - 59	9	(28)	3	(21)	6	(26)		
60 - 69	11	(34)	0	(0)	8	(35)		
70 - 79	6	(19)	7	(50)	6	(26)		
80 +	4	(13)	1	(7)	0	(0)		

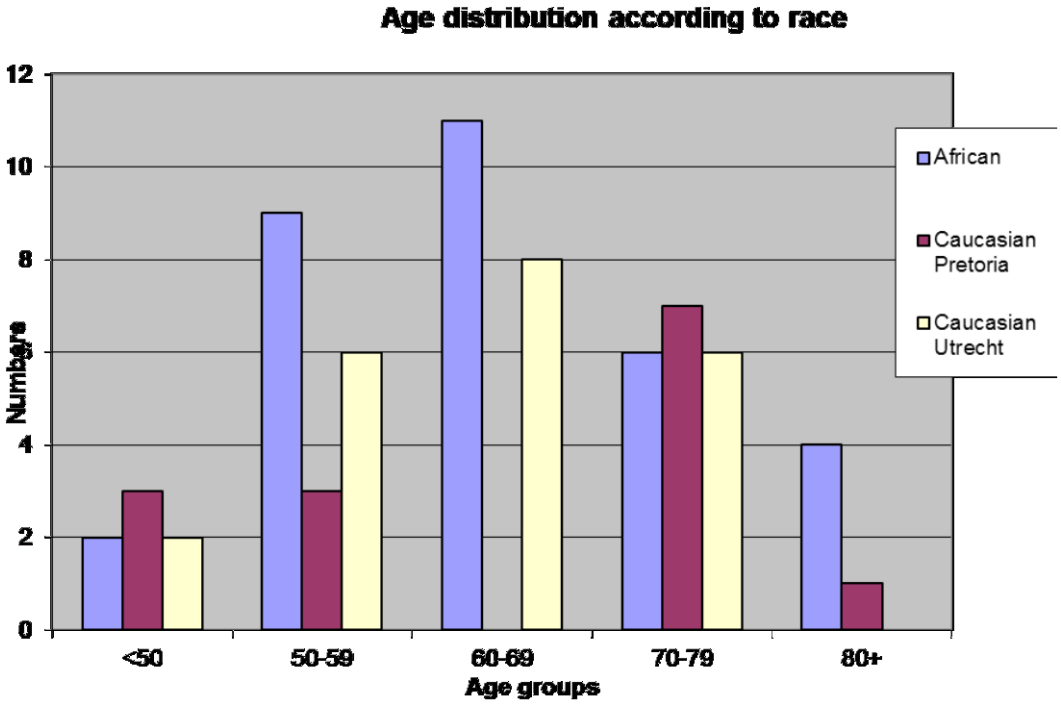

Figure 2.4: Age distribution according to race

FIGO stage distribution using the 1988 staging classification (Prat et al 1996) was known for 65 of 70 patients. Of the 37 Caucasian patients, the large majority (76%) had stage 1 disease, but only 50% of the 32 African women was diagnosed in stage 1. In the African group 28% had stages 3 and 4 disease, while only 11% of Caucasian women had such advanced disease.

The stage distribution and the percentage stage distribution according to race are displayed in table 2.6 and figures 2.2 and 2.3 below.

This important tendency towards higher stage correlates with other studies and with other gynaecological neoplasms studied in the local population. It has been ascribed to a multitude of factors, including poor socio-economic conditions, poor access to health care and lack of (health care) education. There is also the unstudied possibility of more aggressive cancer biology.

Table 2.6: FIGO stage distribution of women per racial group.

	African (%)		Caucasian Pretoria (%)		Caucasian Utrecht (%)		Indian (%)	
Stage 1	16	(50)	10	(71)	18	(78)	-	
Stage 1a	3	(9)	3	(21)	0	(0)		
Stage 1b	8	(25)	4	(29)	11	(47)		
Stage 1c	5	(16)	3	(21)	7	(30)		
Stage 2	3	(9)	1	(7)	3	(13)		
Stage 3	7	(22)	2	(14)	1	(4)	1	(100)
Stage 4	2	(6)	0	(0)	1	(4)		
Unknown	4	(13)	1	(7)	0	(0)		

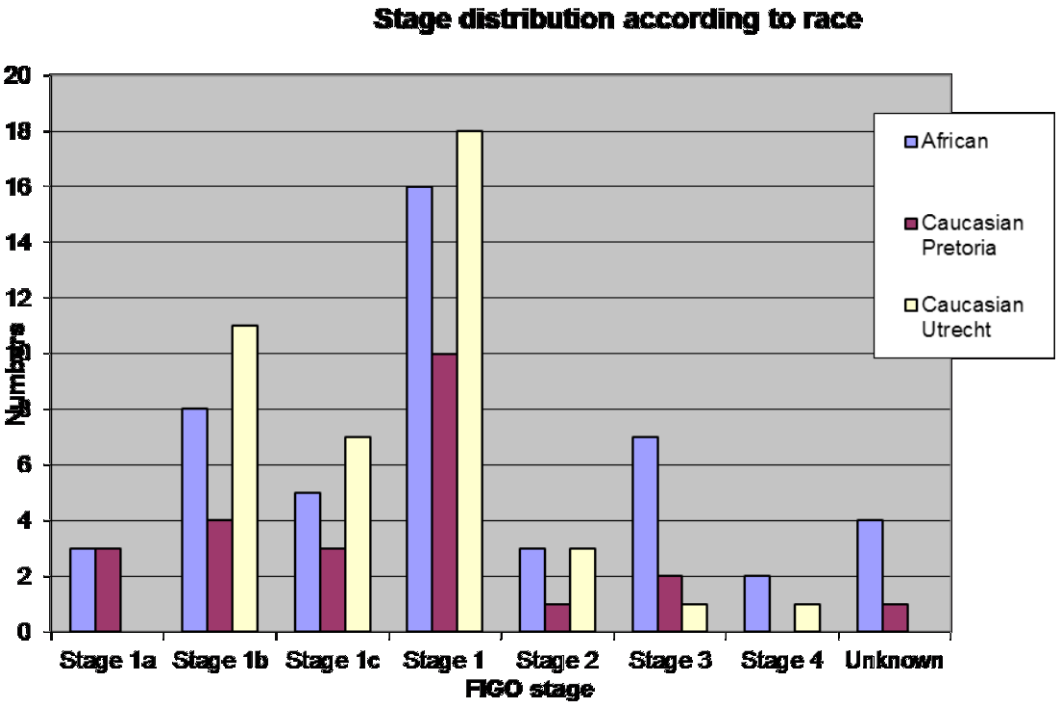

Figure 2.5: FIGO stage distribution according to race

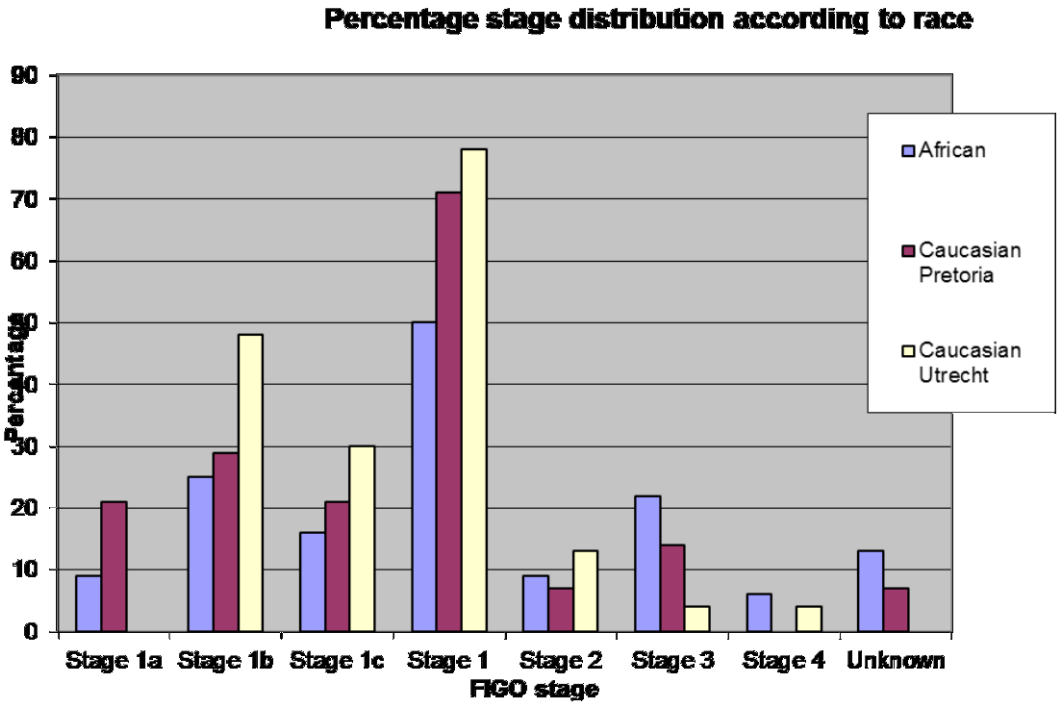

Figure 2.6: Percentage FIGO stage distribution according to race

4.2 Histology data

4.2.1 Histological type

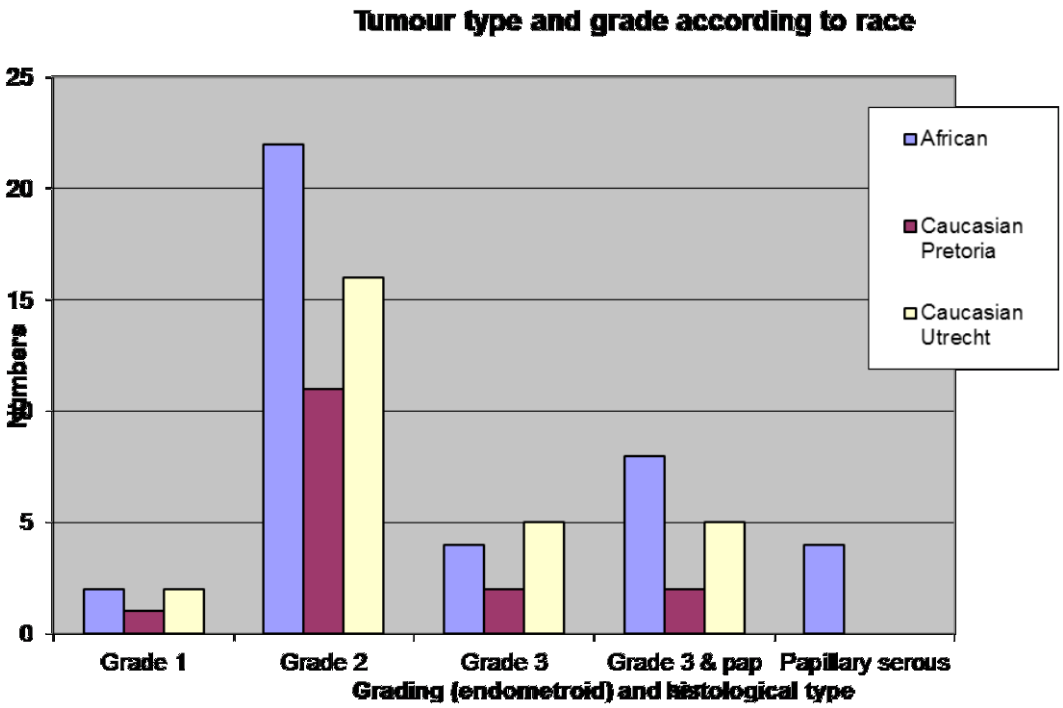

Figure 2.7: Histological type and grading distribution according to race

Four of the 48 patients had papillary serous tumours, while the rest, 44, had endometroid adenocarcinomas of various differentiation grades. The differentiation grades and tumour types of the study population are shown below in table 2.7. In African women, four of 32 tumours (13%) had papillary serous histotypes, while no Caucasian woman had this subtype. Both table 2.7 (below) and figure 2.4 display the tumour type as well as the differentiation grade according to race.

4.2.2 Histological grade

In this group of patients there was a strong tendency towards more poor prognostic pathological types in African women. Seven of the 32 (13%) African women had poorly differentiated tumours and two of 14 (14%) Caucasian women had grade 3 tumours. In all racial groups grade two cellular differentiation grade dominated.

Table 2.7: Histological grade and tumour type per racial group.

	African (%)	Caucasian Pretoria (%)	Caucasian Utrecht (%)	Indian (%)
Grade 1	2 (6)	1 (7)	2 (9)	

Grade 2	22	(69)	11	(79)	16	(70)	1	(100)
Grade 3	4	(13)	2	(14)	5	(21)		
Grade 3 & papillary serous	8	(25)	2	(14)	5	(21)		
Papillary serous	4	(13)	0	(0)	0	(0)		

4.3 Mutation screening

4.3.1 Denaturing gradient gel electrophoresis results

Eighteen tumours with normal myometrium as controls were analysed. All these analyses were repeated on SSCP and all abnormal patterns on SSCP were directly sequenced.

Representative examples of the DGGE results are shown in figures 2.5 to 2.7. The mutation screening was repeated because the sequencing was not completed in the Utrecht laboratory and the Pretoria laboratory did not use and trust the DGGE screening results. Too many results were obtained that was difficult to interpret. However, only two mutations were found in this subset with SSCP that was not predicted by the DGGE screening, both in exon 7.

The results of the DGGE screening tests on tumours and normal tissue of the first 18 Utrecht patients are shown in table 2.8.

Table 2.8: DGGE screening test results: tumours (T1-18) and normal tissue (germline DNA) (N1-18) in the Utrecht subset

	Ex 1	Exon 2	Exon 3	Exon 4	Ex 5(1)	Ex 5(2)	Exon 6	Exon 7	Exon 8	Exon 9
T 1	normal	normal	normal	normal	normal	?	normal	normal	normal	normal
T 2	normal	normal	normal	normal	?	normal	normal	normal	normal	normal
T 3	normal	normal	normal	normal	unsure	unsure	?	normal	normal	unsure
T 4	normal	normal	normal	unsure	normal	normal	normal	normal	shift	normal
T 5	shift	normal	normal	normal	normal	normal	normal	normal	normal	normal
T 6	shift	normal	normal	normal	shift	shift	normal	normal	normal	normal
T 7	normal	normal	normal	normal	normal	shift	normal	normal	normal	normal
T 8	normal	normal	normal	normal	shift	shift	unsure	shift	normal	normal
T 9	normal	normal	normal	normal	normal	shift	normal	normal	normal	normal
T 10	normal	unsure	normal	normal	normal	shift	normal	normal	normal	normal
T 11	normal	normal	normal	normal	?	shift	?	normal	normal	normal
T 12	normal	normal	normal	normal	?	normal	normal	normal	normal	normal
T 13	normal	normal	normal	normal	shift	normal	normal	normal	?	normal
T 14	normal	normal	normal	normal	normal	normal	normal	normal	shift	normal
T 15	normal	normal	normal	normal	normal	shift	normal	normal	normal	normal
T 16	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal

T 17	normal	normal	normal	normal	shift	normal	normal	?	normal	normal
T 18	normal	normal	normal	normal	normal	normal	normal	normal	shift	normal
N 1	normal	normal	normal	normal	?	normal	normal	normal	normal	normal
N 2	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
N 3	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
N 4	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
N 5	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
N 6	normal	normal	normal	normal	normal	normal	?	?	normal	normal
N 7	normal	normal	normal	normal	normal	normal	?	normal	normal	normal
N 8	normal	normal	normal	normal	normal	normal	normal	normal	shift	normal
N 9	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
N 10	normal	normal	normal	normal	normal	normal	normal	?	normal	normal
N 11	normal	normal	normal	normal	normal	shift	normal	normal	normal	normal
N 12	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
N 13	normal	normal	normal	normal	normal	normal	normal	normal	?	normal
N 14	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
N 15	normal	normal	normal	normal	?	normal	normal	normal	normal	normal
N 16	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
N 17	normal	normal	normal	normal	normal	?	normal	normal	normal	normal
N 18	normal	normal	normal	normal	normal	normal	normal	normal	?	normal

shift = definite shift seen on gel
unsure = probable shift
? = uninterpretable result, possible shift
Grey = mutation confirmed

4.3.2 Single strand conformational polymorphism results

Twenty seven of the forty seven tumours of the Pretoria subset showed abnormal migrating on SSCP gels. All were endometroid adenocarcinomas, while no shifts occurred in the papillary serous carcinomas. Shifts were observed in all of the nine exons.

Unfortunately in five of the tumours that displayed shifts, mutation analysis could not be completed as DNA amplification for sequencing was inadequate. Mutation analysis will therefore be reported on the thirty seven remaining endometroid adenocarcinomas, plus the eighteen carcinomas of the DGGE group and the remaining five carcinomas in the Utrecht group that were not screened by DGGE.

Two endometrial hyperplasia samples showed an abnormal migrating pattern on the SSCP gel and were directly sequenced to confirm mutation.

4.4 Sequence analysis

4.4.1 Disease causing mutations

In the Pretoria patients the following was found:

Although shifts occurred in all exons, mutations were not confirmed in exons 2, 4 and 9. A total of 32 mutations were confirmed, 24 different mutations. Three mutations were of unknown significance and one polymorphism was found in four samples.

All these mutations were confirmed to be somatic with none found in the corresponding normal tissue. Only the mutations considered to be disease causing will be discussed further. These results were previously reported, interpreted and discussed in depth by Jamison (2004).

In the Utrecht patients the following was found:

Fifteen mutations were found in 13 of the 23 tumours, with tumours T3 and T11 harbouring two mutations each. No mutations were found in the normal tissue, proving these to be somatic. Of the mutation positive tumours, two were adenosquamous type and four were poorly differentatied tumours. These mutations were also not associated with early stage as one patient with stage 4 disease had two mutations in exon 5. Two mutations of unknown significance were found in T10 and T19.

The distribution of disease causing mutations over the gene is shown in table 2.9 and compared with previously reported frequency. These mutations will be discussed per exon below.

In the endometrial hyperplasia subset of patients the following was found:

Both of the abnormal patterns on gel were confirmed to be mutations in exons 7 and 3 when sequenced. Two mutations were thus found in the group of ten patients with atypical hyperplasia examined (20%). Another mutation of unknown significance was found in HYP9, atypical hyperplasia. Both were observed in the samples with simple atypical hyperplasia, while no mutations were found in the two samples with complex atypical hyperplasia.

Table 2.9: Frequency of disease causing mutations in Pretoria subset according to exon distribution

Exon / Intron	Number of different mutations	Number of tumours with mutation	% of endometroid tumours with mutation	% of all mutations in this exon	Involvement of this exon (%) as previously published*
Exon 1	4	4	11	17.4	7
Exon 2	-	-	0	0	7
Exon 3	1	1	3	4.3	6
Exon 4	1	1	3	4.3	3
Exon 5	5	9	24	39.1	21
Exon 6	1	1	3	4.3	5
Exon 7	3	3	8	13	19
Exon 8	3	4	11	17.4	28
Exon 9	-	-	0	0	0.5
Total	17	23	54 **	100	~100

* Previously published findings estimated from Konopka et al 2002
** Some tumours harboured more than one mutation

Mutation analysis in Pretoria subset

Exon 1

Four disease causing mutations were detected in exon 1, occurring at codons 5, 6, 7 and 17. All four were truncating mutations; two were frameshift mutations causing early termination and one each G-to-T and C-to-T mutations were detected.

Exon 3

The mutation in exon 3 was a C-to-A missense mutation in codon 59.

In the sample numbered EHYP9, a missense mutation was also found. The mutation occurred in exon 3 and the significance is unclear. It could not be determined whether this mutation is germline or somatic and to what extent it would influence DNA replication and protein production. This is a novel synonymous mutation, not previously reported and is considered to be possibly disease causing.

Exon 4

In the Intron 4 a transition G-to-A mutation was detected of which the predicted effect is that of exon skipping.

Exon 5

As previously reported exon five was a mutational hotspot, especially at codon 130 where we found mutations in not less than eight tumours. |Three tumours shared a frameshift mutation (389delG), which would result in termination of the protein at codon 133. Three other tumours had a transition mutation at 388, two had C-to-T and one C-to-G transition, with predicted arginine-to-glysine and arginine-to-stop effects.

Another mutation was found in codon 149 where a C-to-T transition occurred resulting in a predicted truncation of the protein.

Exon 6

In intron six, another mutation was found that would probably result in exon skipping, namely a A-to-C mutation.

Exon 7

Two mutations were detected in exon 7, namely at codon 234 a deletion G resulting in frameshift and at codon 246 a transition C-to-T

Intron 7 again displayed a deletion G mutation, resulting in exon skipping.

In sample numbered EHYP5 (hyperplasia), a nonsense mutation was found in exon 7. The mutation, c.766→T, is presumed to be a somatic mutation although non-tumour DNA was not available for analysis. This mutation will result in protein dysfunction and is considered disease causing.

Exon 8

In exon 8 three frameshift mutations were found in four specimens, one deletion A (codon 288), one insertion A (codon 323) and two patients with deletion ACTT in codon 319.

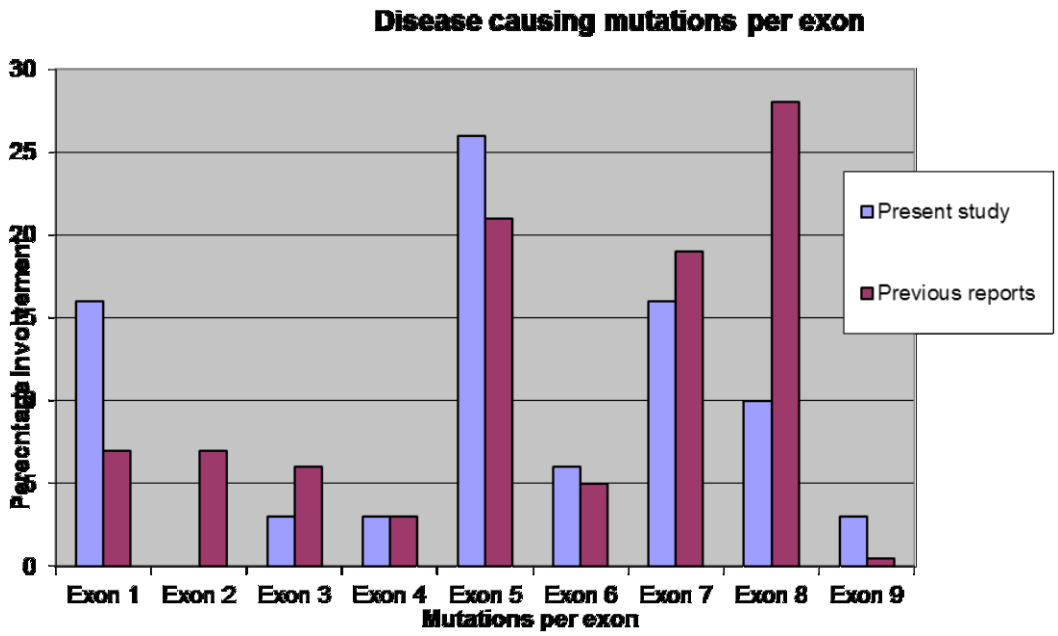

Figure 2.8: Disease causing mutation per exon in Pretoria and Utrecht groups together compared to previous reports

Mutation analysis in Utrecht subset

Exon 1

Two disease causing mutations were found occurring at codons 7 and 26. These were a truncating mutation, G-to-T (demonstrated also in one of the Pretoria group patients) and a frameshift mutation terminating the protein at 53.

Exon 5

Seven mutations were demonstrated occurring in six patients, with T11 having two mutations within this exon. There were four frameshift mutations at codons 130, 135, 142 and 143 resulting in early termination and three transition mutations. The latter occurred at codons 129 and 130 and were G-to-C, C-to-T and G-to-A transitions.

Exon 6

One missence mutation were detected in codon 173, namely a G-to-A transition.

Exon 7

Three mutations were confirmed in exon 7 in the Utrecht subset. These were one frameshift mutation at codon 221 and two identical transitional mutations at codon 233 resulting to a arginine to stop effect.

Exon 8

One of the frameshift mutations detected in the Pretoria group was also present in T4 of the Utrecht dataset (insertion A in codon 323).

Exon 9

Finally a frameshift mutation was detected in codon 346-347 in exon 9. In this tumour CTTC was deleted resultion in early termination.

Table 2.10 Frequency of disease causing mutations in Utrecht subset of endometrial carcinomas according to exon distribution

Exon / Intron	Number of different mutations	Number of tumours with mutation	% of tumours with mutation	% of all mutations in this exon	Involvement of this exon (%) as previously published *
Exon 1	2	2	8.7	13.3	7
Exon 2	-	-	0	-	7
Exon 3	-	-	0	-	6
E / I 4	-	-	0	-	3
Exon 5	7	**6	26	47	21
Exon 6	1	1	4	7	5
E / I 7	3	3	13	20	19
Exon 8	1	1	4	7	28
Exon 9	1	1	4	7	0.5
Total	15	**14	61	~100	~100

* Previously published findings estimated from Konopka et al 2002
** Some tumours harboured more than one mutation

4.4.2 Mutations of unknown significance and polymorphisms

Seven tumours of the Pretoria subset harboured mutations of which the significance is unknown or that are probably non-significant or known polymorphisms. Five of these mutations occurred in tumours shown to harbour at least one other disease causing mutation, while the polymorphism was the only genetic change in tumour END1 and in hyperplasia EHYP9. In tumour END14 a missense mutation was detected as the only genetic anomaly. It is uncertain whether this mutation affects the action of the pten protein and contributes to disease. These mutations are listed in table 2.11 below and will not be discussed further.

It is of importance to note that in this study only PTEN gene mutations were addressed. We did not investigate the incidence of pten-protein aberrations in any way. As discussed protein expression can be measured by semi-quantitative immunohistochemistry or pten protein function.

Table 2.11: Mutations of unknown significance and polymorphisms in endometrial carcinomas

Exon / Intron	Nr of tumours	Nucleotide change	Codon	Interpretation
Exon 1	1	c.44G to T	15	Arg to Iso
Exon 1	1	c.44G to C	15	Arg to Thr
Exon 2	4	IVS2-13delGTTT	N/A	Polymorphism
Exon 2	1	c.97delATT	33	Ile del
Exon 2	1	c.136delTACAGG AACAATATT	46-50	Tyr Arg Asn Asn Ile del
Exon 6	1	c.526delTAT	176	Tyr del

4.5 Correlation between clinical and molecular results

4.5.1 Correlation between PTEN gene mutations and clinical findings in the endometrial cancers

There was no difference found in PTEN mutation frequency according to race when only the endometroid adenocarcinoma group is considered. Including all the tumours, there was a tendency towards a lower frequency of PTEN mutations in African women. Table 2.12 displays the pathogenic mutations per race and stage group.

Interestingly all four tumours with more than one pathogenic mutation (three with two and one with three) occurred in African patients. When all mutations are considered, a tendency towards more mutations in tumours of a higher FIGO stage was also noted. This finding was not reported before. Most previous series contained limited numbers of patients with higher stage disease. It is possible that these genetic abnormalities accumulate with time or that tumours with more severe pten protein dysfunction will tend to be more aggressive and thus be diagnosed in a later stage.

If the latter theory were true, one would expect also a correlation with histological grade. This was not demonstrated in the current study, although it was

previously suggested. The number of mutations per FIGO stage of endometroid cancers is shown in figure 2.6.

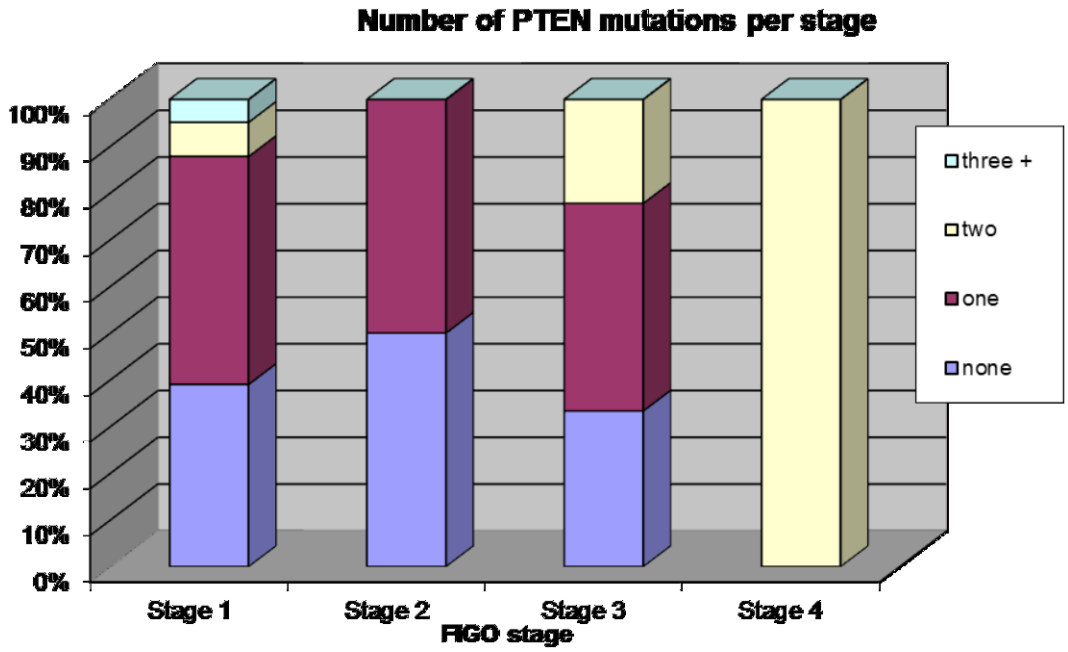

Figure 2.9: The number of PTEN mutations per FIGO stage in endometroid cancers

PTEN mutation positivity did not correlate with any other clinical parameter in this group of patients. The mean age of mutation positive patients was 62,5 years (range 43 to 92) and for mutation negative patients it was 65 years (range 45 to 84).

4.5.2 Correlation between PTEN gene mutations and histology findings in the endometrial cancers

In this group of patients pathogenic PTEN mutations were found in simple atypical hyperplasia (1/10) (10%) and in endometroid adenocarcinomas (20/37) (54%) but not in four papillary serous endometrial carcinomas (0%). Two of three adenosquamous carcinomas displayed one mutation each.

Due to the small sample size, it was not reliable to correlate the pathological subtypes with the presence of PTEN mutations.

PTEN mutations did not correlate with differentiation grade as reported before, but was found with the same frequency in all grades of tumour. This interesting finding is shown in table 2.12.

Table 2.12: Pathogenic PTEN mutations shown according to race and tumour grade (Pretoria)

Race	Differentiation grade	Number of patients *	Tumours with mutations	Percentage with mutations**
TOTAL	Atypical hyperplasia	10	1	10
	Grade 1	5	4	80
	Grade 2	45	23	56
	Grade 3	11	7	64
	Papillary serous	4	0	0
	Total malignancies	**65**	**34**	**52**
AFRICAN	Grade 1	2	1	50
	Grade 2	20	10	50
	Grade 3	4	2	50
	Papillary serous	4	0	0
	Total malignancies	**30**	**13**	**43**
CAUCASIAN PRETORIA	Atypical hyperplasia	10	1	10
	Grade 1	1	1	100
	Grade 2	9	4	44
	Grade 3	2	1	50
	Total malignancies	**12**	**7**	**58**
CAUCASIAN UTRECHT	Atypical hyperplasia	0	0	-
	Grade 1	2	2	100
	Grade 2	16	9	56
	Grade 3	5	4	80
	Total malignancies	**23**	**15**	**65**

* Number with completed mutation analysis
** Only pathogenic mutations included

5. Interpretation and discussion

5.1 Endometrial hyperplasia

Finding PTEN mutations even in simple hyperplasia, confirms the role that inactivation of the protein by genetic mutation plays in the etiology of this disease. This also confirms that this genetic event can occur early in the carcinogenetic pathway.

In the current study it seems PTEN mutations can also occur later in carcinogenesis as the incidence was much higher in cancers than in the hyperplasia. This incidence was reported to be higher if a strict definition of precursor lesions was used, but these results have not been confirmed by other groups or by the current study.

5.2 Endometrial cancer

It was previously reported that PTEN mutations tended to be more common in well-differentiated tumours than in tumours with higher nuclear and architectural grading. In the present study we did not find such a tendency although all grades were well represented in our sampling.

We could confirm the result of previous (small) studies that reported PTEN not to be involved or mutated in papillary serous tumours. This important finding once again confirms that histological type demonstrates molecular differences underlying the processes of tumour genesis.

We found that the frequency of PTEN involvement was the same in all the races when only endometroid cancers were considered. When the serous papillary cancers were added, the frequency of PTEN mutations in African patients was lower.

It appears thus that the frequency of PTEN mutations are not determined by the race, but rather by the tumour type. In the same way that aggressive tumour types are relatively more common in African patients (due to a relative under-representation of endometroid carcinomas), PTEN mutation negative tumours

may be more common in African women (due to an underrepresentation of endometroid PTEN positive tumours).

When the number of mutations per tumour is considered, some interesting and unique findings were made. We found multiple mutations in the PTEN gene in nine tumours and multiple disease causing mutations in six. Seven of these patients were African, while only two of the Caucasian patients in this subset had more than one mutation, one of these patients had stage 4 disease.

This finding suggests that previous clinico-pathological findings of more aggressive tumour behaviour could possibly be explained by more severe inactivation of the pten protein. It is unproven but possible or even probable that more mutations will cause worse impairment of pten protein function. This finding correlates exactly with the findings of aggressive tumour types in African women. The number of mutations per FIGO stage is displayed in figure 2.7 and the FIGO stage distribution per number of mutations in figure 2.6 (paragraph 4.5.1). Although the numbers are small, these column charts demonstrate the tendency towards a higher stage with more mutations.

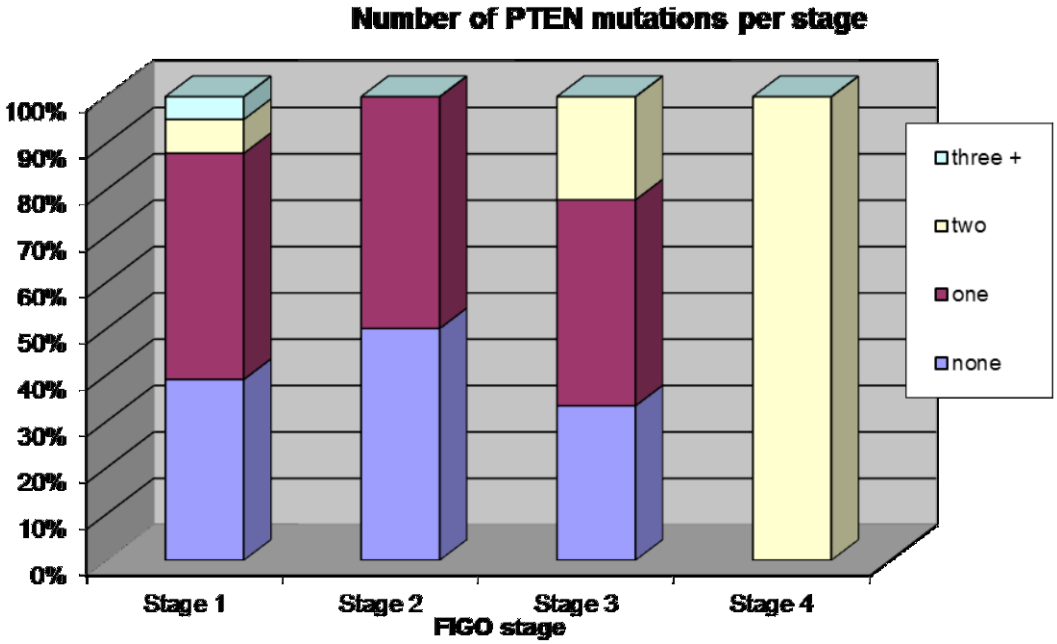

Figure 2.10: FIGO stage distribution per number of PTEN mutations

5.3 Limitations and recommendations for future research

This study was limited to mutation analysis and no attempt was made to study pten protein levels or activity. It would be interesting to correlate gene mutations to protein expression and protein activity.

This study could not demonstrate sufficiently when in the carcinogenetic pathway (early or late) the mutations in the PTEN gene occur. It would be potentially useful to continue this study of the chronology of carcinogenetic mutations in future projects. The findings regarding racial differences should also be confirmed in future studies.

Chapter 3

The role of the tumour suppressor gene PTEN in the etiology of uterine soft tissue tumours

INTRODUCTION

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1 Introduction

1.1 Background

Uterine sarcomas are uncommon genital tract cancers, staged using the International Federation of Gynecology and Obstetrics (FIGO) staging for endometrial cancer and classified using histological subtypes. These tumours are mainly treated by surgery, tend to be aggressive neoplasms and are often diagnosed post-operatively on the histology report.

Although uterine sarcomas are rare (between 2 and 10% of malignancies of the uterine corpus), together they account for between 20 and 30% of the deaths due to malignancies of the corpus (Lurain et al 1997). Despite our efforts to combine surgical therapy with radiation and chemotherapy, we have not been able to lower the mortality due to these tumors. Instead there have been suggestions in the literature that the incidence is increasing and with it the mortality rate. The well-documented difference in incidence and mortality between races also suggests serious previous underreporting in older publications in many parts of the world (Muthupei & Maluleke 1998; Silverberg et al 1990; Harlow et al 1986; Christopherson et al 1972).

Numerous studies suggest that estrogen and Tamoxifen play important roles in the pathogenesis of these tumors (Zelmanovicz et al 1998; Schwartz et al 1991; Bokhman 1983) and previous pelvic irradiation has been estimated to increase the risk for uterine sarcoma by about 5,38 times after 10 to 20 years (Lurain et al 1991), suggesting at least a theoretical risk for an iatrogenic increase in incidence. All these factors lead us to believe that the clinical importance of uterine sarcomas will increase over the next decades.

Uterine sarcoma is a histologically divers group of malignancies, with the two most common tumors the leiomyosarcomas (LMS) and the malignant Mullerian mixed tumors (MMMT) or carcinosarcomas (CS). Uterine leiomyomas and sarcomas originate from the endometrium and myometrium and share many of the same etiological factors. The classical risk factors for the development of uterine sarcomas are listed in table 3.1.

Many of these factors, including age and endogenous or exogenous hyperestrogenism are also shared to some extent by endometrial polyps, endometrial cancer and breast cancer. All these partially related tumours are definitely or potentially hormone responsive. However, many reports also suggest important differences between the different types of sarcomas. The histopathology and pathogenesis are completely different for the two groups of tumours. Leiomyosarcoma arise from smooth muscle fibres in the myometrium or, possibly, in a pre-existing leiomyoma.

It is calculated that only about 0,1% of leiomyomas undergo so-called "sarcomatous degeneration", changing the tumour into a leiomyosarcoma. Leiomyomas thus cannot be considered pre-malignant. Nevertheless, it was thought appropriate to use uterine leiomyoma (and specifically cellular leiomyoma) as the benign counterpart for this study. These two benign and malignant soft tissue tumours of the corpus uteri share both histological origin and many etiological factors.

Table 3.13: Classical risk factors for uterine leiomyomas and sarcomas.

Endogenous hyperestrogenism
Exogenous hyperestrogenism
Tamoxifen use
Hereditary factors
Black race
Co-existing leiomyomas
Advanced age
Previous pelvic radiation

On the other hand the cellular origin of carcinosarcomas has been hotly debated with the current consensus on a monoclonal histogenesis from a toti-potential cell of the endometrium (Guarino et al 1998; Gorai et al 2002). These epidemiological and histopathological data suggest a major overlap in the development of carcinosarcoma and endometrial carcinoma. Carcinosarcoma will therefore be compared on molecular level with endometrial hyperplasia and endometrial carcinomas.

PTEN involvement in the tumorigenesis of both benign and malignant uterine soft tissue tumours is a very real possibility in the light of the abovementioned etiological and histological overlap. It is also possible that PTEN could play a role later in the development of the tumour or in the late stage tumours only, suggesting a role not in carcinogenesis, but in dedifferentiation. This would mimic the type of involvement of PTEN found in glioblastoma (see chapter 1).

In an attempt to help clarify the role of the PTEN gene in the development and progression of uterine mesenchymal tumours, it was decided to study involvement of the gene in uterine leiomyomas and uterine sarcomas.

We were also interested in comparing the involvement of the gene in the different tumours. An important difference between leiomyomas and carcinosarcomas would support the theory that these tumours have completely different pathogenetic pathways. PTEN involvement in carcinosarcoma similar to that found in poorly differentiated endometrial carcinomas would also support the hypothesis that this tumour develops as an extremely poorly differentiated endometrial carcinoma.

Existing knowledge does not to any extent predict the involvement of PTEN in uterine leiomyosarcoma and leiomyoma. This topic was not studied before. It would be expected that the gene will not be involved as it is not considered an important role-player in other sarcomas.

1.2 Research questions and hypothesis

This chapter will focus on existing knowledge of molecular evidence regarding uterine soft tissue tumour genesis, on the histopathology and clinical features of these tumours. An attempt will be made to put the molecular evidence into perspective of existing knowledge on the other aspects.

Additionally, involvement of the tumour suppressor gene PTEN in subsets of patients with benign and malignant uterine soft tissue tumours will be tested.

It is hypothesized that the PTEN gene is involved in the formation of the different uterine soft tissue tumours. It is thus expected that PTEN mutations will

be found in uterine sarcomas and some differences between the tumour types is expected. It is postulated that the gene could be involved even in the benign counterpart, namely uterine leiomyomas.

The research questions for this study as listed in chapter 1 will be:

1. What role does PTEN gene mutation and pten protein inactivation play in the etiology of uterine leiomyomas and uterine sarcomas?
2. What is the frequency of PTEN mutations in uterine soft tissue tumours?
3. Can these mutations be predicted by the finding of micro-satellite instability?
4. Can these mutations also be shown in uterine leiomyomas?
5. How does PTEN mutations correlate with histological type, disease stage and grade?
6. How does the involvement of the PTEN gene differ between the different population groups in South Africa?

2 Literature overview

2.1 Genetic changes in soft tissue tumours of the uterus

2.1.1 Chromosomal abnormalities in uterine leiomyomas and uterine sarcomas

2.1.1.1 Cytogenetic changes in uterine leiomyomas and sarcomas

In the existing published studies on the cytogenetic make-up of uterine leiomyomas, a multitude of sporadic changes have been reported, with about 50% of leiomyomas displaying abnormal cytogenetics. The constant changes are shown in table 3.2.

Table 3.14: Constant changes in the cytogenetics of uterine leiomyomas and possibly affected genes.

Chromosomal change	Gene involved
Deletion (7) (q22, q32)	> 1 gene?

Deletion (10) (q22)	PTEN
Monosomy 10	PTEN
Translocation (12;14)	HMG-I, ER
Also: X,1,3	FH-gene

Few authors have published cytogenetic findings in uterine sarcomas. The involvement of chromosomes 1,7,10 and 11 has been reported (Laxman et al 1993). Genes that are implicated include the genes for the high mobility group proteins (HMG-I), PTEN, CYP17 and the fumarate hydratase gene.

An important overlap is thus seen between the two groups with cytogenetic changes not confined to the malignant tumours. Cytogenetic studies can play an important role in determining clonality and finding new candidate tumour suppressor and oncogenes.

Monoclonality is now accepted for individual leiomyomas, intravenous leiomyomatosis, metastatic leiomyomas (eg. pulmonary), carcinosarcomas and leiomyosarcomas (Gokaslan et al 2005; Fujii & Dinulescu 2005; Gorai et al 1997; Doll et al 2008; Sonoda et al 2000).

2.1.1.2 Alterations of ploidy in uterine sarcomas

Using flow cytometry, alterations in ploidy was studied and found to be frequent in uterine sarcomas. Malmstrom and colleagues found that aneuploidy was associated with high stage, poor differentiation and a poor outcome. It was also associated with high mitotic count and other histologic markers of proliferation and poor differentiation. In this study about 50% of tumours were diploid and these had a significantly better prognosis than the aneuploid tumours (Major et al 1993).

2.1.2 Involvement of specific genetic alterations in leiomyomas and leiomyosarcomas

Uterine sarcomas are rare neoplasms and relatively few studies have addressed specific genomic changes in these tumours.

2.1.2.1 High mobility group (HMG) proteins

This group of highly evolutionary conserved proteins is involved in DNA-binding. Elevated expression of these proteins is associated with cellular transformation. The most important genes involved are the HMGIC gene at 12q15 and the HMGIY at 6p21. Although these genes and their protein products are definitely involved in leiomyomas and other soft tissue tumours the precise mechanism remains uncertain and information is incomplete.

2.1.2.2 CYP17 polymorphism

The CYP17 gene encodes cytochrome P450c17a, which regulates 17alpha-hydroxylase and 17,20-lyase. The gene has two alleles, namely A1 and A2. The A2 alleles are associated with high estrogen and progestogen in premenopausal women. In a study done at the University of Pretoria on 89 African and 56 Caucasian women, the distribution of the alleles in the two groups and also polymorphism distribution was comparable. The CYP17 A2A2 genotype in African women was, however strongly associated with leiomyoma formation and A2A2 women had myomas of larger diameter (Amant et al 2004). The precise meaning and importance of this gene, allele distribution and polymorphisms of the gene is not fully understood and deserves further study.

2.1.2.3 Fumarate hydratase (FH)

The FH gene, also called “MCUL1”, is situated on chromosome 1q42.3-43, consists of ten exons and is involved in the Krebs cycle. Recently it has been found to also function as a tumour suppressor gene in tissue. Germline mutations cause the syndrome called MCUL (multiple cutaneous and uterine leiomyomata), multiple leiomyoma - (ML) or Reed syndrome. It is also the cause of hereditary leiomyoma and renal cell cancer syndrome, called HLRCC. When germline mutations are homozygous, fumarate hydratase deficiency exists.

The involvement of the gene in sporadic common uterine leiomyomas was the focus of a recent study done at the University of Pretoria. The questions asked, were whether low penetrance variants of germline mutations could be the cause and what the involvement of somatic mutations can be. The results of the Pretoria study showed mutations in seven of 28 leiomyomas, two of ten mitotically active leiomyomas and none of 21 leiomyosarcomas.

A total of 3 missense mutations were found in 4 tumours, namely one germline mutation in exon 8 and one somatic mutation each in exons 8 and 10. One nonsense mutation was found in two tumours, namely a somatic mutation in exon 3. Four synonymous or same sense mutations were demonstrated. These mutations were in the coding region of the protein and the importance is unknown. One of these was found in a mitotically active leiomyoma, suggesting that the mutation is not really silent.

This study was conducted on the same tumours that were used for PTEN analysis, and the interaction of the two genes will be discussed. It was the first study to find mutations in the FH gene in sporadic leiomyomas and the first study to involve mainly Black patients. These results have not been published and further study is warranted.

2.1.2.4 K-ras, C-myc

The role of mutations in these proto-oncogenes in uterine sarcomas has only been studied on a limited scale and results are inconclusive (Fotiou et al 1992).

2.1.2.5 HER 2/*neu* or c-*erb*B-2 and Bcl-2

The proto-oncogene bcl-2 is an inhibitor of programmed cell death. It counteracts the action of p53, which induces apoptosis. Morsi and colleagues studied bcl-2 protein expression in normal endometrium. This group observed cyclical changes of bcl-2 expression in normal endometrium and decreased expression levels in hyperplasia and carcinoma (Morsi et al 2000).

Several groups have shown that expression levels correlate negatively with differentiation grade (Geisler et al 1998; Zheng et al 1996). The correlation of the bcl-2 activity with carcinogenesis is still incompletely understood. Ioffe et al (1998) observed that the sub-cellular location of the bcl-2 protein seems important and not the quantity.

2.1.2.6 P 53

The involvement of the p53-gene is usually determined by using p53 overexpression on immunohistochemistry. Abnormal protein products lead to overexpression, pointing towards genetic mutation and thus to involvement of this gene in malignant transformation.

Several groups have studied the expression level of the p53 protein in uterine sarcomas using this method. In uterine sarcomas, like in many other tumours, reports suggest that overexpression is common (Liu et al 1994) and that it correlates with advanced stage disease and recurrence.

2.1.2.7 Microsatellite instability (MSI)

Endometrial stromal sarcoma and carcinosarcomas originate from endometrial stroma and epithelium. Microsatellite instability is frequent in endometrial cancer, and abnormality in DNA repair mechanisms plays an important role in these tumours. Therefore our group was interested to study the frequency and role of microsatellite instability in various subtypes of uterine sarcoma and leiomyoma. It was also interesting for the study on the involvement of the PTEN gene to correlate the findings of the MSI to the PTEN mutation analysis.

The same tumours were therefore studied for both replication errors and PTEN mutations. The results have been published (Amant et al 2001a) and correlation with PTEN results will be discussed in the last chapter.

Loss of heterozygosity (LOH) for chromosome 10q has been shown by Quade and colleagues (1999) to be frequent in LMS. This finding supported the untested hypothesis that the PTEN gene plays an important role in neoplastic transformation in these tumours.

2.1.2.8 PTEN

Multiple interactions of the protein product on cellular level was described and discussed in chapter 1. These include the induction of chemosensitivity, inhibition of Bcl2 expression and interaction with estrogen receptor-alpha and androgen receptors.

The role of the PTEN tumour suppressor gene in endometrial carcinogenesis has been studied extensively, but the involvement of the gene in uterine sarcomas has not received the same attention.

PTEN mutations were the focus of the current study. A detailed literature review of the involvement of the PTEN gene in uterine soft tissue tumours will be discussed below (2.3).

Forty-seven cases of uterine sarcoma and twenty-two cases of mitotically active leiomyomas (MAL) were analysed in the current study using genetic mutation analysis. The details of this study will be discussed in the rest of this chapter. The findings will be interpreted and compared to that of previous findings, similar studies and with the involvement of this gene in other malignant tumours of the genital tract (chapter 5).

2.2. Histology, pathogenesis and prognosis of uterine soft tissue tumours

2.2.1 Uterine leiomyomas

Uterine leiomyomas are extremely common benign soft tissue tumours of the uterus. The histogenesis has not been completely established and these tumours may arise from mature or immature uterine mesenchymal cells.

Table 3.15: Clinical associations and risk factors for uterine leiomyomas

Adenomyosis	Endometrial hyperplasia and carcinoma
Obesity	Diabetes mellitus
Family history	Excessive menstruation
Older age	Non-Caucasian race
Infertility	Hyper-estrogenic states

Leiomyomas occur in between 5 and 25% of younger women and some series have demonstrated up to 50% incidence in uteri at autopsy. Risk factors and clinical parameters associated with the development of these tumours are often quoted but mostly unproven. Some of these are listed in table 3.3. Inheritance play a definite although undefined role, with both population group (African and Afro-American origin) and family history as important risk factors.

Histological parameters of differentiation and especially proliferation are used to differentiate leiomyoma variants from their malignant counterpart, namely leiomyosarcomas. These parameters, as well as the size of the original malignant tumour (Levenback et al 1996) predict malignant behaviour as measured by local and systemic recurrences relatively well. However, all studies with histological review have a significant number of changed diagnoses, indicating low

repeatability and significant over-diagnosis of malignancy in historical samples (Evans et al 1988; Levenback et al 1996).

The most widely accepted histological defining criteria for uterine leiomyosarcomas used today is that of Stanford (Bell et al 1994), which utilizes the degree of cytological atypia, mitotic index and coagulative tumour cell necrosis (CTCN) instead of mitotic index alone as previously propagated. This classification system was evaluated in an outcomes based study of 213 problematic uterine smooth muscle neoplasms and enables differentiation between mitotically active leiomyomas (called 'leiomyoma with increased mitotic index'), atypical uterine leiomyomas and the aggressive and malignant leiomyosarcomas. These criteria accurately predict malignant behaviour, while reducing the false diagnosis of malignancy.

Recently molecular markers raised interest as a means to differentiate leiomyomas from leiomyosarcomas. Useful predictors would correlate well with the histological parameters and with clinical behaviour. Flow cytometric parameters seem promising including ploidy and S-phase fraction, but no definitive molecular marker have been identified that can reliably differentiate between these tumours.

Another new immuno-marker which seems promising is CD10, which stains both normal myometrium and leiomyomas positive, but sarcomas negative (Chu et al 2001).

Clinical parameters and risk factors for the diagnosis of uterine sarcoma in a patient with a "myomatous uterus" are listed in table 3.4. Although used clinically, these risk factors are not reliable and have poor sensitivity and specificity.

Table 3.16: Risk factors for the diagnosis of uterine sarcoma in patients with an enlarged or "myomatous" uterus.

Prolapsed tumour
Increase in size
Necrotic or infected soft tissue tumour
Bleeding tumour

Painful tumour
Unusual or mixed density on ultrasound

2.2.2 Uterine sarcomas

Although uterine sarcomas are commonly defined as a group of malignancies arising from the mesenchymal or connective tissue elements of the uterus suggesting relative homogeneity of origin, these tumours differ widely in histological appearance and origin. The most commonly occurring tumours are carcinosarcomas (CS) or malignant Mullerian mixed tumours, which consist of epithelial and mesenchymal components (~48%) and leiomyosarcomas (LMS) that are pure mesenchymal tumors (~37%) (Levenback et al 1996).

The histogenesis of these two tumour types is probably completely different. Leiomyosarcoma seems to arise from smooth muscle fibres in the myometrium or the myometrial blood vessels, while the current consensus about carcinosarcoma is that it probably originates in the endometrial epithelium (Gorai et al 2002, Fujii et al 2000). Endometrial stromal sarcomas are also pure mesenchymal tumours, probably arising from the endometrial stroma.

Data on ethnic and racial differences have shown not only an increased incidence for all uterine sarcomas, but also a higher mortality in African and Afro-American women. (Mutupei & Maluleke 1998; Silverberg et al 1990). The risk is however different for the two tumours with a reported increase by 60% (1,6 times risk) for leiomyosarcoma, while the risk for CS is about 2,7 times increased in Black women as compared to White women (Harlow et al 1986). As described previously, these aggressive neoplasms also contribute a larger proportion of all uterine malignancies in Non-Caucasians because the incidence of lower risk endometrial carcinomas is lower in these population groups.

It is interesting that most tumors following pelvic irradiation are carcinosarcomas (Levenback et al 1996) and that many researchers report a recent increase in the incidence of these CS rather than LMS (Kahanpaa et al 1986, Major et al 1993). The latter finding correlates with the increase in the occurrence of endometrial cancer.

The most recent histological typing and classification system was published by Scully and co-authors on behalf of the WHO (1994) and is adapted in table 3.5.

The system classifies tumours as pure mesenchymal (among other scarce types also the more common endometrial stromal sarcomas and leiomyosarcomas) and as mixed epithelial and mesenchymal in origin. The latter group consists of tumours with one benign and one malignant part (adenosarcomas and carcinofibromas) and tumours with malignant epithelial and malignant stromal components (carcinosarcomas).

Table 3.17: Histopathologic classification of uterine sarcomas (adapted from Scully RE et al 1994).

Pure malignant mesenchymal uterine tumours	
Endometrial stromal sarcoma	
	Low-grade
	High-grade
Leiomyosarcomas	
	Epitheloid
	Myxoid
Mixed endometrial stromal and smooth muscle tumours	
Other malignant soft tissue tumours	
	Homologous
	Heterologous

Mixed malignant epithelial-mesenchymal uterine tumours	
Adenosarcoma	
	Homologous
	Heterologous
Carcinosarcoma	
	Homologous
	Heterologous
Carcinofibroma	

2.2.2.1 Leiomyosarcoma

Uterine leiomyosarcoma arise from the smooth muscle cells of the uterus and account for about 35% of uterine sarcomas. These tumours are known to have complex cytogenetic abnormalities and rarely originate from previously existing benign neoplasms like leiomyomas although the two tumour types are associated (Evans et al 1988). Generally leiomyosarcomas occur at a younger age than carcinosarcomas with a

peak incidence at around 45 years. The histological criteria for the diagnosis were discussed above (Bell et al 1994).

The widely quoted five year survival rate for uterine leiomyosarcomas of all risk groups together is ~50%. Various studies have suggested and denied many different prognostic factors, including mitotic rate, cellular atypia and age. Tumour size and extra-uterine spread (as reflected in FIGO stage) seem to be obvious prognostic factors and was confirmed in the large-scale study by Evans et al (1988).

2.2.2.2 Carcinosarcoma

These highly malignant tumours are the most common uterine sarcomas, comprising about 55% of the total. Both epithelial and stromal components are malignant and are classified according to type. The most common sarcomatous components are ESS, rhabdomyosarcomas and fibrosarcomas, while the epithelial part consists mainly of endometroid adeno-, serous papillary adeno-, squamous - and undifferentiated carcinoma.

Like in endometrial cancer, age is a significant risk factor for the development of CS, with the incidence rising with older age and the highest risk occurring in the age group above 65 years. Although single cases have been described before the age of 45, this tumour is not typically associated with women in their reproductive age.

It appears from numerous sources and publications that these tumours represent dedifferentiated epithelial neoplasms, probably originating from an early mutation in a pre-existing endometrial carcinoma cell. Clinical data, tumour behaviour patterns and molecular evidence supports the monoclonal theory of tumorigenesis and supports the hypothesis of the epithelial component being the first component from which the mesenchymal cells develop via cellular mutation. This process represents a monoclonal pathway of stepwise dedifferentiation starting from a multipotential cell (Guarino et al 1998; Gorai et al 2002).

Epidemiological and histopathological data also suggest a major overlap in the development of CS and endometrial carcinoma. The current study aims to test this theory in part by examining associations in molecular findings.

Studies utilizing x-inactivation suggests that a small proportion of these tumours originate not from one, but from two endometrial carcinomatous foci, suggesting the so-called "clash theory" in about 5% of the tumours (Zelmanowitz et al 1998).

These biphasic tumours are aggressive and resistant to available therapeutic modalities. The behaviour of the carcinomatous part of the tumour determines prognosis and tumour behaviour and will be found mostly in metastases. Carcinosarcoma has a poor prognosis with an overall survival rate of about 20%.

2.2.2.3 Endometrial stromal sarcoma

Endometrial stromal sarcomas (ESS) are derived from the stromal elements of the endometrium. Tumours from the endometrial stroma can vary from totally benign (endometrial stomal myosis) to highly malignant and poorly differentiated sarcomas (high grade ESS). All these tumours are uncommon and ESS make up only about 10% of the group of uterine sarcomas (Zaloudek & Norris 1994). Hormone responsiveness seems to be certain (like the tissue of origin), may be related to differentiation and reports have seen the light that suggested an improved outcome where initial surgery included adnexectomy.

Survival of all ESS tumours together is generally reported to be better than CS and LMS (Piura et al 1997b). However, survival of high grade ESS is similar to CS (~25% five year survival), while low-grade tumours have an excellent prognosis (Evans et al 1988). Benign variants never metastasize. Generally the clinical behaviour is thus predicted very accurately by histological differentiation, which differs from the predictability of the other uterine smooth muscle tumours.

Nordal and Thoresen (1997) also demonstrated a better overall survival rate for ESS than for the other uterine sarcomas.

2.3 The PTEN gene and uterine leiomyomas and sarcomas

2.3.1 PTEN in normal endometrium and myometrium

The expression of pten in the normal endometrium changes in response to hormonal variations. During the proliferative phase pten is expressed in all tissue types in the uterus, while expression is increased in the early secretory phase and

lowered in the late secretory phase. These changes seem to be confined to the functionally active and hormonally responsive layers of endometrium (Mutter et al 2000). PTEN expression in the endometrial stroma has not been studied.

PTEN expression is also present in normal myometrium and is higher than in even benign neoplasms (Kayisli et al 2007). Although similar levels of pten may be found using immuno-staining, phosphorylation may be very different ,influencing the function (Kovacs et al 2007).

These tests reflect differences in protein production and function, suggesting up- and down-regulation of the protein production. On the other hand somatic PTEN gene mutation is never associated with normal tissue.

2.3.2 Frequency of PTEN alterations in uterine leiomyomas and sarcomas

2.3.2.1 Germline mutations

As described in chapter 1, PTEN was initially found as a result of the mapping of the susceptibility gene for Cowden syndrome and has subsequently also been linked to the Banayan-Zonana and Proteus syndromes. Mice with pten protein knockout develop complex proliferative endometrial lesions pointing to importance in cellular growth regulation in the female reproductive tract (Podsypanina et al 1999). Germline mutations are very uncommon in sporadic endometrial cancer and are not suspected to be involved in sporadic uterine sarcomas and leiomyomas.

2.3.2.2 Somatic mutations

After the finding of LOH on chromosome ten in endometrial cancer, various reports have shown the PTEN or MMAC1 gene, located on 10q23-24 (Steck et al 1997; Li et al 1997a), to be the most commonly mutated tumour suppressor gene in endometrial carcinoma (Tashiro et al 1997; Risinger et al 1997; Kong et al 1997). Quade et al (1999) reported frequent loss of heterozygosity of the short arm of chromosome 10 in uterine leiomyosarcomas as well. These findings suggest that PTEN/MMAC1 inactivating mutations may also play a role in the tumorigenesis of uterine sarcomas.

Kayisli et al recently (2007) reported a study comparing pten expression (immunohistochemistry) in normal myometrium with that in leiomyoma. They found lower expression in leiomyomas using immunohistochemistry and interprets this as a possible involvement of the gene in suppressing apoptosis. This finding does not necessarily implicate genetic mutation and could also be a result of down-regulation via some pathway. These findings cannot be compared directly with the results of mutation analysis.

Using immuno-staining, other researchers found similar pten staining in leiomyoma, atypical myoma and leiomyosarcoma, suggesting no involvement of PTEN in the pathogenesis (Gokaslan et al 2005). Semczuk and co-workers reported similar findings (2008) in a single case report.

The incidence of somatic mutations in endometrial cancer is the highest of any primary malignancy analysed so far, with frequencies reported from 40% to 76% (Nagase et al 1996; Peiffer et al 1995; Risinger et al 1997; Tashiro et al 1997). If carcinosarcomas were thought to be derived from endometrial carcinoma, it would be logical to expect PTEN mutations also in these tumours. It would also then be interesting to compare these findings with mutation analysis of the same gene in leiomyosarcomas and its benign counterpart, leiomyoma. The role of the tumour suppressor gene in uterine ESS has never been studied and very little is known of the carcinogenesis and molecular biology of this tumour.

Although immunostaining became freely available, is easier, can be done on existing slides and is much cheaper, mutation analysis is still the gold standard. This method was thus chosen for the current study. In addition immunostaining is less reproducible than mutation analysis.

3. Materials and methods

3.1 Materials

3.1.1 Sampling and clinical material

All cases of uterine sarcoma diagnosed from 1990 to 2000 at the Pretoria Academic complex (81 cases) were evaluated for the purposes of this study. In all these patients, the diagnosis was made on histology. Criteria for inclusion in this

study were that the histological diagnosis of uterine sarcoma be confirmed and that the histological material be adequate for the purposes of the analysis. Clinical data was collected from the files of the gynaecologic oncology unit or hospital archive when necessary.

3.1.2 Histology reports

The original study group consisted of 47 leiomyosarcomas, 28 carcinosarcomas and six endometrial stromal sarcomas. After careful review of the histology, the following material was available for further analysis:

Twenty-one cases of leiomyosarcoma were reclassified after histological review. Eighteen tumours were classified as mitotically active uterine leiomyoma using the Stanford criteria published in 1994. One case of atypical leiomyoma was diagnosed and two cases of extrauterine LMS. Three cases were excluded due to other reasons, leaving 19 cases of uterine leiomyosarcoma for analysis. Twenty-eight cases of carcinosarcoma were evaluated and the histological diagnosis was confirmed in all cases, except for one case of pure rhabdomyosarcoma. Three additional cases were excluded for technical reasons, leaving 24 CS tumours for further analysis. All of the six original cases of uterine stromal sarcoma was confirmed and remained in the study. (Amant et al 2002a)

The 21 leiomyosarcoma tumours that were reclassified after histological review (most of them to mitotically active leiomyomas) were used as the benign counterpart of uterine sarcomas, postulating that these tumours represent the most "pre-malignant" subsection of the benign mesenchymal tumours. These tumours were thus also included in the study and full mutation analysis was carried out on this material. The theory that these tumours will therefore be most likely to harbour the typical mutations of the malignant counterpart has not been tested before.

3.1.3 Tissue for DNA analysis

Paraffin embedded tissue was retrieved from the pathology archive for analysis. In all cases tissue material from both the tumour and the normal uterine tissue (myometrium) was obtained. After DNA extraction, mutation analysis was done

on both the tissue samples and results were then correlated and compared with available clinical and pathological data.

3.2 Methods

Tumour and normal tissue of 49 cases of uterine sarcoma (19 LMS, 24 CS and six cases with ESS) was analysed. Twenty-one cases of the chosen benign counterpart were also analysed fully (both tumour and normal tissue).

3.2.1 DNA extraction

Micro-dissection of formalin-fixed paraffin-embedded normal and tumour tissue was carried out in collaboration with an anatomic pathologist. After hematoxylin staining, the pathologist indicated normal (myometrial) and tumour (sarcoma or leiomyoma) areas on one slide. This slide was used as a guide to remove normal and tumour tissue separately with a sterile blade from five to ten consecutive sections per patient.

The micro-dissected paraffin embedded tissues were transferred to a microfuge tube and 200µl of extraction buffer (10 mM Tris-HCl, pH 8,3; 50 mM KCl; 0,45% Nonidet P40 and 0,45% Tween20) added where-after it was digested overnight at 56°C with proteinase K (final concentration 200µg / ml). The proteinase K was inactivated by boiling for 10 minutes at 95°C, quenched on ice and spun down.

The resulting supernatant containing the DNA was transferred to new sterile tubes and used or stored at 5°C.

3.2.2 DNA amplification

PTEN-coding sequences were amplified by polymerase chain reaction using the primers described by Davies et al (1999a). The nine exons were amplified in eleven sections, with exons five in two sections and nine in two sections. Intron-based primers were used to minimise the risk of amplifying the processed PTEN pseudogene on chromosome 9, as previously discussed.

PCR was performed in 20µl or 10µl reaction volumes for first round, or second round reactions, respectively. First round reactions containing 4µl of the

tissue extract, 20mM Tris-HCl (pH8.4), 50mM KCl, $MgCl_2$ (1.5mM for exon 8b; 2mM for exons1-7; 2.5mM for exon 8a; 3mM for exon 9), 0.25µM of each dNTP, 0.2µM of each primer and 0.5 units *Taq* DNA Polymerase (Life Technologies, BRL) were amplified for 35cycles consisting of 1 min at 94°C, 1 min at annealing temperature, and 1 min at 72°C, with a final extension step at 72°C for seven minutes. The PCR products were labelled with γ-^{32}P ATP (7000Ci/mmol; ICN) in a second round reaction in which two µl of the first round reaction was amplified in the presence of 0.02 µM (0.42µ Ci) of each end-labelled primer. The primer sequences, the amplification conditions and product lengths are displayed in table 3.5 and are similar to those described in chapter 2.

3.2.3 PTEN mutation analysis

Samples of amplified DNA were screened for mutations using exon-by-exon SSCP analysis. All of the eleven PCR products (exons and parts of exons) that displayed aberrant bands were directly sequenced.

3.2.3.1 Single Strand Conformational Polymorphism

PCR products were diluted 1:10 with denaturing buffer, denatured at 95°C (5 minutes), quenced on ice and 3 µl product was loaded on a 0.5 X Mutation Detection Enhancement (MDE) gel. The gels were run at 8 Watts, 14-20 h in 0,6 X TBE buffer and read after drying using exposure to medical X-ray film (Fuji) as described in chapter 2.

Table 3.18: Primers and optimised conditions used for amplification and mutation detection by SSCP in uterine soft tissue tumours

Exon	Primer name	Primer sequence	Product length (bp)	PCR conditions: Temp (°C) MgCl2 (mM)	SSCP conditions (hours at 8W)
1	PTEN 1F PTEN 1R	caagtccagagccatttcc cccacgttctaagagagtga	233	58 2.0	16
2	PTEN 2F PTEN 2R	ttcttttagtttgattgctg gtatctttttctgtggcttag	239	50 2.0	16
3	PTEN 3F PTEN 3R	ctgtcttttggtttttctt caagcagataactttcactta	213	50 2.0	15
4	PTEN 4F PTEN 4R	tataaagattcaggcaatgtt cagtctatcgggtttaagtta	190	50 2.0	15
5A	PTEN 5AF PTEN 5AR	ttgttaattaaaaattcaagag gcacatatcattacaccagt	217	48 2.0	15
5B	PTEN 5BF	tgaccaatggctaagtgaa	248	50	16

	PTEN 5AR	aaaagaaacccaaaatctgtt		2.0	
6	PTEN 6F PTEN 6R	cccagttaccatagcaat taagaaaactgttccaataca	275	50 2.0	16
7	PTEN 7F PTEN 7R	ttgacagttaaaggcatttc cttattttggatatttctcc	264	50 2.0	16
8A	PTEN 8AF PTEN 8AR	ttcatttctttttctttttcttt ggttggctttgtctttctt	238	53 2.5	15,5
8B	PTEN 8BF PTEN 8BR	ccaggaccagaggaaac cacatacatacaagtcacaa	235	56 1.5	16
9	PTEN 9F PTEN 9R	agtcatatttgtgggtttt ttattttcatggtgttttatc	268	48 3.0	17

3.2.3.2 Sequence analysis

Direct DNA sequencing was performed using Sequenase PCR product Sequencing Kit (Amersham Life Sciences) as prescribed by the manufacturer. Sequenced samples were diluted and heat denatured and 3 µl was loaded on a 6% denaturing polyacrylamide gel. Electrophoresis was performed in 1X TBE buffer at 60 Watts and results read as described before.

4. Results

4.1 Clinical data

Data used in sections 4.4.1 and 4.4.2 is based in part on the clinico-pathological study done on the same patient population by Amant et al, published in the European Journal of Gynaecological Oncology (2001b).

4.1.1 Age distribution

The mean age at diagnosis of the nineteen patients with leiomyosarcoma was 57 years, of the twenty-four patients with carcinosarcoma it was 65 years and of the six women with endometrial stromal sarcoma it was about 55 years (Amant et al 2001b). This finding that carcinosarcoma occurred at a significantly higher age than the other sarcomas correlates with the findings of other reviews (Evans et al 1988; Muthuphei & Maluleke 1998).

The fact that CS also occurs in the elderly similar to endometrial carcinoma, supports the theory that this tumour represents a form of poorly differentiated endometrial carcinoma, rather than a myometrial tumour or a true primary mesenchymal tumour or sarcoma.

4.1.2 Menopausal status

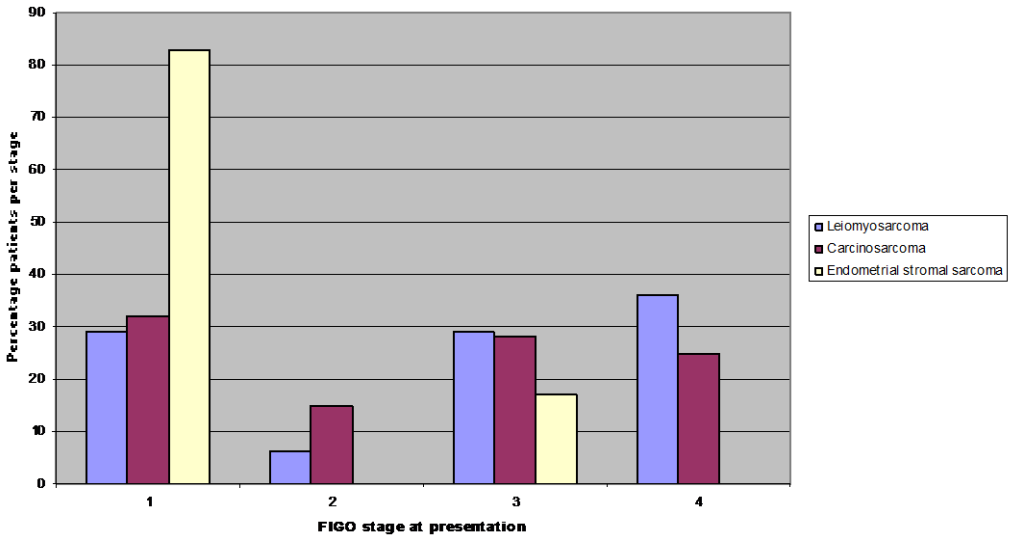
According to the available data, about a third of the patients presenting with LMS and ESS were pre-menopausal at the time of diagnosis. In contrast to this finding, only one patient (~4%) with CS was pre-menopausal at the time of diagnosis. These data could be interpreted in the same way as the data on age distribution.

4.1.3 Stage distribution

In this study, about 25% of patients with leiomyosarcomas presented in FIGO stage I, about 25% in stage III, and around 30% in stage IV. FIGO stage II describes patients where the uterine tumour grows down into the uterine cervix, thus a stage highly unlikely for a solid tumour. The distribution pattern was almost identical for carcinosarcomas, with around 10% of patients with both these tumours unstaged on the information we had available. The stage distribution is shown in figure 3.1. Patients with endometrial stromal sarcoma presented with earlier stage disease, with more than 80% of tumours in stage I. Evans et al (1988) ascribe this phenomenon to the perception that these tumours cause uterine bleeding at an earlier stage and patients therefore present earlier. It is however also possible that these tumours remain at an earlier stage for longer and are thus diagnosed at an earlier stage. This finding could simply reflect the less aggressive tumour behaviour.

Clinical data on age and menopausal state for LMS and ESS corresponded well. On the other hand, the data on tumour behaviour (stage distribution) for LMS and CS correlated more closely, while ESS had more favourable clinical features. This suggests that some factors in the pathogenesis of LMS and ESS are similar but are not shared by CS. On the other hand the prognostic features of CS and LMS did not differ much, while the prognosis for ESS is better.

Figure 3.11: Stage distribution according to tumour type



4.1.4 Symptoms

Vaginal bleeding is considered a symptom associated with either cervical or endometrial pathology. On the other hand general lower abdominal pain and the awareness of uterine enlargement are considered symptoms commonly caused by pressure or a mass. In our study significantly more patients with carcinosarcoma than leiomyosarcoma patients complained of endometrial disease type symptoms (~87% vs 53% had abnormal vaginal bleeding), while significantly more patients with leiomyosarcoma complained of pain or pressure symptoms (74% vs 29%).

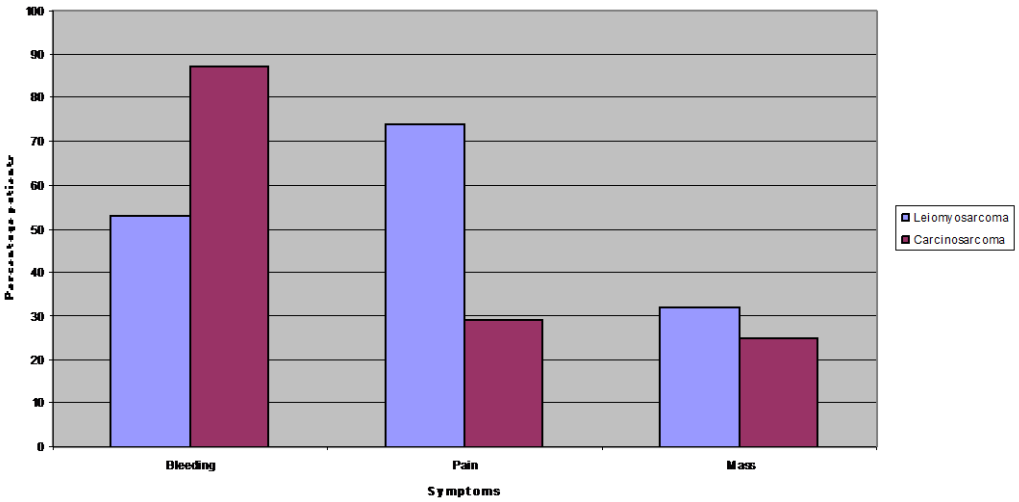
We postulate that this difference reflects in part the difference in the origin and position of the tumours. See figure 3.2.

4.1.5 Differences between population groups

In an important population based study, Plaxe and Saltzstein reported that African and Afro-American women develop significantly less low risk tumours and in fact have the same incidence of high risk endometrial cancer subtypes (1997). Many other studies have confirmed this much higher risk for corpus carcinoma in Caucasian than other races, but not an important difference in total survival (Liu et al 1995; Schiff et al 1997), due to a higher proportion of high-risk tumours in mainly African races.

Uterine sarcomas have also been shown convincingly to have a higher incidence in Black women and higher mortality rates (Madison et al 1998; Arrastia et al 1997). Uterine sarcomas form a bigger proportion of all uterine malignancies in African populations, but it is unclarified whether this is due to a lower incidence of more indolent endometrial carcinomas or due to a truly higher incidence rate.

Figure 3.12: Symptoms according to tumour type.



If carcinosarcoma were considered one of the poorly differentiated carcinomas these findings would suggest at least a similar incidence for this tumour in Black than in White females. The fact that these tumours occur at a much higher incidence in African and in Afro-American women is not yet well explained.

The incidence of these tumours and the outcome in different South African race groups has not been studied comprehensively or in a population-based way, but our study again suggests a higher incidence in Black women. Forty-three cases occurred in African patients, three in Caucasian patients (two carcinosarcomas and one leiomyosarcoma) (about 6%) and one case (LMS) in a coloured patient (mixed ancestry). The distribution pattern of endometrial cancer was about one third (~30%) Caucasian and two thirds African. For these two tumour types, the Unit will be expected to have a similar drainage area. This is a postulate.

It is widely accepted that leiomyoma occurs more commonly in African and Afro-American women. It is also well described that there is an association between leiomyoma and leiomyosarcoma, although the latter does not commonly develop from the first. It is therefore no surprise that leiomyosarcoma also seem to occur more often in Black than White women, as is the case in our study. The reasons for these disparities are, as discussed above, poorly understood.

4.2 Histology data

Seleye-Fubara and Uzoigwe (2007) showed that among uterine sarcomas carcinosarcoma was the most common (36%), followed by leiomyosarcoma. In their study 4% of all gynaecologic malignancies were uterine sarcomas.

In our review of nine years, carcinosarcoma was also the most common sarcoma (24 of 47) and represented 51%. This was followed by leimyosarcoma (19 patients, 40%) and endometrial stromal sarcoma (6 patients, 8%). We do not have accurate data on the total number of uterine malignancies over this period.

4.2.1 Leiomyosarcoma

The diagnostic criteria used in the review of histology slides were discussed above (3.1.2). Poor prognostic features on histology are included in the criteria for classification as a sarcoma and naturally would have a high incidence in the LMS tumours. These features include coagulative tumour cell necrosis (CTCN), lymphovascular invasion (LVS) and cellular atypia. Respectively these features were demonstrated in 16, seven and 18 of the 19 leiomyosarcomas. Additionally spread of the disease outside the uterus was histologically confirmed in some patients resulting in upstaging. This included spread to the omentum (two patients), adnexae (four patients), lymph nodes (two patients) and the intestines (also two patients).

4.2.2 Carcinosarcoma

Carcinosarcomas are poorly differentiated tumours and all 24 tumours displayed some poor prognostic features. Lymphovascular spread was present in the majority of tumours (13 of 24), coagulative tumour cell necrosis in 21 tumours (84%) and all tumours had cellular atypia. Extra-uterine spread was confirmed in the omentum in three cases, adnexae in six cases, while one case each had spread

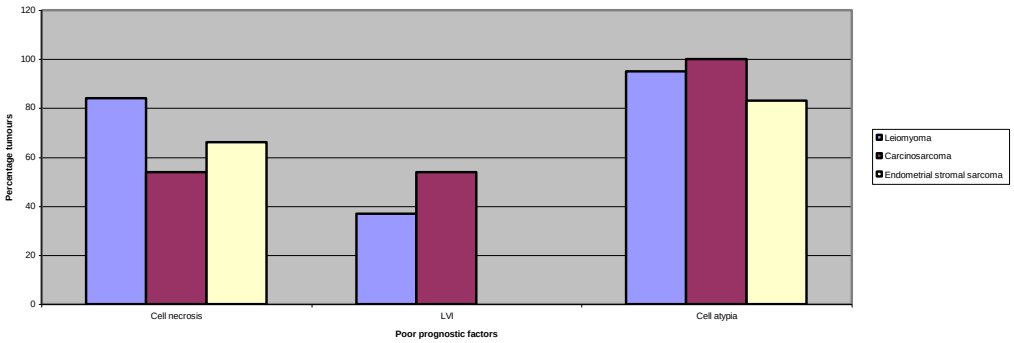
to the lymph nodes, lung, intestines and peritoneum. The comparison with other tumour types are shown in figure 3.3.

The sarcomatous elements were also classified according to the Scully system quoted above (table 3.4) into homologous and heterologuous types. Three tumours had homologous sarcoma components, 17 had heterologous elements and four could not be classified.

4.2.3 Endometrial stromal sarcoma

Five of the six tumours were classified as high-grade tumours, with CTCN present in four of six patients and significant atypia in five. One patient had extra-uterine spread with ascites at the time of diagnosis. All others were diagnosed in FIGO stage I.

Figure 3.13: Poor prognostic features according to tumour type.



4.3 Mutation screening

4.3.1 Single Strand Conformational Polymorphism (SSCP) results

All aberrations on the screening test by SSCP were followed up by sequence analysis. The results will be discussed here.

4.4 Sequence analysis

4.4.1 Non-malignant tissue samples

No mutations were found in any of the twenty-one leiomyomas included in this study. A significant role for PTEN in this benign and potentially pre-malignant tumour type is therefore excluded by this study in spite of the small numbers.

All the samples of normal myometrium representing normal or germline DNA, tested negative for PTEN mutation as well. This excludes germline or inherited mutation as an underlying causative factor and confirms that mutations demonstrated in the tumour DNA are indeed novel somatic mutations, usually disease causing.

4.4.2 Leiomyosarcomas

One PTEN mutation was found in one of the nineteen leiomyosarcomas. This nonsense mutation is a C-to-T transition, resulting in protein truncation. The mutation was absent in the normal tissue (myometrium) confirming a somatic mutation. The mutation occurred in exon 5 and affected codon 130, which is a mutational hotspot (Myers et al 1997a; Bonneau & Longy 2000).

This mutation is considered definitely significant (disease causing) as codon 130 lies within the phosphatase core area and mutations here would affect the phosphatase activity of the protein product. The phosphatase activity of the pten-protein is the core of its tumour suppressor activity, as discussed in chapter 1.

Table 3.19: Mutations in the PTEN gene in leiomyosarcomas.

Tumour	Mutation type	Exon	Nucleotide change	Effect
LMS 42	nonsense	5	c.388C to T	Arg130Stop

In a separate study of micro-satellite instability and loss of heterozygosity on these same tumour samples, no MSI were demonstrated in LMS 42. Failure of post-replication repair does not seem to be an important factor in tumorigenesis in this tumour type. Neither was any focus of LOH identified to flag a possible novel gene that could be involved.

4.4.3 Carcinosarcomas

We were interested to see whether PTEN mutations could be demonstrated in carcinosarcomas with endometroid epithelial components, as this would support a shared etiopathogenesis with endometrial carcinoma.

We found four mutations in carcinosarcomas, all somatic with absence of mutations in the normal DNA represented by normal myometrium. This method

of including normal DNA in mutation analysis also guarded us from demonstrating artefact mutations (PCR-induced, pseudogene induced or mutations caused by the method of paraffin extraction) as all these are likely to also be present in the normal DNA.

All three tumours harbouring the four mutations had an endometroid adenocarcinoma epithelial component. In no CS with any other epithelial component could any mutation in PTEN be demonstrated.

One tumour (CS 5) had two mutations in the PTEN gene, both significant. Both mutations were frameshift mutations, one in exon 7 and one in exon 8. Both these mutations in themselves will produce a truncated non-functional protein product and are therefore considered significant and disease causing. It is not known whether these mutations involved both alleles. This mutational pattern can be a cause for microsatellite instability as discussed earlier in this chapter (1.2.7). Indeed this tumour, CS 5, did demonstrate MSI as discussed above.

One missense mutation was found in tumour CS 15 (G-to-A in codon 130) and one missense mutation in tumour CS 19 (G-to-A in codon 15). The latter is a unique mutation not previously reported. Both of these mutations are thought to be disease causing.

Both of these tumours had endometroid epithelial component. Neither of these tumours displayed MSI or LOH in any of the chosen genetic tests.

Table 3.20: Mutations in the PTEN gene in carcinosarcomas.

Tumour	Mutation type	Exon	Nucleotide change	Effect
CS 5	frameshift	7	c.800delA	Stop at 275
CS 5	frameshift	8	c.968delA	Stop at 343
CS 15	missense	5	c.389G to A	Arg130Gln
CS 19	missense	1	c.44G to A	Arg15Lys

4.4.4 Endometrial stro

No mutations were found in any of the six endometrial stromal sarcomas included in this study or in the normal myometrium

representing normal DNA. We could not demonstrate any involvement of the PTEN gene in the tumorigenesis of this very uncommon mesenchymal tumour. A significant role for PTEN in this tumour type is not suspected.

4.4.5 Polymorphisms and pten-protein aberration

We did not find any polymorphisms in the PTEN gene. In this study only PTEN gene mutations were addressed. We did not investigate the occurrence of pten-protein aberrations, protein expression as measured by semi-quantitative immunohistochemistry or pten-protein function.

Influence of the gene mutations on protein length is a predictable event influencing protein function in a mutation specific way.

4.5 Correlation between clinical findings and molecular results

No PTEN mutations were found in any leiomyoma.

The one leiomyosarcoma that had a mutated copy of the PTEN gene had no differentiating clinical characteristics.

One of four Caucasian patients with CS had a PTEN mutation, while two of nineteen African patients with the same tumour were shown to have a PTEN mutation. Due to the small numbers, this difference cannot be considered significant. However, studies on endometroid endometrial carcinoma have indicated a much lower involvement of the PTEN gene in African women than in Caucasians. The findings of our own study of endometroid adenocarcinoma have been discussed in chapter 2 and displayed in table 2.9.

Some authors (Maxwell et al 1996) found an incidence as low as 5% for PTEN mutations in African-American women with endometrial cancer, suggesting a very small role for the PTEN gene in African women. This racial disparity described in endometrial cancer would be expected to persist into the product of genetic progression to a phenotypically divers tumour, namely the carcinosarcoma with endometroid epithelial component.

No PTEN mutations were found in any of the six endometrial stromal sarcomas.

Due to the small number of mutations, no attempt was made to correlate these findings with other clinical findings, like age or stage.

4.6 Correlation between histology findings and molecular results

Due to the small sample size and the low incidence of mutations, it was not statistically reliable to correlate the pathological sarcoma types with the presence or absence of PTEN mutations. Our observations and subjective interpretation are described.

In this dataset, the incidence of PTEN mutations ranged from none (all endometrial stromal sarcomas) to one in 19 or ~6% (all leiomyosarcomas) and three in 23 or ~14% (all carcinosarcomas). The highest incidence was three in eighteen or 17% in all CS tumours with an endometroid carcinoma element.

Although the numbers are too small for any statistical analysis, these findings possibly represent a correlation between the genotype and the phenotype of the tumour. It would be logical that the genetic aberrations found in tumour cells would also determine the phenotypic and histologic appearance of the tumour (Fujii et al 2000).

5. Interpretation and discussion

5.1 Benign myometrium

No PTEN mutations were found in any of the tissue samples of benign germline tissue. This is what was expected as uterine sarcomas and leiomyomas have never been implicated as tumour types that occur in patients with the PTEN associated syndromes.

In our study these samples also served as controls for the method and to confirm that any mutations found are indeed somatic.

5.2 Leiomyomas, leiomyosarcomas and endometrial stromal sarcomas

Like in other mesenchymal tumours, we did not find important involvement of the PTEN gene in any of the uterine mesenchymal tumours. We could not detect any PTEN mutations in any of the endometrial stromal sarcomas or in any of the uterine leiomyomas. We detected only one mutation in the nineteen leiomyosarcomas examined. In this rare case, PTEN mutation seems to be involved in the lack of growth control and thus somewhere in the change from normal cell to a cell with neoplastic behaviour.

5.3 Carcinosarcomas

Carcinosarcomas differ from the other uterine sarcomas. These tumours probably originate from the epithelial cells and are thus rather carcinomas with de-differentiation. The findings of this study confirm that these tumours differ also on molecular level from the other sarcomas and from the benign mesenchymal tumours. Indeed CS seems to have more in common on molecular level with endometrial carcinoma than with LMS.

All carcinosarcomas are poorly differentiated tumours and as such similar results were expected as in poorly differentiated endometrial carcinoma. We found PTEN mutations in 3 of 23 tumours (13%). In our own dataset of endometrial carcinoma (chapter 2), three of ten grade 3 endometrial carcinomas (30%) displayed PTEN mutations, which seem a bit higher than in the sarcoma group. Non-mutational involvement of this tumour suppressor gene is expected in many more tumours.

Considering only those with endometroid epithelium, we found around a 17% incidence of PTEN mutations in CS with endometroid epithelium. In poorly differentiated endometroid carcinoma, we found a 50% incidence in the small subset of six patients, with identical incidences in both population groups. Our findings in endometroid carcinosarcomas correlates well with that reported in poorly differentiated endometroid adenocarcinoma of the endometrium by Maxwell et al (2000).

The incidence of PTEN mutation in African women with uterine sarcoma with an endometroid carcinoma component was ~12% (two of 17 tumours), while the incidence in Caucasians was ~30% (one of three tumours). Although in keeping with previous suggestions of racial disparity, our sample size was far too small to confirm that this trend extends to the sarcomas. In addition our findings in endometrial carcinoma did not support disparity between population groups.

The finding of two disease causing and protein truncation mutations in one tumour (CS 5) deserves mentioning. This finding in itself is highly significant and proves that PTEN somatic mutations can be involved in the tumorigenesis of carcinosarcomas. It also demonstrates that the inactivity of this protein can be associated with aggressive tumour growth (without normal inhibition) and aggressive tumour types in the uterus. Previous reports often reported an association with well differentiated types and good prognosis in the uterus while in other tissue types the opposite may be true (eg. brain tumours).

The association of these two mutations with the genetic finding of MSI is also of significance. Indeed it is believed that this severe inactivation of the pten protein will be the direct cause of the MSI, which is not usually associated with a single disease causing PTEN mutation.

Due to the small numbers assessed and the small number of mutations, it was not considered accurate to try to relate the presence or absence of mutations to other clinical and pathological variables.

5.4 Strenghts, limitations and recommendations

This study was one of the first in the world to study sarcoma and uterine sarcoma. It is definitely the first South African study and the only one to include both Caucasian and Black patients. It was also the first study of PTEN involvement in uterine leiomyoma.

Similar to the study of endometrial carcinoma, this study was limited to mutation analysis. No attempt was made to study pten protein levels or activity. It would be interesting to correlate gene mutations to protein expression and activity.

The findings in CS provide further strong support for the hypothesis that the pathogenesis of CS and endometrial carcinomas overlaps. Carcinosarcoma seems to be the least differentiated of all uterine carcinomas. On the other hand the different uterine sarcomas share very little in terms of etiology. It will be interesting to compare more molecular findings between the different uterine neoplasms.

Carcinosarcoma of the uterus is the first non-endometrioid, and the first non-epithelial gynaecological cancer where PTEN-mutations are found in such a high percentage of cases. To our knowledge this is also the first sarcoma where PTEN is found to play an important role.

The findings of this study shows important involvement of this tumour suppressor gene in the development of carcinosarcomas of the uterus. We could not, however, demonstrate when in the carcinogenetic pathway (early or late) these mutations occur. It would be hugely interesting to answer this question, but a suitable scientific model of study is outstanding. The most probable pre-cursor lesion for carcinosarcoma is atypical endometrial hyperplasia or early endometrial carcinoma.

The significance of all the interesting findings of this study is limited by small numbers. Unfortunately sarcomas are rare neoplasms and the methods used for mutation analysis are tedious. It would be useful to compare our findings to similar studies in future to complete the picture of PTEN involvement.

Chapter 4

The role of the tumour suppressor gene PTEN in the etiology of endometroid ovarian tumours

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1 Introduction

1.1 Background

The relative rarity of early stage ovarian epithelial cancer, the absence of established ovarian cancer precursors and the histological diversity of ovarian cancer types are all factors that make it difficult to study the carcinogenetic process in the ovary. The ovary is also an inaccessible organ and tissue is not freely available. Few studies have focussed on the molecular characteristics of borderline ovarian tumours and even fewer on benign tumours. This is probably because most researchers did not believe that these tumours are important precursors of malignant disease in the ovary and therefore did not see such molecular studies as potentially informative.

Rather, most information seems to point towards different genetic pathways leading to distinct tumour types, some immediately highly malignant and others benign. It is widely believed that the three groups of ovarian tumours (epithelial, stromal and germ cell tumours) have distinct development pathways and etiologies. It is not clear whether the different epithelial tumour types also have different pathogenesis. Recently, however, molecular findings started to emerge that links tumour type and phenotype very strongly with molecular findings and genotype.

These findings suggest that for epithelial tumours, morphology may be a guide to the genetic developmental pathway. This theory would suggest that serous tumours of various malignancies share more (genetically) with each other than serous and mucinous tumours of the same malignancy. According to this theory, it would make more sense to, for example, study mucinous adenomas together with mucinous adenocarcinomas and mucinous tumours of low malignant potential (LMP) and less sense to study serous and mucinous adenocarcinomas together (Sanseverino et al 2005).

Endometroid adenocarcinoma of the ovary on histology closely resembles the same tumour type in the endometrium. Various authors described and suggested that these tumours also share many genetic and molecular features.

In order to investigate the role of the PTEN gene in the ovary, it is therefore logical to first probe the involvement of the tumour suppressor in endometroid adenocarcinoma of the ovary. A potentially useful model to examine ovarian oncogenesis (where PTEN is likely to be involved) is ovarian endometriosis and the progression and transformation into atypia and then endometroid, clear cell and other associated cancers.

Endometriosis is a common disorder estimated to affect between 7 and 10% of the female population in reproductive age and ~20% of female patients presenting with infertility (Wheeler 1989). The etiology of endometriosis is not well understood.

Endometriosis is a heterogeneous disease and it is thought that different etiological factors are of importance in the different types. Different forms that have been described include peritoneal disease with only superficial invasion, uterosacral or posterior cervical disease (typically deeply invasive) and ovarian lesions (endometriomas). Myometrial invasion by endometrial glands is called adenomyosis and has been recognized as a separate disease previously, but probably shares some etiological factors with endometriosis and frequently co-exists. Clinically most patients have a single form of the disease rather than a field effect.

Ovarian epithelial carcinoma is a much less common condition than endometriosis, affecting between 1% and 4% of women, but with an aggressive clinical course. Ovarian epithelial cancer is an important cause of gynaecological cancer mortality. Most patients still present with late stage disease and despite the developments of the last decades with regard to chemotherapy, will succumb of their disease.

While most ovarian malignancies were not classically considered hormone sensitive, ovarian endometriomas are extremely sensitive to ovarian steroid hormones. Unopposed estrogen and tamoxifen have a strong stimulatory effect on endometrial tissue and unopposed stimulation is known to induce carcinoma of this tissue type (Prasad et al 2005). Some concern therefore exists regarding the

use of unopposed estrogen therapy (and unopposed endogenous estrogen) in patients with ovarian endometriomas, a condition that is frequently undiagnosed.

Numerous studies do suggest that estrogen and also tamoxifen play important roles in the pathogenesis of all these tumours (Zelmanovicz et al 1998; Schwartz et al 1991; Bokhman 1983; McCluggage et al 2000) suggesting at least a theoretical risk for an iatrogenic increase in incidence. All these factors lead us to believe that the incidence and clinical importance of endometroid ovarian adenocarcinoma will increase over the next decades.

Ovarian endometroid carcinoma displays exactly the same architecture than uterine endometroid adenocarcinomas, and is therefore most probably also estrogen sensitive, especially in the well-differentiated subtypes. Some authors have shown less steroid hormone receptors in these tumours than in the endometriotic lesion, a finding that is probably to be expected, as most tissue types lose receptors as they dedifferentiate and become more malignant. In addition hormone receptor development and positivity depends upon hormone supply.

Endometrioma of the ovary thus forms the ideal benign counterpart for endometroid adenocarcinoma of the ovary for the purposes of a study of malignant progression. The benign and malignant tumours share many architectural characteristics, a lot of evidence has pointed towards the benign tumour being the precursor of the malignant one and the two tumours probably share many etiological factors.

1.2 Research questions and hypothesis

It is the purpose of this chapter to explore existing evidence of involvement of the PTEN gene in ovarian carcinogenesis and to put this into perspective of existing knowledge of tumorigenesis in this organ. Additionally, a model to study involvement of the tumour suppressor gene in the ovary will be tested, hoping to add to accumulating molecular data for putative ovarian cancer precursors.

The model to be tested is that ovarian endometriosis and atypical endometriosis can be used as a precursor lesion or benign counterpart to endometroid

ovarian adenocarcinoma, hoping to find PTEN mutations even early in the process.

The hypothesis is that the PTEN gene is involved (early) in ovarian carcinogenesis in a subgroup of ovarian cancers, namely in endometroid adenocarcinomas. It is expected to find genetic aberrations early in this process, like in endometrial cancer.

The research questions for this study as listed in chapter 1 are:

1. What role does PTEN gene mutation and pten protein inactivation play in the etiology of ovarian endometroid adenocarcinoma?
2. What is the frequency of PTEN mutations in these tumours?
3. Can (ovarian) endometriosis be used as the benign counterpart or pre-malignant lesion of ovarian endometroid adenocarcinoma?
4. Do PTEN mutations also occur in ovarian endometriosis?
5. Do PTEN mutations correlate with histological type, disease stage and grade?

2 Literature overview

2.1 Endometriosis as neoplasm and pre-malignant condition in the ovary

2.1.1 The etiology of endometriosis

Despite the common occurrence of endometriosis, little is known about its etiology. The most widely accepted theory is that pelvic endometriosis arises from implantation of disseminated menstrual blood into peritoneal and pelvic structures. The incidence of endometriosis is undoubtedly higher in patients with high volume regurgitation but the other factors associated with a higher risk of implantation and uncontrolled growth of these cells are not so well described.

Genetic, hormonal and immunologic factors play important roles, but important specific genetic alterations on cellular or germline genetic level has not

been identified. It is thought that many of these etiological factors may be shared between benign proliferative disorders, benign tumours and some malignancies of the female genital tract (Guarch et al 2001; McCluggage et al 2000).

2.1.2 The pathophysiology of endometriosis and ovarian cancer

It is generally believed that multiple genetic, environmental and immunological factors as well as angiogenic and endocrine factors influence the development and progression of endometriosis. Similar factors probably lead to the malignant transformation of a subset of endometriotic lesions (Vigano et al 2006).

Almost all authors who studied the immune system and endometriosis, found **immunity** to be altered in women with endometriosis. The immune changes are mostly local but involve both humoral and cellular immunity. These immune system alterations favour the invasive behaviour of ovarian neoplasms and will favour malignant transformation of ovarian endometriosis. Ovarian cancer progression and dissemination is also seriously enhanced by immune suppression.

On the other hand important associations have recently been found between various **autoimmune diseases** (Sjogrens, systemic lupus erythematosus and thyroiditis) and endometriosis by showing an increased incidence (even 20 times) of these diseases in endometriosis sufferers (Ness 2003).

Some authors believe that chronic **inflammation** plays an important role in the genesis of endometriosis or ovarian cancer (Ness & Cottreau 2000). Many also mention previous work related to talc and asbestos exposure, pelvic inflammatory disease and the protective effect of tubal ligation and hysterectomy. These factors are believed to impact via the inflammatory reaction, cytokines, prostaglandins, inflammatory cell change and oxidative stress.

The association between **steroid hormone** levels, endometriosis and ovarian cancer is less clear. Most evidence is epidemiological and suggests that a relative excess of estrogen may be a factor in the formation of endometriosis and ovarian cancer. Progestogen could be a protective hormone, probably (partly) via its stimulation of apoptosis and its estrogen-opposing effect. This notion is supported by the preventative effect of the oral contraceptive pill, pregnancies and prolonged

breast-feeding in epidemiological studies. Additionally it seems that unopposed estrogen is a risk factor for both endometriosis and ovarian cancer.

Tamoxifen has been linked to endometriosis progression, endometrial proliferative disorders within endometriosis and endometroid adenocarcinoma in endometriosis. Borderline endometroid neoplasia and ovarian adenofibroma has also been described (McCluggage et al 2000).

Berchuck and co-workers (2004) sought to investigate the protective effect of progesterone further. They found a protective effect in the +331G/A progesterone receptor promoter polymorphism against ovarian clear cell and endometroid carcinomas and against endometriosis. This polymorphism was previously associated with an increased breast and endometrial cancer risk.

An association with other endocrine disorders also seems feasible and has been reported for both endometriosis and ovarian cancer. Examples include polycystic ovary disease, increased waist to hip ratio, increased insulin-like growth factor levels (IGF-1) and many other growth factors (Ness 2003).

Table 4.21: Classical risk factors for endometriosis.

Endogenous hyperestrogenism	Race
Unopposed exogenous estrogen	Age
Tamoxifen treatment	Infertility
Hereditary factors	Delayed childbearing
Menstrual factors	Outflow obstruction

Several authors have paid attention to the relation of **angiogenesis** and **growth factors** to the development of both endometriosis and related ovarian cancers. Deguchi and co-authors (2000) investigated VEGF and platelet derived endothelial-derived growth factor and related it to microvessel count. No relation could however be demonstrated between these factors and malignant progression. Del Carmen and co-authors (2003) could demonstrate an increase in VEGF in endometriosis associated ovarian cancer when compared with benign endometriosis (25 cases), while Healy et al (1998) proposed that the endometrium of women with endometriosis has an increased ability to proliferate and implant because of

enhanced endothelial cell proliferation. Fujimoto and colleagues (1999) reviewed angiogenesis and various angiogenic factors in endometriosis.

It is thus clear that endometriosis and ovarian cancer, which are both monoclonal diseases (Wells 2004; Catasus et al 2004), share many of the classical and theoretical etiological factors.

2.1.3 The association between endometriosis and malignancy

2.1.3.1 Gynaecological cancer

An excess incidence of ovarian cancer in infertile women, women stimulated with ovulation induction hormones and women with endometriosis has long been suspected, described, discussed and sometimes disputed. It is not possible to identify the single or most important etiological factor in these women, as many have more than one potential reason to have an elevated ovarian cancer risk (Ghourab 2001; Varma et al 2004).

A number of pathology-based studies have recently seen the light that examined the incidence of malignancy in endometriosis or that examined the incidence of endometriosis in ovarian cancer. Alternatively authors simply searched their databases for patients with a combination of the two diagnoses and then examined or reviewed all these patients (Modesitt et al 2002). Two of these studies will be discussed to demonstrate the findings.

Stern and colleagues (2001) recently published a series of 1000 consecutive cases of surgically proven endometriosis to establish the risk for co-existent cancer in this cohort. This carefully done pathology study does not at all address the risk to develop cancer in the future and shows a startling incidence of 5,5% of co-existing cancer in all patients diagnosed with endometriosis. This is in contrast with the 0,7% reported in previous pathology based pelvic endometriosis follow-up studies (Nishida et al 2000). Five percent of their patients with ovarian endometriosis on histology had co-existing ovarian cancer, while only 1% of patients with extra-ovarian endometriosis had co-existing ovarian cancer.

The group of Stern also report an intimate association between extra-ovarian endometriosis foci and the development of extra-ovarian clear cell or endometroid

adenocarcinoma and adenosarcomas. An increased incidence of co-existent endometrial pathology and cancer is also reported in patients with endometriosis associated extra-uterine cancer.

Ogawa and his co-workers from Japan (1999) did the opposite. They published a study involving 127 consecutive ovarian cancers in which they searched for evidence of endometriosis (typical and atypical) and demonstrated this pre-cursor in a surprisingly high number of 37 patients. The series is atypical compared to those done in Western countries as it consisted of 43 patients with clear cell carcinoma, 7 with endometroid and 60 with serous papillary and 17 with mucinous carcinoma. The clear cell type, which is much more common in Japan, was most strongly associated with endometriosis.

This Japanese group also investigated markers of inflammation and could not confirm inflammation to be an important factor in transition or carcinogenesis. They could demonstrate histological transition from typical to atypical endometriosis and then to carcinoma in a surprisingly large proportion of specimens (22/37 and 23/37) and defined atypia as a separate entity to inflammation. They disputed the importance and incidence of inflammatory atypia and could not demonstrate this phenomenon in their specimens.

In studies describing ovarian neoplasms in young women on ovulation induction drugs, authors report mainly serous tumours of tumours with low malignant potential (Ghourab 2001). Most reports and case reports of endometriosis associated ovarian cancer describe especially endometroid and clear cell adenocarcinoma, adenosarcoma and endometrial stromal sarcomas (Fishman et al 1996; Kavanagh & Wharton 1990; LaGrenade & Silverberg 1988; Heaps et al 1990).

It therefore seems that the importance of endometriosis as a precursor to ovarian cancer is still largely unknown and probably differs in different populations. The disease is a much more important precursor of endometroid and clear cell carcinoma than of the other histological types. It is however very likely to be underestimated in historical studies and the importance will probably increase.

2.1.3.2 Non-gynaecological cancer

Having endometriosis has been linked non-convincingly to an increased incidence to develop various non-gynaecological cancers at a later stage. These cancers include malignant melanoma, breast cancer and haematological cancers (non-Hodgkin's lymphoma or NHL).

However, large differences between these studies exist (Swiersz 2002), with more cancers and higher risks reported after histologically proven or hospital discharge diagnosis (Brinton et al 1997) than after self-reported endometriosis (Olson et al 2002). These differences may reflect the inaccuracy in self-reported diagnosis, a stronger association with more severe disease, population differences or inherent methodological problems.

2.1.4 Evidence that endometriosis can be a pre-malignant disease

Recently, a lot of evidence for ovarian endometriosis as a very important etiological factor for ovarian cancer has been published and it is now certain that a strong association exist between particularly long-standing ovarian endometriosis and endometroid, as well as clear cell adenocarcinoma of the ovary.

Many authors have shown convincing evidence in a large percentage of endometroid carcinomas that these neoplasms develop from ovarian endometriosis. Atypical endometriosis can be demonstrated in the large majority of cases with endometroid carcinoma (Guo et al 2001; Wells 2004).

2.1.5 The malignant potential of different forms of endometriosis

Although increased susceptibility in women with endometriosis to develop ovarian clear cell and endometroid cancer is now well proven and widely accepted, the magnitude of this risk is not known (Varma et al 2004). All pathological studies reporting the incidence of malignancy in patients with endometriosis have inherent biases (Steed et al 2004). All these studies also demonstrate different factors that may lead to gross underreporting of endometriosis-associated ovarian and extra-ovarian malignancy. These reports therefore provide us with some insight and useful data to compare different subgroups, but cannot provide accurate incidence rates.

Brinton and his group (1997), tried to quantify risk in a large Swedish study involving 20 686 women who had a hospital discharge diagnosis of endometriosis. They reported a relative risk of 4,2 for ovarian cancer in women followed up for more than ten years after diagnosis of ovarian endometriosis. This finding was in spite of a high incidence of salpingo-oophorectomy. Unfortunately this group did not report on the histological types.

It is thus clear from the available data that **ovarian endometriosis** carries a much higher risk than extra-ovarian endometriosis for the development of cancer in general and specifically for the development of ovarian neoplasm.

The development of extra-ovarian neoplasms is even less well studied and the incidence of malignancy in this subgroup of patients is clearly also unknown.

2.1.6 Endometriosis as a genetic disease

Evidence is accumulating supporting a strong genetic component to endometriosis. It is most probably a polygenic disease caused by multiple genes interacting with the environment. Acquired chromosome and gene-specific alterations probably accumulate causing clonal expansion of cells with altered invasive and growth potential. (Somatic genetic changes.)

Inherited genetic factors also play an important role, although no clear Mendelian pattern of inheritance should be expected in multifactorial diseases. These inherited factors are very likely to influence susceptibility to endometriosis, explaining the increased incidence in family members to some extent. Allelic differences in drug-metabolising enzymes are inherited and have recently been implicated in the development of endometriosis (Bischoff & Simpson 2000).

2.2 Histopathology of endometroid ovarian lesions and ovarian neoplasms

Early stage ovarian epithelial cancer is relatively rare and the histology of pre-malignant stages has not been well defined. In spite of many attempts to describe precursor lesions morphologically (Resta et al 1993), most of these ‘lesions’ also occur frequently in perfectly normal ovaries and thus the value of such histological entities are not widely accepted. In another attempt to describe

precancerous lesions, Piek, Kenemans and Verheijen (2004) described some pre-malignant changes in the fallopian tubes of patients with BRCA1 mutations, as well as abnormal ovarian surface epithelial cells from women in this group. These lesions again were not easily identified, often limited to small inclusion cysts and were not universally recognised as an abnormal or pathological finding.

Although originally disputed by many critics, these findings have been repeated by other investigators (Colgan et al 2001; Olivier et al 2004; Paley et al 2001) and seem logical.

The colorectal model of tumorigenesis that has been well established (Fearon & Vogelstein 1990) and described, does not seem to apply in ovarian cancer. This paradigm suggests that malignancies arise only after an accumulation of genetic mutations, each of which will give rise to a separate premalignant or benign tumour or histological entity.

This difficulty with the premalignant precursor has led to the notion that ovarian cancer predominantly arise *de novo* and not through benign or borderline precursors. In ovarian cancer accumulating genetic anomalies may be needed, but probably does not correlate with identifiable histological entities like polyps or adenomas. Therefore molecular genetic studies offer the best hope to understand the carcinogenetic process. These studies may also provide us with a better understanding of the relation between genetic alterations and tumour types.

2.2.1 Endometriosis and related non-malignant lesions

Pelvic endometriosis is described and defined as a lesion consisting of endometrial glandular cells and endometrial stromal cells. These lesions have been shown to be monoclonal in origin (Ness 2002).

Proliferation of endometriosis occurs mainly under the influence of steroid hormones and is common in pre-menopausal women. Proliferative endometriosis has also been described in post-menopausal women and occurs typically under the influence of tamoxifen or unopposed estrogen therapy, especially high dosage or estrogen implants.

The definition, description and frequency of atypical endometriosis is less certain. These lesions are more frequent in endometriomas in the ovary and are considered to be pre-malignant. Malignant transformation of endometriosis is well-documented and also occurs more frequently in the ovary (Fishman et al 1996) than in extra-ovarian endometriosis. The pathology of these lesions will be described under 2.2.2.

Prefumo and co-workers from Genova in Italy (2002) studied epithelial abnormalities in cystic ovarian endometriosis. This group found high incidences of metaplasia (12%), hyperplasia (9,4%), atypia (6%) and of carcinoma (4%) in 388 patients with ovarian endometriomas. More cysts were derived from the left and more changes occurred in older patients.

Nishida and colleagues (2000) found atypia in 12% of cases in a Japanese group of patients.

2.2.2 Endometriosis related malignant lesions

Sampson (1925) developed criteria for the diagnosis of malignancy developing in endometriosis that was later changed by Scott (1953). These criteria for endometriosis related malignancy are still widely accepted and are also used in this study. The criteria include the co-existence of carcinoma and endometriosis in the same ovary, presence of endometrial stroma surrounding epithelial glands, exclusion of a second malignant tumour metastatic to the ovary and morphologic contiguity between the malignancy and the endometriosis.

The sites of origin are classified as ovarian or extra-ovarian, with the first being by far the most common. Etiological factors for these two subtypes seem to differ with unopposed estrogen treatment strongly associated with extra-ovarian neoplasms (Leiserowitz et al 2003) and not really with ovarian malignancies.

In the ovary the most common types of related cancer are clear cell and endometroid adenocarcinoma. Other described associated cancers in the ovary include adenosarcoma, endometrial stromal sarcoma, mucinous and papillary (serous) adenocarcinoma. Extra-ovarian endometriosis related malignancies are

more likely to be endometroid, adenosquamous, papillary or non-specified adenocarcinoma as well as adenosarcomas or endometrial stromal sarcoma.

Endometriosis associated cancers that typically occur at a younger age, are mostly endometroid or clear cell type, typically are diagnosed in early stage and have a relatively good prognosis (Leiserowitz et al 2003). Many recent reviews and studies confirm (some of) these findings, including those by Modesitt and co-workers (2002), Erzen et al (2001) and Takahashi et al (2001).

It is probable that the etiology of disease occurring in older women is often overlooked, that the clinical picture is therefore not so well described and the importance of endometriosis related cancer underestimated.

The team of Yoshikawa (2000) did a review of 15 published reports of endometriosis related ovarian neoplasms and found a prevalence of firstly clear cell carcinomas (39%), followed by endometroid carcinomas (21%), serous and mucinous carcinomas (each 3%). In Western literature however, endometroid carcinomas are much more common than clear cell (25% vs. 7%) which differs from Japan where clear cell dominates endometroid (20% vs. 10%).

2.2.3 Ovarian neoplasms

Ovarian cancers consist of three types, namely epithelial cancers, stromal tumours and germ cell tumours. Epithelial cancers are divided into serous, mucinous, endometroid, clear cell, transitional, squamous, mixed and undifferentiated types. Endometriosis related cancers originate from the epithelial component (glandular) of the endometriotic implant or more rarely from the stromal component (endometrial stroma).

It is interesting to remember that the endometriotic implant is monoclonal with both epithelial and stromal components originating from the multipotent desquamated cell. Metaplasia of cell types after implantation is associated with dedifferentiation which can lead to the formation of tumours with an alternative histological appearance. Metaplasia has been convincingly demonstrated in large numbers of endometriotic implants and some authors have linked this to inflammation (Ness 2000).

2.2.3.1 Ovarian endometroid carcinoma

This tumour is defined as a primary epithelial ovarian tumor with a histological appearance similar to endometroid carcinoma of the endometrium. Stantesson first described it in 1961 and recorded an incidence of about 24% at the FIGO cancer committee in Stockholm. Also similar to endometrial cancer, the degree of cellular atypia divides the group into well, moderately and poorly differentiated groups, with well differentiated tumours more common in association with ovarian endometriosis and associated with improved prognosis.

2.2.3.2 Ovarian clear cell carcinoma

Clear cell carcinoma is commonly associated with ovarian endometriosis and the incidence is much higher in Japan for unknown reasons. This tumour is sometimes considered a variant of endometroid adenocarcinoma, but has very distinct histological features distinguishing it from the latter. The prognosis is similar to that of endometroid carcinomas.

2.2.3.3 Mucinous adenocarcinoma and mullerian mucinous borderline tumour (MMBT)

This tumour was previously described as a mucinous tumour with low malignant potential which resembles the endocervical epithelium. Recently a malignant counterpart for this tumour was described by Lee and Nucci (2003) as endocervical-like type epithelial carcinoma. These tumours were either mucinous or of mixed epithelial origin and linked convincingly to ovarian endometriosis in the majority of patients. This variant of mucinous ovarian carcinoma is thus the most recently described endometriosis-related ovarian carcinoma. Again this tumour type occurs after metaplastic cellular changes in endometriotic implants.

2.2.3.4 Endometrial stromal sarcoma

Endometrial stromal sarcoma (ESS) is a mesenchymal malignancy originating from the stromal part of endometriosis. Fukunaga (2000) investigated 327 cases of ovarian endometriosis and demonstrated smooth muscle metaplasia in 18% of cases. This study was one of the first to demonstrate significant epithelial metaplasia in endometriotic lesions. They could not link metaplasia to neoplastic transformation in their specimens.

ESS is more common in extra-ovarian endometriosis than in ovarian lesions, possibly reflecting the increased hormone sensitivity of the pelvic peritoneum vs. the ovary (Kovac et al 2005; Fukunaga et al 1998). This finding could also reflect the tissue preference of cells. Stromal cells may prefer to grow on the peritoneum rather than the ovary.

2.2.3.5 Other non-endometroid epithelial carcinomas

Other non-endometroid carcinomas include papillary serous adenocarcinoma (the most common subtype in the absence of endometriosis, accounting for about 10% of endometriosis related cases), adenosquamous carcinoma, undifferentiated and mixed carcinoma (Scully et al 1995; Pecorelli et al 1999). The latter three subtypes are extremely rare and are therefore inadequately studied. All these subtypes are more common in extra-ovarian endometriosis than in ovarian endometriosis.

Ovarian serous adenocarcinoma has been studied extensively, but not a lot is known about the pre-cursor lesions. In order to better understand the histogenesis of serous ovarian cancer and to describe pre-cursors, some authors (Piek et al 2001; Jongsma et al 2002) have chosen prophylactically removed ovaries and tubes from women with BRCA1 and BRCA2 mutations. This model makes sense in that it probably comprises one of the groups with the best theoretical chances to have precancerous lesions or precursors. They may thus harbour genetic changes with or without histological lesions that is shared by serous carcinoma but not by normal tissue.

2.3 Genetic changes in endometriosis and ovarian neoplasms

Epithelial ovarian cancer are categorised into distinct morphological groups based on the appearance of the epithelium. Current data indicate that each histological subtype is associated with unique molecular and genetic alterations, which probably determines the morphology.

It also seems that the change in endometriosis associated with morphological atypia, hyperplasia and other epithelial changes is a result of cellular genetic changes and thus carcinogenesis is an accumulation of genetic mistakes of which

the order seems unimportant. In this model the later genetic mistakes could be alterations giving the tumour the ability to invade and metastasise.

Currently, no single genetic model for ovarian tumorigenesis is widely accepted. Given the wide variety of tumours, no single model is likely to ever be applicable to all different histological types. This is in contrast to the widely accepted model developed for colorectal carcinoma discussed above (Fearon & Vogelstein 1990). Rather than developing a sequenced model, the possibly important genetic mistakes will be considered in the next section and specifically their involvement in endometriosis related ovarian cancers.

2.3.1 Cytogenetic changes in endometriosis and ovarian carcinoma

Simple numeric chromosomal changes have long been described in a variety of ovarian tumours. Most reports of cytogenetic changes are found in the older literature. In benign and borderline epithelial tumours non-specific numeric changes occur, with aneuploidy implicated in more aggressive tumour biology, while granulosa cell tumours most often contain monosomy 22, trisomy 12 and 14. Tumours of low malignant potential and early lesions often contain gains at 3q, 8q, 20q.

2.3.2 Specific genetic alterations in endometriosis and ovarian carcinoma

2.3.2.1 K-ras

Activation of the ras proto-oncogene family, mostly by point mutation, occurs relatively infrequently in gynaecological cancers. In endometrial cancer mutations occur in about 30% of tumours, mostly in the *K-ras* and have also been demonstrated in precursor lesions, suggesting involvement at the early stages of carcinogenesis (Mutter 1999). Various scientists have investigated the role of the *K-ras* gene and protein in endometriosis, in ovarian endometroid adenocarcinoma and in malignant transformation.

Otsuka and colleagues (2004) analysed ovarian clear cell cancer, endometriosis and atypical endometriosis lesions for *K-ras* mutations. They found *K-ras* mutations in the clear cell carcinoma but not in the benign or atypical lesions, suggesting K-ras to be an important initiator of malignancy. The same

group (Okuda 2003) reported finding *K-ras* mutations in only one of 27 patients with endometroid ovarian adenocarcinoma. These findings support the notion that genotype determines phenotype or that different genetic alterations are involved in different histological types.

Dinulescu and her group (2005) induced peritoneal endometriosis in mice by inducing oncogenic *K-ras* via an adenoviral vector. This study is discussed further under 4.5.

2.3.2.2 Beta-catenin

Beta-catenin is another gene established to play an important role in early and well-differentiated endometrial cancer. Gene mutation is associated with the early carcinogenetic mechanism and with favourable pathology and outcome in this neoplasm (Doll et al 2008).

The gene has not been studied extensively in ovarian cancer. Examining eight borderline endometroid ovarian tumours, Oliva et al (2006), demonstrated beta-catenin mutations in seven of the cases. All of these tumours had normal immunostaining for beta-catenin and only one tumour had a PTEN mutation.

2.3.2.3 HER 2/*neu* or c-*erb*B-2

In endometrial cancer the majority of published results on HER 2/*neu* suggest an association with higher grade and higher stage at diagnosis, suggesting relatively late involvement in this neoplasm. HER 2/*neu* expression has also been linked to non-endometroid forms of endometrial cancer (Wang et al 2005).

The involvement of this oncogene in endometriosis was studied by Schneider and co-workers (1998) who did not find any expression. Importance in ovarian cancer is currently unclear, but available evidence on involvement in the malignant forms does not suggest direct involvement in carcinogenesis (Mhawech et al 2002), but rather prognostic value (De Graeff et al 2008).

On the other hand Wiener and colleagues (1996) from Texas transfected ovarian cancer cells (from a cell line) with the specific protein tyrosine kinase (PTK) of the HER 2/neu receptor and found that this caused an increase in specific protein tyrosine phosphatases (PTP's) (PTP-H1, PTP-1B and PTP-alpha).

The importance of this study is that it shows interaction, involvement and expression of multiple PTP's in both normal and malignant ovarian tissue. These workers demonstrated differential expression of some PTP's between malignant and normal tissue. They demonstrated that we are only beginning to unravel the intricacies of the protein tyrosine phosphatase cascade and to appreciate its importance in cell growth regulation.

2.3.2.4 P 53

Involvement of the P53 gene is usually shown by immunohistochemical demonstration of overexpression of the p53 protein or by mutation analysis of the gene itself. Both these methods are considered valid although mutation analysis is generally more accurate. In most tumours p53 overexpression or mutation is found in aggressive types or in late stage disease.

Niwa and co-workers showed in 1994 alterations in the P53 gene (by mutation analysis) in 42% of serous and 42% of endometroid ovarian adenocarcinomas. Most of these patients had late stage disease. Other histologic types had less involvement, but the difference was not significant.

In a study utilizing both methods and showing correlating results, Okuda and co-authors (2003) report frequent involvement of the P53 gene in ovarian endometroid adenocarcinoma (65%, n=27), but not in clear cell ovarian carcinoma (0%, n=37). This confirms previous findings, including that of the group of Nezhat (2002), who compared findings in endometriosis, endometroid, clear cell and serous papillary ovarian carcinomas. They found no p53 staining in the benign and between 37 and 55% positivity in the various malignant lesions.

Qian and Shi (2001), who published their results in the Chinese Journal of Oncology, did a similar study using benign, atypical and malignant transformed endometriosis lesions. These authors stated in their abstract that they found most p53 staining in the malignant lesions, less in the atypical lesions and even less in the benign tissue. They also reportedly demonstrated an increase in the transformation area between benign and malignant. The numbers are not mentioned in the abstract, but the authors quote the p-values with significant differences between the levels of protein expression in the different groups.

Results of these studies are surprisingly consistent, reflecting most probably an important role for the P53 gene in malignant transformation of ovarian endometriosis. In endometrial cancer, P53 mutation is more common in serous papillary adenocarcinoma (even as an early event) and probably occurs as a later event only in the majority of endometroid adenocarcinomas. Protein p53 expression is a poor prognostic marker in most tumours and probably also in ovarian carcinoma.

Endometrial carcinomas positive for p53 staining, are associated with adenomyosis lesions that are also positive for the oncogene, suggesting either a field effect or an adenomyotic precursor to the endometrial cancer (Taskin et al 1996).

2.3.2.5 BRCA 1 and BRCA 2

Familial ovarian cancer syndrome, like familial breast-ovarian cancer syndrome is usually caused by a germline inherited mutation in one of these tumour suppressor genes. Various authors have attempted to compare outcome of ovarian cancer in this subset with that of sporadic cancer. Although most authors found a slightly improved survival for familial cases, this finding is not universal. Involvement of the BRCA genes in sporadic breast and ovarian cancer started receiving research attention in the last decade.

While initial studies found BRCA gene mutations in very few sporadic cancers, it is becoming clear that these tumour suppressors play an important role in tumorigenesis and have a very intricate interaction with other known oncogenes and tumour suppressor genes. Gene inactivation can be by mutation or by promoter methylation, i.e. epigenetic function loss.

Press and co-workers (2008) recently found BRCA1 inactivation in 18 of 49 (37%) ovarian cancers. All these tumours were high grade tumours and were associated with P53 involvement or PTEN inactivation. These authors suggest a classification of ovarian neoplasms according to the type of BRCA1 involvement. This classification will have no current clinical relevance and has not been shown to correlate with prognosis either.

2.3.2.6 Bcl-2

The proto-oncogene bcl-2 inhibits programmed cell death by counteracting the action of p53, which induces apoptosis. Several groups have included bcl-2 staining in their immunohistochemical studies on malignant transformation of ovarian endometriosis. However all these authors have produced results that are either non-significant (Nezhat et al 2002; Mhawech et al 2002; Kusuki et al 2001) or difficult to interpret (Qian & Shi 2001).

2.3.2.7 DNA repair genes, micro-satellite instability (MSI) and loss of heterozygosity (LOH)

MSI is frequent (15% to 34%) in sporadic endometrial cancer and it has been shown that the finding of MSI correlates strongly with methylation of the hMLH1 promoter region causing inactivation of the hMLH1 gene (Salvesen et al 2000). The result on cellular level is the same as a mutation in the gene causing defects in the DNA repair system.

In endometrial cancer MSI has been found almost exclusively in the endometroid adenocarcinoma histological subtype (see also chapter 2). Investigators looking at MSI in ovarian endometroid carcinoma and endometriosis have found higher rates of MSI in endometriosis associated cancers and in endometroid adenocarcinomas than in non-endometroid ovarian cancer. The rates were however lower than in uterine endometrial cancer (Catasus et al 2004). Nakayama et al (2001) could not demonstrate MSI in four specimens of endometriosis in any of seven tumour suppressor loci.

Martini and co-workers (2002) were able to demonstrate hypermethylation of both hMLH1 and PTEN with inactivation of protein expression in atypical endometriosis cases as well as in endometroid cancer specimens.

LOH at the PTEN locus is a frequent finding in clear cell carcinomas (around 30%) (Ho et al 2009; Hashiguchi et al 2006) and in endometroid adenocarcinomas (~60%) (Kolasa et al 2006). It is usually incompletely explained by PTEN mutation.

2.3.2.8 PTEN

The incidence of somatic mutations in the PTEN gene in endometroid endometrial cancer is the highest of any primary malignancy analysed so far. The frequencies reported vary from approximately 40% to even 76%. It is suspected that the gene also plays a major role in the ovarian counterpart, endometroid ovarian cancer.

Various researchers have looked at PTEN involvement in both ovarian cancer and endometriosis by determining loss of heterozygosity on 10q23.3 and by immunohistochemistry. Studies using direct mutation analysis are scarce. These results will be reviewed in the next section.

Not only the incidence of PTEN mutations but also the timing thereof and the interaction with other genetic changes in endometriosis and related cancer is of interest. The place of PTEN in the genetic pathway and in the genetic cascade of carcinogenesis possibly differs between different tumour types. The current knowledge on the involvement of the pten-protein in these pathways will be discussed.

2.3.2.9 Phenotype and genotype in ovarian cancer

Ovarian neoplasms display a wide range of histological phenotypic patterns. An important question is whether the histological pattern and genotype are related and can be predicted by the genetic aberrations and vice versa. Currently we know that genetic alterations are tumour specific and the incidences of the changes are different in different cancers, but we cannot predict or type cancers on the grounds of genotype alone (yet).

While simple chromosomal numeric changes have been displayed mainly in benign and borderline epithelial tumours and in stromal (granulosa cell) tumours, invasive (epithelial) ovarian cancers show complex chromosomal changes involving genes that regulate cell proliferation, apoptosis and that play a role in the tyrosine kinase signalling cascade (Diebold 2001). More aggressive neoplasms typically have more gene aberrations.

The currently known genetic alterations and their pattern of occurrence therefore are not sufficient explanation of the wide phenotypic variability of ovarian tumours.

2.4 The PTEN gene in endometriosis and ovarian neoplasms

2.4.1 PTEN germline mutations in ovarian cancer

PTEN was initially found as a result of the mapping of the susceptibility gene for Cowden syndrome and has subsequently also been linked to the Banayan-Zonana and Proteus syndromes. Mice with pten knockout develop complex proliferative endometrial lesions pointing to importance in cellular growth regulation in the female reproductive tract (Podsypanina et al 1999). Germline mutations are very uncommon in sporadic endometrial cancer and are not suspected to be involved in sporadic ovarian carcinoma and endometriosis.

Ovarian tumours are more frequent in Cowden's disease but are generally stromal types (Papageorgiou & Stratakis 2002). Interestingly, the first report of ovarian dysgerminoma in a patient with Cowden syndrome was published in August 2008 (Cho et al 2008). This finding implicates PTEN in the etiology of germ cell ovarian cancer as well. These tumours were not included in this study.

2.4.2 PTEN somatic mutations and pten protein expression

It has been mentioned that PTEN mutation analysis is an imperfect predictor of pten-activity. Investigators working on the role of PTEN in many (other) tumours have shown that most mutations that were found in tumours caused functional impairment of the pten-protein mainly by truncating the product and were therefore considered to be disease causing. PTEN mutation will almost always lead to abnormal pten protein, but the opposite is not true.

The investigation of pten protein expression by immuno-histochemistry therefore produces different results to PTEN mutation analysis. It seems that many factors influence expression of the pten protein and that the involvement of this gene and protein is very widespread. In ovarian cancer the majority of tumours which showed impaired pten-function do not have PTEN mutations. Abnormality of pten expression is thus quite common and many authors think that abnormal function plays an important role in either tumorigenesis or progression and that it correlates with decreased survival.

Studies of PTEN mutation in ovarian tumours are extremely limited and this is one of the major contributions of the current study. The findings of studies examining both pten expression and PTEN mutations will be discussed here.

2.4.3 PTEN in normal endometrium and ovary

PTEN expression in the normal endometrium changes in response to hormonal variations. During the proliferative phase PTEN is expressed in all tissue types, while expression is increased in the early secretory phase and lowered in the late secretory phase. These changes seem to be confined to the functionally active and hormonally responsive layers of endometrium (Mutter et al 2000). Kovacs and colleagues (2007) showed changes in the level of phosphorylation of PTEN during the cycle, although the total levels did not change. The changes followed those described by Muller and suggest that activation of survival signals may contribute to the development of myometrial tumours. PTEN expression in the endometrial stroma and ovary has not been studied.

2.4.4 PTEN in ovarian endometriosis

The involvement of the PTEN gene is extremely difficult to explore in (ovarian) endometriosis. Specifically gene mutation analysis necessitates micro-dissection to isolate tumour tissue from normal tissue and a decent amount of tissue is needed to produce intact DNA for PCR-analysis. Endometriotic tissue tends to consist of small pieces of tumour with a lot of haemorrhage and hemosiderin and densely adhered to normal ovary with fibrosis.

Immuno-histohemical analysis is much easier and is now favoured by many authors due to its ease. Results should however be interpreted with caution. Cirpan (2007) reports on a large recent study of immuno-staining for pten protein in 63 patients, 33 with endometriosis and 30 with ovarian carcinoma. They found similar staining in endometroid ovarian cancer and in ovarian endometriosis and conclude that pten defective endometriomas may be pre-malignant. If this finding is confirmed by other studies, this may be an important clinical application for this molecular analysis.

In one of few studies employing mutation analysis, Obata and Hoshiai (2000) could not detect any PTEN mutations in a small subset of atypical ovarian

endometriosis lesions. In a study with similar methodology to the current one, Sato et al (2000) reported a 20% incidence of PTEN mutations on both endometroid ovarian cancer (n=20) and ovarian endometriosis (n=34).

2.4.5 PTEN in ovarian epithelial cancer

Many investigators looking primarily for PTEN mutations in primary ovarian cancer found PTEN to play a minor or no role in ovarian carcinogenesis. When all tumour types are considered together, results are not impressive. Schondorf and co-workers (2000) found two mutations in a group of 86 specimens (2,3%) and detected these mutations in recurrent ovarian cancers and not in primary cancers. The group of Yokomizo (1998) detected two mutations (homozygous deletions) in 31 unselected ovarian cancers and seven cell lines (4,8%). The group of Maxwell, Risinger and Berchuck, who did much of the initial PTEN work in endometrial cancers (Maxwell et al 1998), also reported on primary ovarian cancers (50) and cell lines (11), finding the same results (0/61).

Chen and co-workers (2004) showed mutated PTEN in only 7% of ovarian cancers, but they only tested for exon 5 aberrations. This group investigated mRNA expression as well, which correlated inversely with stage and differentiation. This led them to conclude that PTEN expression is involved in both tumorigenesis and progression in ovarian tumours.

Using immunohistochemistry, the group of Lee (2005) found more reduced pten protein in carcinomas than in borderline tumours but could not find a relation with either stage or apoptotic index. In borderline endometroid carcinomas, Oliva and co-workers (2006) found one mutation in seven cases.

Groups that focused only on ovarian endometroid (and clear cell) carcinomas showed a higher incidence of PTEN involvement. Obata et al (1998) found mutations in 21% (of 34 tumours) and Sato et al (2000) in 20% (total of 20 tumours). The group of Catasus (2004) could demonstrate PTEN mutations in only 3 of 21 endometroid carcinomas. These three studies probably represent the total available literature reporting PTEN mutation analysis in this tumour type published in the English scientific literature.

Recently a Dutch group (De Graeff et al 2008) found improved survival and low stage in patients with negative PTEN staining in the ovarian tumours. PTEN inactivation also correlated with non-serous tumour type. This study is the exact opposite of what was described by Schondorf and co-workers (2003) when they found that decreased pten expression accompanies ovarian cancer progression. Chen et al (2004) describes abnormal (or down-regulated) expression of pten as "closely associated with tumorigenesis and pathobiological behaviors" in ovarian endometroid cancer.

The answer about the associations between genetic or molecular findings and clinical course undoubtedly lies in the intricate interaction of the different genes and seem far more complex than initially thought. Additionally the finding of a prognostic marker does not necessarily lead to an improvement of management and outcome.

2.4.6 PTEN and loss of heterozygosity (LOH)

After the finding of LOH on chromosome ten in endometrial cancer, various researchers have searched for similar findings at the 10q23.3 locus, the location of the PTEN gene in endometriosis and endometroid ovarian carcinoma.

2.4.6.1 Frequency of LOH (10q23) in ovarian lesions

Thomas and Campbell (2000) report a frequency of 15-20% LOH in various locations in endometriosis, but not in normal endometrium.

The group of Sato (2000), investigated the involvement of PTEN in endometriosis and related cancers by determining LOH at locus 10q23.3. They found LOH in 56,5% endometriosis cysts, in 42% of endometroid ovarian cancers and 27% clear cell ovarian cancers. Not all these were explained by PTEN mutations.

Obata and Hoshiai (2000) also investigated LOH at 10q23 and PTEN mutations, and found a 43% incidence of LOH at 10q23 in ovarian endometroid and 28% in serous carcinomas and 40% in atypical ovarian endometriosis. Only about 50% of the cases with LOH had PTEN mutations that explained the LOH.

The results of these authors and several others suggest a frequency of loss of heterozygosity of markers around and within the PTEN area of between 30 and 50%. The difference between the different tumour types suggest that genetic alterations leading to LOH 10q23 plays an important role in the early events leading to malignant transformation of endometriotic lesions.

The group of Lin (1998) found in synchronous ovarian and endometrial cancers an incidence of LOH in endometrial cancers of 43% and in simultaneous ovarian tumours an incidence of 50%, which is higher than most other authors, but might be explained by the histological types included (especially clear cell carcinomas).

2.4.6.2 LOH (10q23) vs. PTEN mutations in ovarian lesions

From the above discussion it is clear that LOH at the 10q23 locus cannot be explained by PTEN mutations alone. Possible explanations for this phenomenon include the existence of alternative tumour suppressor genes in the vicinity of the PTEN area and disruption of PTEN (and pten) by allelic loss, intragenic mutation and epigenetic silencing.

Kurose and colleagues (2001) published results of an important study investigating the relation between PTEN mutation, pten-protein expression, the finding of LOH (10q23) and the expression of important presumed downstream protein targets of PTEN in the tyrosine kinase cascade. They investigated primary ovarian cancers for these genetic alterations and found that the Akt-pathway was clearly involved in many but not all cancers. This group found decreased or absent pten staining in as many as 78% of ovarian cancer cases, suggesting a most important role for this tumour suppressor protein in spite of a low incidence of mutation positivity (6% in this study) in primary ovarian cancer. Their results for LOH were 45% positive, and they found an association between LOH 10q23 and decrease immunostaining for pten-protein, suggesting an important role for epigenetic silencing of PTEN-expression, a very significant finding.

Other studies quoting LOH vs. PTEN mutation numbers include those of Fujji et al (2002) who found LOH (10q) 4/17, MSI in 4/17 and PTEN mutations in 6/17 cases. LOH correlated well with PTEN mutation. This study was done on patients

was synchronous uterine and ovarian endometroid cancer and the incidences are therefore surprisingly low, possibly indicating less involvement in late stage or aggressive cancers.

In atypical endometriosis, Obata and Hoshiai (2000) found LOH in 40%, but no PTEN mutations. They found LOH in 43% of endometroid cancers, 28% of serous cancers and somatic PTEN mutations in 21% of endometroid cancers. This study's results confirm the impression of LOH in the region 10q23 being the most prominent change, an early event and the most common in endometroid cancer. PTEN is most commonly involved in endometroid carcinoma and does not explain all the LOH findings.

Saito and co-workers (2000) found allelic imbalance (AI) in the 10q23.3 region of 12/31 ovarian cancers (39%). Again this group only found PTEN mutations in 9% of the cases. They found a large number of cases without PTEN mutations that showed AI in the exact region of the PTEN gene. This group postulated that AI of the 10q23.3 region also causes inactivation of another, hitherto unknown, tumour suppressor gene in close proximity of the PTEN gene.

Suzuki, Unoki and Nakamura (2001) induced expression of two novel genes, DUSP1 and BTG1 genes by introducing exogenous PTEN into endometrial cancer cell lines, and have recently also described single gene polymorphisms in these genes. These genes and the polymorphisms may explain some of the cases of hitherto unexplained loss of heterozygosity and may be useful to examine associations between genetic alterations and disease susceptibility.

2.4.6.3 Importance of LOH (10q23) in ovarian lesions

It has been suggested that LOH or PTEN mutation analysis can be useful to determine whether a synchronous ovarian tumour is metastatic from the endometrium or whether it is a simultaneous new primary tumour as suggested by the group of Lin (1998). Fujii and co-workers used combined analyses of LOH, PTEN mutation and MSI to determine clonality with very interesting results. They micro-dissected 17 synchronous cases and showed monoclonality in 35% (some with genetic progression) and polyclonality in 47%; in 18% clonality could not be determined beyond doubt.

2.4.7 PTEN and other genetic changes

It is clear that an accumulation of genetic alterations is needed to cause cancer via inactivation of apoptosis pathways or stimulation of growth. The precise role of PTEN in the tyrosine kinase signalling cascade and the role and importance of this cascade in carcinogenesis in general will take many more decades to decipher.

Recently the group of Dinulescu (2005) published an interesting study linking the involvement of the K-ras and pten proteins in a mouse model of ovarian carcinogenesis. These authors also used the ovarian endometriosis model and caused endometroid ovarian carcinomas by activation and inactivation of the two genes respectively. They found that either oncogenic K-ras expression or pten-deletion caused pre-cancerous lesions with specifically endometroid histology, while the combination of the two mutations always caused aggressive endometroid ovarian cancer.

This is the first study to show that two alterations in the tyrosine kinase-signalling cascade can potentially be more dangerous than one. This study also importantly demonstrated a 100% penetrance of the two cumulative genetic alterations in the same pathway that inevitably lead to cancer. All the mice in this study developed endometriosis after injection of oncogenic K-ras (adenovirus vector) and all developed endometroid ovarian adenocarcinoma after knockout of the second gene in the pathway, namely PTEN.

Because K-ras mutations have not been demonstrated in a large number of endometriotic lesions or in endometroid ovarian cancers, this study must reflect one of many methods in which inactivation of one of the apoptosis pathways can cause benign proliferative and malignant tissue change in the ovary. It is interesting that a sequence of genetic events that have a totally predictable outcome might not actually play an important role in the development of the same disease in humans.

2.4.8 PTEN involvement in the genetic sequence of carcinogenesis

Considering endometriosis related ovarian cancers, a number of investigators have published findings suggesting that PTEN inactivation occurs early in the carcinogenetic pathway. These authors have reported finding PTEN mutations and

LOH (23q) in ovarian endometriomas and then also often the same aberrations in the related cancer (Sato et al 2000).

These findings are convincing and correlate well with the findings in endometrial endometroid cancers. The current conclusion is thus that PTEN mutation is an early event and happens in the cancer precursor (mainly endometrioma), inactivating the normal apoptotic pathway.

Considering the even bigger roll of PTEN in various ovarian tumours as suggested by many samples that show evidence of depleted pten-protein expression and LOH without PTEN mutation, it seems obvious that PTEN aberrations and even more so anomalies of the tyrosine kinase signalling cascade play a huge role in carcinogenesis in the ovary. In the other tumour types, PTEN and pten-protein inactivation may be a later event than PTEN mutation in endometriosis.

It seems that PTEN mutation plays a more pronounced role in endometroid ovarian carcinomas developing from endometriomas while inactivation of the tyrosine kinase signalling cascade in other ways are more important in alternative epithelial tumour types.

2.4.9 PTEN related genetic anomalies

2.4.9.1 The tyrosine kinase-signalling cascade

Mok and colleagues (1995) thought that expression of various proteins that control tyrosine phophorylation may be important. They detected abnormal expression of a protein called PTPN6 (protein tyrosine phophatase, nonreceptor type 6) using immunoblotting analysis in the vast majority of ovarian carcinomas selected for the experiment (10/11).

2.4.9.2 Akt or Protein kinase B (PKB)

This serine/ treonine kinase, has been shown to be an extremely important physiological mediator of the effects of insulin, several growth stimuli and growth factors and it protects cells against natural cell death. Activated PKB/Akt will provide a cell survival signal that will inhibit stress induced apoptosis and

therefore Akt is a known cell survival promotor (Kulik et al 1997) and as such an opponent of PTEN in the tyrosine kinase pathway.

The activation of this kinase is a complex process and when activated PKB/Akt is considered to be a proto-oncogene that induces cellular transformation to neoplastic cells. Focal adhesion kinase (FAK) is also an important upstream mediator of the PKB/Akt pathway, regulated among others by PIP3 that is the key component of this control pathway.

Kurose et al (2001) demonstrated P-Akt immunostaining in 57% of ovarian cancers and showed an inverse correlation between PTEN expression and P-Akt expression.

Yuan and colleagues (2000) examined primary ovarian cancer specimens and found activation of Akt1 and Akt2 by demonstrating elevated levels in 33 of 91 specimens and found frequent activation as well. They also demonstrated an association with high grade and stage and with increased PI 3-kinase activity. When PI 3-kinase was inhibited, apoptosis was induced in ovarian cancer cells. This is an important potential clinical application for research into the tyrosine kinase cascade.

3. Materials and methods

PTEN involvement in the tumorigenesis of both benign and malignant ovarian tumours seems probable in the light of the research findings discussed above. The research described in this section is an attempt to help clarify the role of the PTEN gene in the development and progression of ovarian endometroid tumours, including ovarian endometriomas, atypical endometriosis and endometroid adenocarcinoma of the ovary. It is hoped that our findings will contribute to the accumulating molecular data for putative ovarian cancer precursors and will help understand the role of the PTEN gene in the carcinogenetic pathway in general.

It was decided to use endometriosis and ovarian endometriomas specifically, to study ovarian oncogenesis as it was thought and previously reported that involvement of the PTEN gene would be greatest in this tumour type. The sequence of tumour development in this model is also almost unique in the ovary

and the only model of which the different pathological modalities are all available in archival material.

We were interested in the sequence of involvement of the gene in the different parts of the ovarian lesion. Finding a difference and evidence of progression from benign to atypical to malignant would support our theory of progression and may also support our theory of early involvement of the gene in endometriotic lesions of the ovary.

3.1 Materials

3.1.1 Sampling and clinical material

We retrospectively searched the clinical files for all available patients known to have the diagnosis of endometroid ovarian adenocarcinoma over a four year period. Fifteen patients were identified. Archival material was then collected and the diagnosis reviewed to select patients with simultaneous ovarian endometriomas and endometroid ovarian cancer. Patient selection was thus based on availability and on review of the original diagnosis. Clinical information was collected from the clinical files.

Paraffin embedded tissue of ovarian endometrioma and ovarian endometroid adenocarcinoma was retrieved for analysis.

3.1.2 Histology reports

After careful review of the histology, seven patients were selected who had endometroid ovarian adenocarcinoma probably developing from ovarian endometrioma. In all these patients both benign and malignant lesions were described and potentially available to be studied.

After hematoxylin staining all pathological slides were reviewed systematically and areas of **benign endometriosis**, areas with cellular or structural atypia (**atypical endometriosis**) and **endometroid adenocarcinoma** were marked. Laser micro-dissection of formalin-fixed paraffin-embedded normal and tumour tissue was done using the PALM micro-laser dissection instrument to avoid cellular contamination between different tissue types.

DNA from benign endometriotic lesions, of atypical endometriosis and of malignant tumour was obtained where possible and appropriate. Mutation analysis was done on all the tissue samples and results were then correlated with available clinical and pathological data.

3.1.3 Tissue for DNA analysis

In three patients both the ovarian endometriosis and the endometroid ovarian carcinoma lesions rendered enough pcr-product on DNA amplification to do mutation analysis. In these patients the progression could be studied from benign to malignant lesions.

Four other patients had enough DNA to study the ovarian endometroid adenocarcinoma lesions but insufficient DNA from the benign lesions. We could therefore not complete a reliable mutation analysis on these samples.

This problem demonstrates again the difficulties encountered to study of ovarian carcinogenesis. Again, immunohistochemistry is much easier and cheaper to perform, but with less exact results.

3.2 Methods

3.2.1 DNA extraction

The micro-dissected tissue samples were carefully transferred to micro-tubes where it was treated with the extraction buffer (10mM Tris-HCL, pH8.0; 0,45% Nonidet P40; 0,45% Tween-20) and 0,2mg/ml Proteinase K (Roche) was added to the tissue and after overnight digestion at 55°C, the proteinase K was inactivated by boiling (5 minutes at 95°C). The DNA solutions were quenched on ice and centrifuged. The supernatant, containing the DNA was transferred to new sterile tubes and used or stored at 5°C. Following DNA extraction, the GenomiPhi kit (AEC Amersham) was used to amplify the whole genome because of the very small amount of DNA obtained.

3.2.2 DNA amplification

PTEN-coding sequences were amplified by polymerase chain reaction using the primers described by Davies et al (1999). The nine exons were amplified in eleven sections, with exons five in two sections and eight in two sections. Intron-based

primers were used to minimise the risk of amplifying the processed PTEN pseudogene on chromosome 9, as described by Dahia et al. (1998).

3.2.3 PTEN mutation analysis

PCR was performed in 20µl or 10µl reaction volumes for first round, or second round reactions, respectively. First round reactions containing 4 µl of the tissue extract, 20mM Tris-HCl (pH8.4), 50mM KCl, $MgCl_2$ (1.5mM for exon 8b; 2mM for exons1-7; 2.5mM for exon 8a; 3mM for exon 9), 0.25µM of each dNTP, 0.2µM of each primer and 0.5 units *Taq* DNA Polymerase (Life Technologies, BRL) were amplified for 35cycles consisting of 1 min at 94°C, 1 min at annealing temperature, and 1 min at 72°C, with a final extension step at 72°C for seven minutes. The PCR products were labelled with γ-^{32}P ATP (7000Ci/mmol; ICN) in a second round reaction in which two µl of the first round reaction was amplified in the presence of 0.02 µM (0.42µ Ci) of each end-labelled primer.

3.2.3.1 Sequence analysis

Direct DNA sequencing was performed on all the DNA samples obtained using the BigDye Terminator V3.1 cycle sequencing kit (Applied Biosystems) as prescribed by the manufacturer. Sequenced samples were analysed using the ABI 3130 system.

4. Results

4.1 Clinical data

Due to the nature of the identification and selection of cases and the small sample size, no analysis of clinical data will be done. The results are shown to confirm reasonable spread and typical pattern of the cases. Only the results of the seven selected cases are shown.

4.1.1 Age distribution and population group

Age at diagnosis ranged from 32 years to 76 years. Age distribution is shown in figure 4.1.

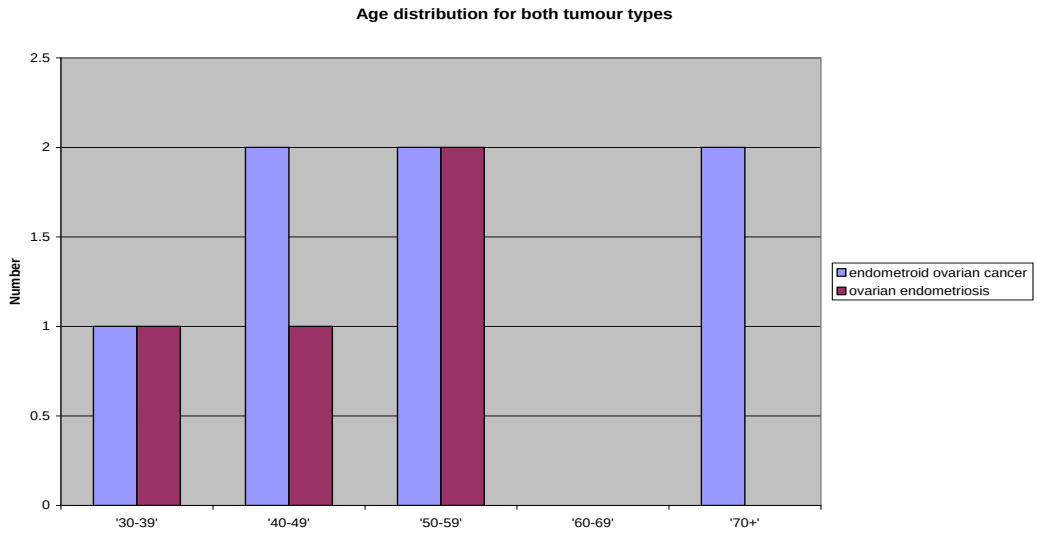

Figure 4.14: Age distribution of ovarian endometroid lesions.

Six of the seven patients were Caucasian and one patient was of mixed origin (coloured). This finding is probably biased due to case selection, but may also reflect the racial distribution of this tumour type.

4.1.2 Menopausal status and associated disease

Three patients were pre-menopausal at the time of diagnosis, while another one was using unopposed estrogen. The other three patients were post-menopausal and not on hormone therapy. This tumour type is known to be more prevalent in younger groups and may be hormone sensitive.

Hysterectomy is known to reduce the risk of epithelial ovarian cancer, but is not known whether it reduces endometroid ovarian cancer risk or the risk of cancer in existing endometriosis. Three of the seven patients had previous hysterectomy.

Two patients were diabetic and two were hypertensive. One young patient (40 years) previously underwent colon cancer surgery.

Two patients were previously treated for infertility and four were previously diagnosed to have endometriosis. Of the seven patients, four had no previous pregnancies and one patient had one child. Pregnancy data is shown in figure 4.2.

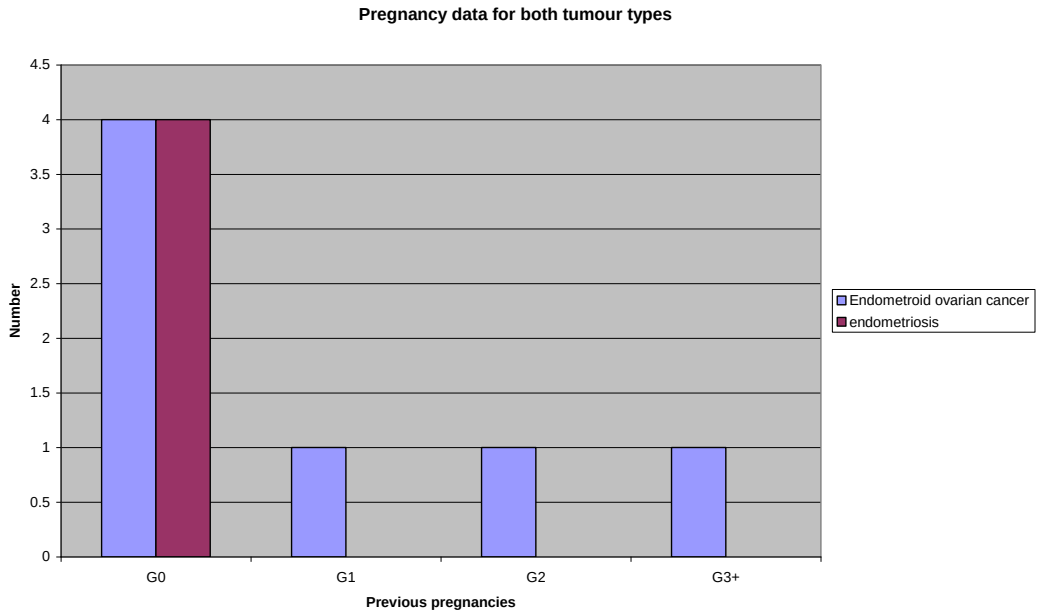

Figure 4.15: Pregnancy data for ovarian endometroid lesions.

Hysterectomy is known to reduce the risk of epithelial ovarian cancer, but it is not known whether it reduces endometroid ovarian cancer risk or the risk of cancer in existing ovarian endometriosis. Three of the seven patients had previous hysterectomy.

Two patients were diabetic and two were hypertensive. One young patient (40 years) previously underwent colon cancer surgery.

4.2 Histology data

4.2.1 Stage distribution and differentiation grade

The FIGO stage and histological differentiaition grade is shown in table 4.2.

Table 4.22: Histological and clinical parameters.

Study number	Differentiation grade	FIGO stage	Synchronous endometriosis	Co-morbidity
EOC 01	2	2	Ovarian	Infertility
EOC 02	2	2		Colorectal Ca
EOC 03	3	2		DM, HT
EOC 04	1	2	Ovarian	DM
EOC 05	3	4		HT

EOC 06	2	?	Peritoneal	Unopposed estrogen
EOC 07	1	2	Ovarian	Infertility

Other than the typical picture of papillary serous carcinoma, most patients had disease confined to the pelvis at the time of surgery. Five of seven patients were diagnosed in stage 2 and only one patient had disseminated disease.

In spite of the early stage at presentation, four patients died of disease within the four to five years follow up.

The three patients that survived all had pre-existing endometriosis.

4.2.2 Tumour size

Endometroid ovarian cancer tends to form a more solid and larger primary tumour than papillary serous carcinoma. The tumour sizes varied from 1cm to 15cm, average 6 cm.

4.3 Mutation screening

Due to problems with the PCR amplification due to both low DNA quality and insufficient DNA quantity, all samples were sequenced directly without mutation screening. The results will be discussed here.

4.4 Sequence analysis

4.4.1 Non-malignant tissue samples

No PTEN mutations were found in any of the endometriosis samples.

In the one patient who had a PTEN mutation in the ovarian cancer, the associated endometriotic lesion did not display the same mutation, suggesting PTEN mutation was an event associated with malignant behaviour in this tumour.

In the second patient no concurrent endometriotic lesion was available for analysis; this patient was elderly (76 years).

4.4.2 Endometroid ovarian carcinoma

Two patients displayed somatic mutations in the ovarian malignant tumours. Both mutations are considered to be disease causing.

Table 4.23: Mutations in the PTEN gene in endometroid ovarian carcinomas.

Tumour	Mutation type	Exon	Nucleotide change	Effect
EOC 4	nonsense	5	c.388C to T	Arg130Stop
EOC 3	frameshift	6	c.497delT	Stop at 178

4.4.3 Polymorphisms

We did not find any polymorphisms in the PTEN gene in this small group of tumours.

4.5 Correlation between PTEN gene mutations and clinico-pathological findings

The group of tumours where mutation analysis could be completed were too small to correlate these to clinical and pathological findings.

The one mutation positive tumour were poorly differentiated and the other well differentiated, while both patients were diabetic, had stage 2 disease and were post-menopausal (ages 58 and 76).

5. Interpretation and importance

5.1 Ovarian endometriosis

It was expected that PTEN mutations may be present in this benign counterpart of endometroid ovarian cancer if it was indeed found in the malignant tumour. No mutations were however found in this group of tumours. The importance of this finding is severely limited by the small amount of DNA that was available for analysis.

Some other mutation analysis studies in the literature have concordant findings (Obata et al 1998), while others found some mutations (Sato et al 2000) and PTEN involvement on immunohistochemistry.

In one patient the availability of DNA from the benign tumour made it possible to determine that the PTEN mutation was indeed only present in the malignant tissue. This finding is of some importance as it points towards PTEN mutation being a late event in carcinogenesis in this tumour.

We could not correlate PTEN mutation with the finding of cellular atypia. It will be much easier to study the timing of the involvement of the PTEN gene with a method that does not require DNA extraction from these very small lesions.

5.2 Endometroid ovarian carcinoma

This study demonstrated that PTEN mutation plays a role in early stage endometroid ovarian cancer of different etiology. We found evidence of involvement of PTEN in a significant part of this small study population. Two of seven tumours (~29%) had mutations.

The findings concurred with the groups of Obata (1998), Sato et al (2000) and Catasus (2004) who found mutations in between 14 and 24% of endometroid carcinomas. These findings are discussed above (2.4.5) in more detail.

5.3 Epithelial ovarian carcinoma

It is becoming increasingly clear that the developmental pathways for the three major subtypes of ovarian epithelial cancer are fundamentally different. The subtypes are serous, mucinous and endometroid carcinoma, with the molecular characteristics of the latter studied in this project. Evidence is accumulating to demonstrate that serous papillary tumours often arise from the lumen of the fallopian tube, while the etiology of mucinous tumours remains a dilemma.

It is known that endometroid ovarian carcinoma can arise from ectopic endometrial implants that undergo malignant transformation, but it is unclear what proportion arise *de novo* via cellular metaplasia of the ovarian surface epithelium. In the same way that the PTEN gene is involved in malignant transformation of endometrial cells in the endometrium, this gene is probably intimately involved in somatic mutation and transformation of endometrial implants on the ovary.

While PTEN mutation plays a limited role in the majority of ovarian tumours, this study and some others have demonstrated that it is intimately involved in the pathogenesis of endometroid ovarian carcinoma. The precise nature, level and chronology of involvement will remain the subject of study in the near future.

Generally the incidence of PTEN mutations reported in a group of ovarian epithelial cancers is determined by the proportion of endometroid and clear cell tumours in the study.

5.4 Strengths, limitations and recommendations

This study was one of the first in the world to study PTEN involvement in ovarian carcinoma and endometriosis. As far as we could establish, it is the only South African study.

The findings of this study shows important involvement of this tumour suppressor gene in the development of ovarian endometroid carcinoma. We could not, however, demonstrate when in the carcinogenetic pathway (early or late) these mutations occur. It would be hugely interesting to answer this question, but a suitable scientific model of study is outstanding.

The significance of the findings of this study is limited by small numbers but the results are in line with the findings of other research groups. When all the mutation analysis studies are considered together, results are concordant and about 20% of endometroid ovarian cancers show mutations (16 of 82). The results of endometriosis studies are less consistent (demonstrating the difficulty to perform these studies) and results vary between 0% and 20%).

Similar to the studies of endometrial carcinoma and uterine sarcoma, this study was limited to mutation analysis. No attempt was made to study pten protein levels or activity. It would be interesting to correlate gene mutations to protein expression and activity in future.

It is interesting to compare the findings of this study to that of the endometrial carcinoma and hyperplasia studies done on the same population and described in chapter 2. The results of the different tumour types will be correlated and discussed in the final chapter.

Chapter 5

The role of the tumour suppressor gene PTEN in the etiology of cancers of the female genital tract: Concluding remarks

INTRODUCTION

RESEARCH FINDINGS

HYPOTHESIS TESTING

CONTRIBUTIONS AND LIMITATIONS

RECOMMENDATIONS AND IMPACT

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1 Introduction

1.1 Background

Knowledge of the role of the PTEN gene and its protein product (pten) in the cell cycle and growth control is ever expanding. Understanding of the related proteins and cell growth control mechanisms also increases as more information on cell biology becomes available. As these mechanisms are discovered, the intricate interactions of different molecules become clearer. This research area is very large and can be intimidating. It has been extremely inspiring and exciting to be involved in this field as a clinician.

In the first chapter of this dissertation, the current information about the place of PTEN and its aberrations in the carcinogenetic pathway was summarized. Particular emphasis was placed on pre-existing knowledge on its role in the female genital tract. No attempt will be made to repeat or summarize that discussion here.

The opening chapter forms the basis of the interpreted and focused literature reviews that introduce the following chapters. This review and discussion of the related knowledge of clinical and pathological features, tumorigenesis and histology, create an essential background for the research that follows.

The chosen research model was to study both the malignant tumour and the closest available pre-malignant lesion or benign counterpart. The idea of this carcinogenesis model was to demonstrate different levels of involvement of the studied tumour suppressor, PTEN, in the evolving steps. The involvement of aberrations in the tumour suppressor gene in abnormal cell growth control was studied by mutational analysis of the nine exons of the gene.

1.2 Research questions and hypotheses

1.2.1 Research questions

The research questions as listed in the introductory chapter are essentially the same for the different tumour types. The central question is: What role does PTEN

gene mutation and pten protein inactivation play in the etiology of the studied female genital tract neoplasms?

The carcinogenetic model that was chosen will also be evaluated and defended. In addition the value of mutation analysis as a method to study gene involvement will be discussed.

The specific questions used to answer the central question are repeated here for ease of discussion.

1.2.1.1 Endometrial hyperplasia and carcinoma

1. What role do PTEN gene mutation and pten protein inactivation play in the etiology of endometrial carcinoma?

2. What is the frequency of PTEN mutations in endometrial cancers and pre-cancers?

3. When in the carcinogenetic process do these mutations occur?

4. How do PTEN mutations correlate with disease stage and grade?

5. How does the involvement of the PTEN gene differ between the different population groups in South Africa?

6. How does the involvement of the gene differ between South African and European patients?

1.2.1.2 Uterine soft tissue tumours

1. What role do PTEN gene mutation and pten protein inactivation play in the etiology of uterine leiomyomas and uterine sarcomas?

2. What is the frequency of PTEN mutations in uterine soft tissue tumours?

3. Can these mutations be predicted by the finding of micro-satellite instability?

4. Can these mutations also be shown in uterine leiomyomas?

5. How do PTEN mutations correlate with histological type, disease stage and grade?

6. How does the involvement of the PTEN gene differ between the different population groups in South Africa?

1.2.1.3 Endometroid ovarian cancer

1. What role do PTEN gene mutation and pten protein inactivation play in the etiology of ovarian endometroid adenocarcinoma?

2. What is the frequency of PTEN mutations in these tumours?

3. Can (ovarian) endometriosis be used as the benign counterpart or pre-malignant lesion of ovarian endometroid adenocarcinoma?

4. Do PTEN mutations also occur in ovarian endometriosis?

5. Do PTEN mutations correlate with histological type, disease stage and grade?

1.2.2 Hypotheses

Three hypotheses were set to be tested in this dissertation, which defined the role of the PTEN tumour suppressor gene in the etiology of cancers of the (upper) female genital tract.

The first hypothesis is that the PTEN gene is intimately involved in endometrial carcinogenesis and may be involved in the development of endometrial hyperplasia.

The second hypothesis is that the PTEN gene is involved in the formation of the different uterine soft tissue tumours.

The third hypothesis is that the PTEN gene is involved in ovarian carcinogenesis in a subgroup of ovarian cancers, namely in endometroid adenocarcinomas.

1.3 Outline

This chapter will be used to summarize the research findings and to discuss it in the context of current knowledge. The findings in the different tumours will be brought into perspective. The research questions listed will be used to structure this discussion.

The central hypothesis of this thesis is that PTEN plays an important role in tumours of the female genital tract. Although this hypothesis was proven, the involvement is highly selective and the hypotheses of the different studies will be discussed separately.

Concluding remarks will focus on the contribution of this work to the local and international research arenas, limitations of the different research projects and on recommendations for further investigations. The potential impact that knowledge of PTEN involvement can have on translational research and clinical practise including new anti-neoplastic drugs will also be discussed.

2 Research findings

2.1 Clinical findings

2.1.1 Age at diagnosis

The average age at diagnosis of endometroid endometrial cancer was 65 years with a tendency towards earlier diagnosis in African women and older age in Caucasians. Of the sarcomas, carcinosarcoma occurred at an older age (average age about the same as endometrial carcinoma) and the other two tumour types were diagnosed about ten years younger.

Endometroid ovarian carcinoma was diagnosed in an almost bimodal pattern with an average age of 55 years. Case selection would have influenced this pattern, but although the series cannot be considered representative, this is concurrent with published data.

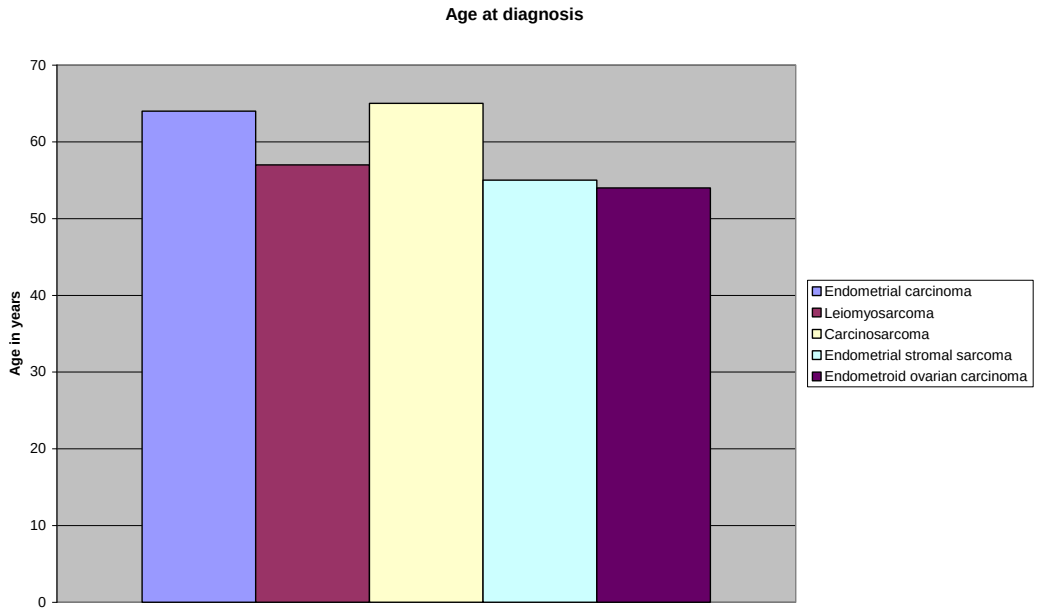

Figure 5.16: Average ages at diagnosis of all malignant tumours included in PTEN mutation analysis.

The average ages for the different tumours are shown in figure 5.1.

2.2.2 Stage distribution

Tumour biology, symptomatology, access to and quality of health care and many other factors influence the stage at which cancer presents. FIGO stage was available for the large majority of malignant tumours is this study and is displayed in figure 5.2. Huge differences in stage at diagnosis exist. In this study, these differences probably reflect mostly tumour related factors and the stage distribution is typical of the tumour types as published in other overviews.

Endometrial carcinoma is often diagnosed in early stage, confined to the uterus, while the uterine sarcomas have a more varied stage distribution. In this study the endometroid ovarian carcinomas were usually early stage (stage 2, or confined to the pelvic area), which is not typical for epithelial ovarian cancer. This is probably due to case selection bias and to the inherently different tumour growth pattern of endometroid carcinomas.

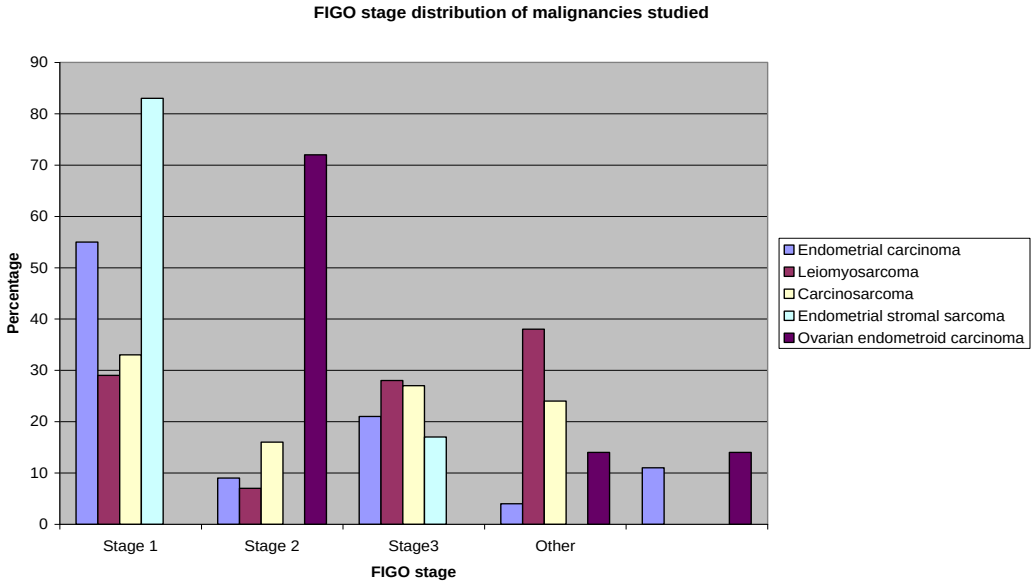

Figure 5.17: FIGO stage distribution of all malignant tumours included in PTEN mutation analysis

2.2 Histology findings

2.1.1 Differentiation grade

The distribution pattern of endometroid carcinoma and uterine sarcomas reflect an inability to predict the prognosis of these tumours on the grounds of morphology and a limited ability to stratify these malignancies using histology.

While endometrial carcinoma is graded as “moderately differentiated” in the large majority of cases, sarcomas are not really graded or classified and are usually considered poorly differentiated by nature of the diagnosis.

It is hoped that molecular stratification will fare better in the future in predicting outcome on the grounds of cellular biology or behaviour. The results are shown in figure 5.3

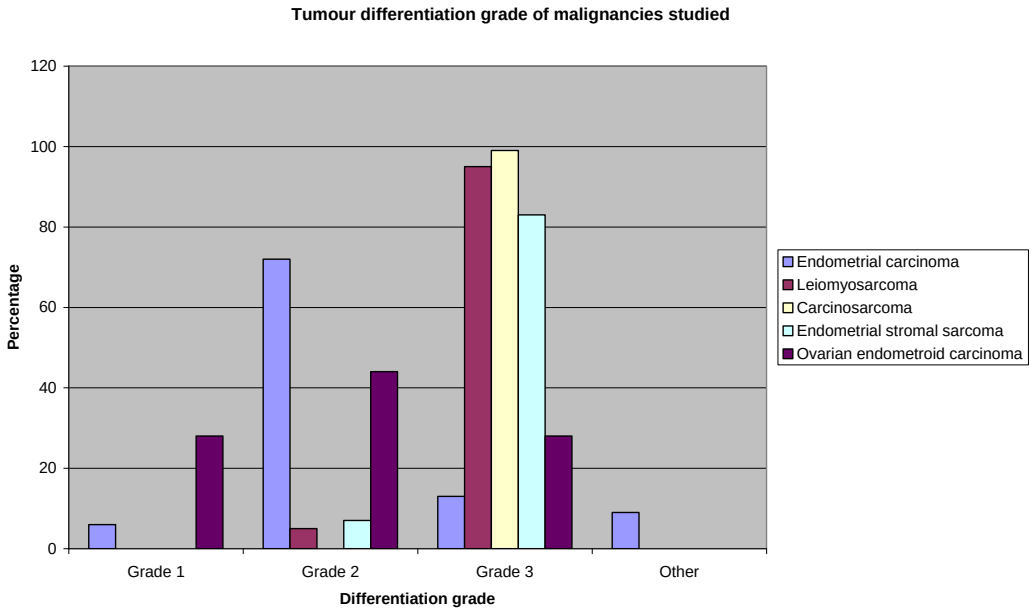

Figure 5.18: Histological differentiation grades of all malignant tumours included in PTEN mutation analysis.

2.3 PTEN mutation analysis

The role of PTEN gene mutation in the chain of events leading to a clone of invasive malignant cells was analysed in specific gynaecologic tumours and tissues using polymerase chain reaction based mutation analysis.

The results obtained from these experiments will be used to answer the different research questions for each tumour type as asked in the introductory chapter and in the respective studies.

2.3.1 Frequency of mutations

2.3.1.1 Endometrial carcinoma

In endometroid adenocarcinoma PTEN-mutations occurred in more than half of all patients investigated and many tumours had more than one mutation. We reported a frequency of 54% disease causing mutations in endometroid carcinoma, which is in keeping with the frequencies reported by other investigators as discussed in the second chapter. In addition seven mutations and polymorphisms were found which have an unknown significance and impact on pten protein function.

In non-endometroid carcinoma, we found no mutations in four tumours. This finding is consistent with other reports in published literature, although the subgroup was too small for interpretation.

2.3.1.2 Uterine soft tissue tumours

Very little was known about the involvement of PTEN in uterine tumours other than endometrial cancer at the onset of this study.

Carcinosarcoma

In the current study of uterine soft tissue tumours, involvement by gene mutation in the PTEN gene was demonstrated only in carcinosarcoma. The frequency of mutations in this tumour was four mutations in three of the 24 carcinosarcomas or 12,5%. The incidence is thus much lower than that found even in poorly differentiated endometroid endometrial carcinomas in this and other studies.

This finding is highly significant and supports the hypothesis of the endometrial origin of these tumours. It also supports the notions that a strong link exist between this gene and endometroid differentiation and that morphology is strongly linked to cellular genetics.

Leiomyosarcoma

We demonstrated one PTEN mutation in a uterine leiomyosarcoma, while no mutations were found in the group of atypical leiomyomas. The incidence in this study is thus respectively 5% in LMS (one in 19 tumours) and 0% in leiomyomas (nil in 21 tumours).

We can conclude that PTEN does not play an important role in smooth muscle tumours of the uterus. This finding underlines the significant differences between LMS and CS, linking the latter strongly to endometrial neoplasia rather than to myometrial smooth muscle tumours.

Endometrial stromal sarcoma

We did not find any mutation in the six ESS samples that were studied (0%). The notion that PTEN is involved in epithelial rather than mesothelial neoplasms is supported by this finding. Although a small study, this is a scarce tumour type and the finding is of significance.

Summary

PTEN involvement in uterine soft tissue tumours are linked to an epithelial component and most strongly to the endometroid epithelial component.

2.3.1.3 Endometroid ovarian cancer

We used a model of ovarian endometriosis and atypical endometriosis developing into endometroid ovarian adenocarcinoma in a small group of patients. We did indeed find PTEN mutations in two of the seven (29%) tumours analysed completely.

This finding confirms PTEN involvement in this tumour type, which is probably less important than the role of this gene in the endometrium. The sample size does not allow any further analysis or deduction.

Again this finding suggests that PTEN involvement is linked to endometroid epithelial morphology. The incidence of PTEN mutations in the malignant tumour types investigated in this study is summarized in Figure 5.4.

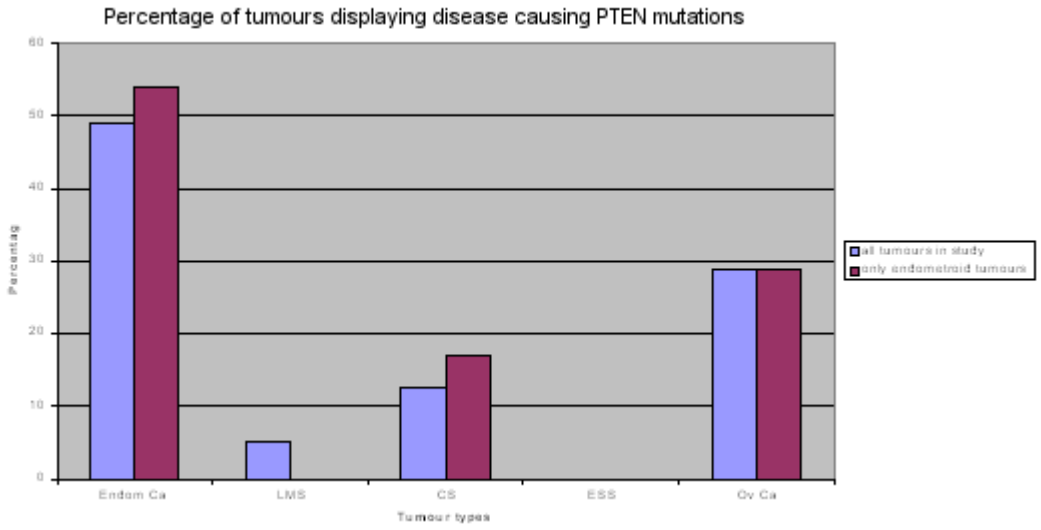

Figure 5.19: Percentage of malignant tumours in this study that displayed mutations considered to be disease causing

2.3.2 Timing of PTEN mutations and mutations in pre-cursor lesions

2.3.2.1 Endometrial carcinoma

Pathogenic PTEN mutations were found both in simple atypical hyperplasia (10%) and in endometroid adenocarcinomas (54%). This finding confirms that

inactivation of the protein by genetic mutation plays a role in the etiology of this disease. It also confirms that this genetic event can occur early in the carcinogenetic pathway.

In the current study it was shown that PTEN mutations can also occur later in the carcinogenetic process or even later on in the already malignant tumour as the malignant cells accumulate more genetic mutations. The incidence was much higher in cancers than in the hyperplasia.

A correlation was found between higher stage and more than one PTEN mutation. Interestingly, all four tumours with more than one pathogenic mutation occurred in African patients. It is postulated that in this population group higher stage also correlates with poor access to health care and thus longer disease duration. It is thought that these genetic abnormalities may accumulate over time causing more severe pten protein dysfunction. Previous series contained very few patients with high stage or long-standing disease.

It would be very interesting to test this finding in a larger number of patients with late stage disease.

2.3.2.2 Uterine soft tissue tumours

No mutations occurred in the benign counterpart of leiomyosarcoma, namely leiomyomas. It is postulated that if PTEN mutation does occur, it is only one step in the whole cascade of cellular genetic anomalies leading to loss of cell growth control. It is improbable that this will be either important or early in this type of neoplasm.

The incidence of PTEN mutations in endometrial hyperplasia should be compared to that found in carcinosarcomas asit is believed that this sarcoma is an type of or an analogue of poorly differentiated endometrial carcinoma. With an incidence of 10% in hyperplasia and 12,5% in carcinosarcoma, it must be postulated that PTEN mutations probably occur early in the carcinogenetic process and is then followed by other cellular mutations which render these cells aggressive and invasive. The other mutations possibly cause a different morphology and differentiation. Generally PTEN mutation frequency does not

predict aggressive biology, but rather non-aggressive behaviour. In carcinosarcoma the cells may also dedifferentiate into sarcoma cells either early in the process or after the occurrence of the PTEN mutation. This sequence is still unknown and was not tested by the current study.

The correct precursor or counterpart for ESS is probably stromal myosis, but this tumour was not available for analysis. In figure 5.5 the data from endometrial hyperplasia is used for comparison.

2.3.2.3 Endometroid ovarian cancer

We could not demonstrate PTEN involvement in the ovarian endometriosis lesions examined. Neither could we find the known PTEN mutations that occurred in the endometroid ovarian carcinoma in the neighbouring endometriosis implant.

These findings seem to support the theory that PTEN mutation can be involved in ovarian carcinogenesis, but probably is neither common nor an essential step. It was found by others that involvement is more common in endometroid carcinomas than in other epithelial types.

It is suggested that involvement of this gene in endometriosis and in endometroid ovarian cancer be studied further. Such a study can utilize newly available immunohistochemistry to avoid the difficulties encountered during the current analysis.

The PTEN mutation analysis data in benign versus malignant tumours are summarised in figure 5.5 but interpretation should be cautious and the table should be read with the text.

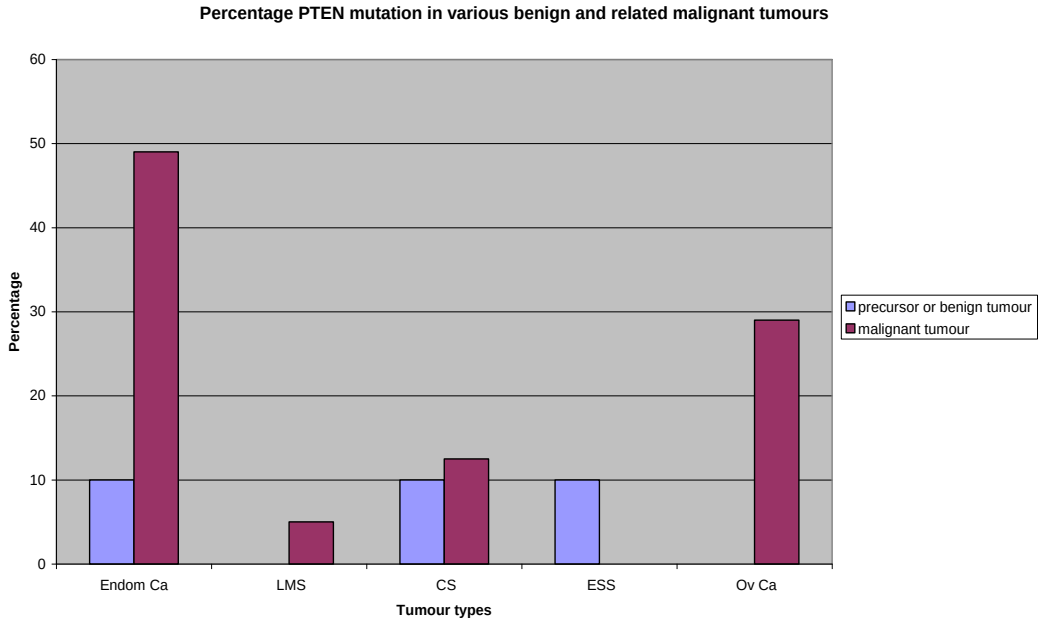

Figure 5.20: Percentage of precursor lesions or benign tumours vs. related malignant tumours that displayed mutations considered to be disease causing.

2.3.3 Correlation with stage, type, grade and other genetic findings

2.3.3.1 Endometrial carcinoma

As mentioned, we found a tendency towards more than one PTEN mutation in tumours with a higher stage. We did not find a higher incidence of PTEN mutations in higher stage disease and previous studies included too few high stage tumours to comment on this aspect.

Although previously suggested, the current study did not demonstrate a correlation with histological grade. There was also no difference found in PTEN mutation frequency in different population groups when only the endometroid adenocarcinoma group was considered. When all tumour types were included, there was a tendency towards a lower frequency of PTEN mutations in African women, which is of huge interest.

In this study pathogenic PTEN mutations were found in simple atypical hyperplasia and in endometroid adenocarcinomas but not in any of four papillary serous endometrial carcinomas.

Again these findings underline the strong link between phenotype and genotype but do not prove causality. It confirms the disparity in tumour type distribution or morphology between different population groups.

Microsatellite instability was tested in this same cohort, and was previously reported (Jamison 2004).

2.3.3.2 Uterine soft tissue tumours

We found important differences between different tumour types in this group, with only carcinosarcoma displaying important PTEN involvement and with mutations only demonstrated in tumours with an endometroid epithelial component. As discussed before, these tumour types do not allow comparison of different histological grades.

Microsatellite and LOH analysis was performed on the same group of tumours and previously published (Amant et el 2001). It is of interest to compare these results to the findings of PTEN mutation analysis.

The only leiomyosarcoma that harboured a PTEN mutation (LMS 42) did not have any LOH or MSI, while 9 tumours had either or both of these findings in at least one of the examined loci.

CS 15 had two PTEN mutations and displayed MSI in all of the loci examined. The other two carcinosarcomas that had PTEN mutations (CS 15, CS 19) had neither LOH nor MSI. In total only five tumours were LOH positive and only three had MSI. Only one endometrial stromal sarcoma had MSI.

MSI and LOH did not correlate with PTEN mutation at all, although the one tumour with two mutations had a high frequency of MSI.

2.3.3.3 Endometroid ovarian cancer

The current study focused only on the endometroid ovarian tumour type. In contrast with previous findings in epithelial ovarian cancer (consisting mostly of papillary serous cancer), we found important PTEN involvement in this study. This finding correlates well with previous and subsequent studies of endometroid tumours.

We did not find any correlation between stage or grade and mutation status.

2.3.4 Differences between population groups

2.3.4.1 Endometrial carcinoma

In endometrial carcinoma, African race correlated with higher stage, non-endometroid type, and younger age at diagnosis. These differences may influence the distribution of PTEN results.

Maxwell and co-workers (1996) found an incidence as low as 5% for PTEN mutations in African American women with endometrial cancer, which suggested a very small role for the PTEN gene in African women. We also found a lower overall incidence of PTEN mutations in African women, but NOT when only endometroid carcinomas were considered. These results suggest once again that morphology correlates better with genotype than other factors like population group and stage.

2.3.4.2 Uterine soft tissue tumours

The vast majority (>90%) of sarcomas of all types in this study occurred in African women. It is thus not possible to draw any conclusions about genetic disparity between different groups from this study.

It is known that uterine sarcomas occur much more frequently in African women and our study (of consecutive endometrial carcinomas and consecutive sarcomas in the same unit) confirms this difference.

3 Hypothesis testing

The answers to the various research questions discussed above are used to test the different hypotheses.

3.1 Endometrial tumours

The hypothesis is that the PTEN gene is intimately involved in endometrial carcinogenesis and may be involved in endometrial hyperplasia.

The PTEN gene is indeed intimately involved in endometrial carcinogenesis and specifically in endometroid carcinoma tumorigenesis. Involvement is probably early in the process as shown by mutation positive hyperplasia and can

extend to later involvement as was shown by multiple mutations in some late stage tumours. PTEN mutation is a common but not essential step in oncogenesis in this cancer. PTEN involvement my also be non-mutational as will be discussed below.

3.2 Uterine soft tissue tumours

It is hypothesized that the PTEN gene is involved in the formation of the different uterine soft tissue tumours.

The PTEN gene is involved very selectively in uterine soft tissue tumours. It is not involved in benign soft tissue tumours and is not significantly involved in leiomyosarcoma or endometrial stromal sarcoma. It is involved significantly in uterine carcinosarcoma and specifically in those tumours with an endometroid epithelial component. Mutations were less common than in endometrial tumours and were not an essential part of oncogenesis.

3.3 Ovarian endometroid carcinoma

The hypothesis to be tested in this study was that the PTEN gene is involved in carcinogenesis in endometroid ovarian adenocarcinomas.

The PTEN gene is indeed involved in ovarian oncogenesis in this type of epithelial ovarian carcinoma. Again mutations occurred but were not an essential step in carcinogenesis. We could not sufficiently test the involvement of the gene in benign ovarian endometroid lesions and thus cannot comment on the chronology of mutations.

4 Contributions and limitations

4.1 The carcinogenetic model

As discussed in the first chapter of this thesis, investigation into the carcinogenetic process on cellular level is advanced. At the same time, however, we are only at the very threshold of understanding.

Various models exist to study carcinogenesis or to derive from research results, the importance and timing of different events in the process. The use of

cancer precursors is common, although many assumptions are made in the interpretation of results. We used precursor lesions and benign counterparts in this study. Generally the model was useful and practical, although interpretations are made with care. We consider this model and its use in all the studied neoplasms as one of the contributions of this dissertation.

4.1.1 Endometrial hyperplasia and endometrial carcinoma

There is little uncertainty that endometrial intra-epithelial neoplasia and atypical hyperplasia are both endometrial carcinoma pre-cursor lesions. The latter is probably the important pre-cursor of endometroid carcinoma, while the former is associated with non-endometroid and specifically with serous subtypes.

As such endometrial hyperplasia was a suitable pre-malignant or benign counterpart for this study. Although it was reasonably easy to obtain enough DNA for analysis, we (and all other authors) were unable to obtain material from the same patients who had cancer.

It is believed that valid conclusions can be drawn from this model. We did not have a pre-cursor lesion available for non-endometroid carcinoma, but also did not demonstrate any PTEN involvement in the type.

4.1.2 Uterine leiomyoma and leiomyosarcoma

Atypical leiomyoma resembles leiomyosarcoma so much on histology, that all of the tumours in this study were initially classified as LMS. As such no better pre-cursor is available to study this carcinogenetic process. On the other hand, it is unlikely that LMS develops from any known non-malignant tumour. All evidence points towards this tumour developing de novo from a single mutated neoplastic cell. We could not demonstrate a difference between involvement of PTEN in LM and LMS.

4.1.3 Uterine carcinosarcoma

All evidence points towards this tumour originating from endometrial cells rather than the myometrium. As such leiomyoma is not a suitable benign counterpart. The findings of the study confirm this conclusion. For the discussion and comparison endometrial hyperplasia was therefore used as the more suitable pre-

cursor lesion or carcinogenetic model. As far as we are aware, this approach has not been used before, can be criticized but seems the most logical one. No alternative pre-cursor lesion for this tumour type has been postulated or described.

4.1.4 Ovarian endometriosis and ovarian endometroid carcinoma

The evidence linking ovarian endometriosis to an increased risk to develop endometroid ovarian carcinoma was discussed at length in chapter 4 and will not be repeated here. There is little doubt that this condition has neoplastic behaviour and can undergo further cellular change that can cause malignancy.

Although it is a very valid pre-cursor lesion, we had great difficulty to collect enough material for DNA based gene testing. It was especially difficult to collect uncontaminated cellular material from the same patient to study separately from the carcinoma. This practical problem limited the significance of our study.

4.2 The female upper genital tract

Most studies on PTEN involvement in gynaecological cancers involved the study of a single organ or tumour type. To study the involvement of a single tumour suppressor gene in the whole of the female upper genital tract was a very interesting endeavour. Generally tissues from the upper genital tract are derived from embrionically related tissue and are exposed to similar carcinogens. In contrast carcinogenesis of the lower tract is dominated by viral and infective carcinogens.

It was considered theoretically logical to study the upper genital tract tumours as a group using similar techniques for all the neoplasms for more than one reason.

In the first place it would be interesting to compare results found in different tissues. In the second place it was logical that similar tumour types would have a comparable genetic make-up. It was found in many instances that the genomic pattern would predict the cellular structure or tumour type.

On the other hand, many previous authors have found different risk factors for the tumours and malignancies in the different organs. No basis for theoretical

similarities between tumourgenesis in the different organs can be found in familial or inherited cancer susceptibility models. The evidence linking the fallopian tube to carcinogenesis resulting in ovarian epithelial cancer is an obvious exception.

The findings of the current study support the differences between tumour types rather than organs, because direct comparison was possible.

The integrated and combined approach is considered an important contribution of this study when compared to other PTEN studies.

4.3 PTEN involvement

The extremely complicated nature of cell growth control is still very poorly understood. Any single genetic change in this cascade can only be important if the protein product has a ripple effect by interaction with other proteins. This is true for the PTEN gene and its protein. The interaction of pten with the multitude of other proteins in the cascade is certain. Although these interactions are partly understood, the details still need further elucidation.

These facts demonstrate how incomplete any carcinogenetic study today will be if not read together with the myriad of other knowledge available about the topic. It is evident that the current study also lacks perspective, importance, relevance and conclusion if read on its own.

4.3.1 Role of PTEN in the carcinogenetic pathway

The role played by an intact PTEN gene in the maintenance of normal cellular growth is described in detail in chapter 1. It will suffice to point out here that the place of pten as protein and lipid kinase in the cascade is very complex. The intricate interactions with other proteins make the effect of the mutated gene's inferior product difficult to quantify or even estimate.

Recent work by various authors have demonstrated that estrogen (and tamoxifen) interacts with and inhibits pten protein expression (Zhang et al 2010; Turbiner et al 2008). The effect is probably via the NFkappaB-dependent pathway and results in activation of PI3K/Akt pathway. This effect is thus here postulated to be key to the proliferation of endometriosis and estrogen dependent

endometroid carcinoma cells and is in keeping with the theory and findings of the current study.

4.3.2 PTEN mutation analysis

It is of huge importance to demonstrate mutations in the gene and it was the only available test when this study was initiated. Mutation analysis, however, does not fully describe or explore the effect of the mutations on the protein product and on the pathway. Also, if no mutation is found, it does not exclude a defective protein product or another implicated role for PTEN in the carcinogenesis of the specific tumour, often via another protein in the pathway.

In spite of these limitations, mutation analysis is still the only accurate method to demonstrate gene involvement rather than epigenetic or alternative protein inactivation.

4.3.3 Alternative tests of PTEN involvement

4.3.3.1 Immunohistochemistry

Mono- and polyclonal antibodies are used to bind and stain specific proteins in the cell. This method is widely used and often relies on over-expression of abnormal proteins after gene mutation. The stains then show positivity in case of mutation as happens in p53 involvement in tumorigenesis. Point mutations of the p53 gene lead to over-expression of the mutant protein product which has a much longer half-life than the wild type. P53 staining then implies involvement of the TSG in the malignant process.

Immunohistochemistry is semi-quantitative and is usually interpreted as mild, moderately or strongly positive.

Genetic mutation which inactivates the protein expression completely should stain negative. In the case of inactivated tumour suppressor genes, immuno-staining will be negative and negative staining will imply involvement of the gene in such a case. This is the usual method of PTEN.

Immunohistochemical staining is much simpler than mutation analysis. It can be done on slides and historical slides. There is no need for tissue blocks and for micro-dissection. However, staining for the PTEN protein does not always

correlate well with genetic abnormality and remains an inferior method to examine the involvement of the gene.

Pallares and co-workers (2005) compared four commercially available immunohistochemical tests for pten protein activity. The tests were correlated with PTEN gene abnormalities and with immunohistochemical expression of phosphorylated AKT. (This is an indirect measure of impaired PTEN function.) No correlation was found between the results of the four tests, one which stained the nucleus and three which stained the cytoplasm! Only one of these four tests correlated with alterations found in the PTEN gene and with phosphorylated AKT. Two of the tests specifically stained cells known to be transfected with wild type (normal) PTEN, positively. These findings severely question the reproducibility of immunostaining while it is known that mutation analysis is very reproducible.

To fully appreciate the role of a specific tumour suppressor in carcinogenesis and in loss of growth control, one needs to consider gene tests (mutation analysis of the exons and introns), direct protein tests (including protein truncation and functional analysis), tissue protein expression tests (immunohistochemistry or FISH) and even protein function tests. In addition the linked substances in the involved pathway should also be investigated.

Until more reliable methods become available the results of tests should be carefully interpreted. Both clinical and research use should be done with great caution.

4.4 Epigenetics vs genetics

4.4.1 Defining epigenetics

Over the last decade it became clear that the understanding of the human genome would be the beginning rather than the end of the understanding of the role of "genetics" in human disease. It became clear that not only the molecular make-up of the genes (mutations or normality), but also the differential expression of these genes play a pivotal role.

Gene expression or "activity" can be measured to some extent by measurements of the protein product of the gene in the tissue. This method

generally is difficult to quantify and the protein product of a mutated gene can be invisible or overexpressed, which complicates interpretation.

Epigenetics is the science of the make-up of the molecules that influence the structure of the DNA. Generally the parts of the chromatin that is "open" will be active in the specific cell at a set time. Most parts of the chromatin of cells will be inactive at any set time, especially in normal mature cells. Cells that are re-programmed to be stem cells, will typically display mainly epigenetic abnormality.

This process that determines the level of activity of the different genes is central to the understanding of cell differentiation. It determines how a cell would change from a multi-potential stem cell to a differentiated cell that forms part of an organ. All normal cells in the body have the same genetic make-up, while the cells and organs differ dramatically in structure and function.

Some authorities postulate that the dedifferentiation of cells to become cancerous cells with uncontrolled growth represents a change back to the stem cell phase. In this phase cells are multipotential and more of the chromatin is exposed and thus active.

This theory will fit the notion expressed in chapter 3 that carcinosarcomas originate from one cell that differentiates into two different cell lines. This would mean that the cell, in the process of carcinogenesis, regains its multipotential (embrionic) ability only temporarily and then differentiates back into a mature and differentiated cell.

On the other hand chemicals that have been shown to revert the adult cell back to a stem cell (like 5AzaCR) are already used with success in haematological malignancies (Peter Jones, personal communication).

4.4.2 DNA methylation as part of epigenetics

The methyl-groups between the DNA strands are the main mechanism of control of the chromatin packaging in the cellular nucleus. Areas of loss of methylation can cause loose chromatin packaging and could represent a change back to embrionic cells, which may be the pre-cursors of cancer cells. On the other hand

hypermethylation will cause chromatin packaging to be closer and this has been demonstrated to be an important gene silencing mechanism. This mechanism is of extreme importance in the silencing of tumour suppressor genes, resulting in loss of function of the gene, without genetic change.

In cancer genetic study over the last decades, a oncogenic genetic change was considered to be "frequent" in a tumour type if mutation of the gene occurred in ~30% of cases of a specific malignancy. PTEN mutation in endometrial cancer is an elegant example. "Infrequent changes" were quoted as between 5 and 10% of tumours, with an example being PTEN mutations in uterine sarcomas. These "infrequently" involved genes are now found to be involved much more frequently, but via the hypermethylation gene-silencing route rather than via genetic mutation.

If gene silencing by hypermethylation is therefore studied, it is demonstrated that these epigenetic changes occur more frequently than genetic changes in the same genes.

4.4.3 MicroRNA and gene expression

MicroRNAs or miRNA represent a new class of genes influencing gene expression by down regulating gene expression. As such this class is particularly important in the inactivation of tumour suppressor genes without gene mutation. It is known that abnormal expression of some miRNAs impact on cell survival. Recently Yang et al (2008) studied the expression of some of these genes and demonstrated that, importantly, miRNA-214 is associated with platinum resistance via the direct down-regulation of PTEN.

4.4.4 Epigenetics and Knudson's theory

As discussed in Chapter 1, the theory of Knudson hypothesis is that multiple mutations will be needed for a normal cell to escape cell growth control, as genetic silencing necessitates the silencing of both alleles and more that one mechanism of control needs to be escaped.

It is now clear that cancer cells typically have between ten and fifteen mutations that can be demonstrated in the genome of the tumour. These mutations

do not need to affect the same gene in both alleles, as a more subtle effect will also influence cell growth control when multiple genetic defects are present.

Additionally it is now postulated that more than 300 genes per tumour can be hypermethylated, making expression of these genes impossible. Silencing of genes due to epigenetic change rather than by mutation will become the focus of research in the new era.

It is also implicated that while one allele can be silenced by a mutation, the other allele can be silenced by hypermethylation at the same locus. This would be in support of the initial theory of Knudson.

5. Impact of the study and recommendations

Knowledge about the control of both cellular differentiation and cell growth is at the foundation of the understanding of tumour genesis. Both these disciplines are advancing rapidly and were boosted tremendously by the human genome project. The study of carcinogenesis will in a sense always follow the advances made in cell biology and cellular growth control, rather than the other way around.

5.1 Impact

5.1.1 Molecular study

Molecular testing methods

Technological advances dictate the possibilities for the study of cell and tumour formation, behaviour and genetics. In this way, many fantastic theories in the past were only proven or disproven generations later when the technology became available. Today technology often prescribes which tests are done rather than the intelligent theoretical framework. In this way carcinogenetic studies often become a mindless testing of all available genes or proteins as is displayed in the era of multi-array testing.

The answer to these potential threats to the study of cancer genesis must lie in the formation of multi-disciplinary discussion and research groups. Ideally groups should have input about the theoretical nature of the question, the clinical dilemma and about availability and limitations of current technology. The most

productive and creative groups already display these characteristics and smaller groups and individuals can hardly compete.

Epigenetics

As the focus change from genetics to epigenetics, the extremely exciting characteristic of the epigenome is that it is NOT instable. This means that knowledge about the epigenetics will become hugely important. Changes which may be carcinogenetic are not permanent as is the case with gene mutation. Rather these epigenetic changes are prone to change and are thus also prone to genetic modulation and for the development of future designer drugs.

Combination of genetic alterations

As information accumulates about the involvement of different genes in specific human cancers, the new challenge will be to investigate and understand combinations of gene and protein changes needed to form specific phenotypes and to lead to invasive cancers. In some exiting work in female genital tract tumours, Mizumoto and colleagues (2006) transformed endometrial cells into first immortal and then tumourigenic cell lines by introducing oncogenes and silencing tumour suppressors. They found that the inhibition of PTEN led to growth capacity that was not dependent on cell anchorage.

Ogawa and co-workers (2005) similarly investigated the combination of gene pathway alterations in 12 cell lines and demonstrated that either upregulation of PI3/AKT cascade or RAS/MAPK pathways is crucial for endometrial carcinogenesis.

5.1.2 Improved diagnosis and stratification

Several studies were discussed which already show that improved knowledge of the interaction of the tumour-causing genes in cancer will improve our ability to predict prognosis even if outcome is not improved. This would allow for selective use of more aggressive treatment protocols in deserving patient populations, a strategy which may limit unnecessary treatment and improve the outcome of patient with aggressive tumour types.

In addition gene mutation analysis can be used to study the differential origins of tumours in cases of synchronous endometroid cancer of the uterus and ovary. Ricci et al (2003) demonstrated superior results to that of histopathology only.

Simple immunohistochemical tests using both the PTEN and the AKT stains have already been shown to predict survival accurately (Uegaki et al 2005, Terakawa et al 2003). Advanced endometrial carcinoma staining positive for PTEN and negative for AKT has a better prognosis than the others although this finding was not universal (Wang et al 2005) which may be partly explained by different PTEN immunostains. There is a strong inverse reactivity of PTEN and AKT stains. The implication is that intact PTEN function correlates with improved outcome as expected.

Unfortunately studies to predict the response of (metastatic or recurrent) endometrial cancer to drugs and hormone treatments are not always successful. Ma and colleagues could correlate neither hormone receptor status nor tumour suppressor IHC with response to Letrozole (2004).

5.1.3 Predicting treatment response

Athough the dualistic model of endometrial cancer (described by Bokhman) is widely accepted, molecular genetics provided the data to support the model and to accurately classify patients. Altered genes are associated with myometrial invasion and also with response to treatment (Abal et al 2006).

PTEN activation was recently shown to be essential to the therapeutic effect of trastuzumab in women with ErbB2-positive breast cancer. It was shown that loss of PTEN accurately predicts resistance to this expensive designer drug (Nagata et al 2004). This finding has immediate importance but also holds promise for future therapeutic applications.

The development of similar drugs which could activate the PTEN phosphatase and in turn down-regulate the PI3K pathway is keenly awaited. (Crowder, Lombardi & Ellis 2004.)

5.1.4 Novel treatment and improved outcome

Obviously understanding carcinogenesis is the basis of cancer prevention and treatment. With its intricate involvement in the inhibition of cellular growth, the understanding of PTEN involvement will also enhance our ability to fight tumour progression (Gadducci 2008).

There is no doubt that the PTEN gene and its normal product will have a similar function in the ovary and in ovarian cancer cells to that displayed in other tissues. By transferring the PTEN gene into ovarian cancer cell lines using adenovirus, Minaguchi et al (1999) showed again that the gene causes significant growth inhibition. The effect on growth was via apoptosis and arrest in the G1 phase of the cell cycle. This study shows potential for adenovirus mediated gene therapy using the PTEN gene.

PTEN negative cells are successfully transfected with wild-type PTEN in vitro and in cell lines. Wu and co-workers (2008) demonstrated reversal of malignant behaviour in ovarian cancer cell lines by introducing wild type PTEN into the cells. It is expected that genetic modulation of active cancer cells in vivo will become possible and may lead to improved outcomes.

AKT inhibition and PTEN re-activation is expected to be available treatment modalities in the near future and will probably revert cells to less malignant growth patterns and re-introduce chemo-sensitivity (Yang et al 2008).

It may also become possible to up-regulate PTEN activity chemically as suggested by the findings of Lin and co-workers (2008) when the effect of valproic acid was studied.

Ongoing work involving the ellipticines also targets the PTEN/PI3K and Akt pathway. These small molecule inhibiting drugs may eventually prove useful in tumours with high Akt kinase activity or disrupted PTEN (Jin et al 2004).

5.2 Recommendations

While protein expression studies have severe limitations, studies such as immuno-histochemical staining for a specific protein have the advantage of including gene

suppression by other methods than mutation. These studies may be more interesting and more accurate in many instances than mutation analysis only. Ideally the relation between these tests should be studied extensively before a choice of method is made.

This study did not investigate gene expression, but only genetic mutations in the tumour cells. It will be important but difficult and expensive to further investigate the contribution of both genetic change and epigenetic change in the future.

The finding of important PTEN involvement coupled with the finding that wild-type gene presence correlates with improved outcome necessitates investment into translational research in this field.

Immuno-histochemical methods need refinement and improvement before drug use can be based on this investigation.

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