Chapter 1

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

Introduction to the study

Justification of the study

Theory base and general literature overview

Delineation of the research

Chapter 1 16
The role of the tumour suppressor gene PTEN in the etiology of cancers of the female genital tract general introduction
1 Introduction to the study 18
1.1 Background 18
1.2 Research questions 20
1.2.1 Endometrial hyperplasia and carcinoma 20
1.2.2 Uterine soft tissue tumours 21
1.2.3 Endometroid ovarian cancer 21
1.3 Objectives 22
2 Justification of the study 22
2.1 Motivation 22
2.2 Significance 23
3 Theory base and general literature overview 23
3.1. Genetic changes as part of the carcinogenetic process 23
3.1.1 DNA repair genes 24
3.1.2 Proto-oncogenes and oncogenes 25
3.1.3 Tumour suppressor genes 28
3.2. The function of the phosphatases and kinases 31
3.2.1 Protein phosphatases and protein kinases 31
3.2.2 The lipid kinases and phosphatases in the phospholipid pathway 35
3.3. PTEN: The tumour suppressor gene and its protein product 36
3.3.1 The isolation of PTEN as a novel tumour suppressor gene 36

3.3.2 PTEN as a protein phosphatase and lipid phosphatase 38
3.3.3 The interaction of PTEN with known kinases, phosphatases and growth factors 39
3.3.4 PTEN and the control of cellular growth 41
3.3.5 Biological activities of PTEN not directly related to growth 42
3.3.6 Interaction of PTEN with other tumour suppressor genes, oncogenes and chemotherapeutic agents 44
3.3.7 Interaction of PTEN with steroid hormone receptors 44
3.4 The involvement of PTEN in non-gynaecologic neoplasms 45
3.4.1 Brain tumours 45
3.4.2 Breast cancer 45
3.4.3 Other malignancies 46
3.5 The involvement of PTEN in the etiology of cancers of the female genital tract 46
3.5.1 Neoplasms of the uterine epithelium 47
3.5.2 Neoplasms of the uterine soft tissue 47
4 Delineation of the research 48
4.1 Tumours of the female genital tract 48
4.1.1 Etiology of tumours of the upper vs lower female genital tract 48
4.1.2 Models of neoplastic transformation 48
4.2 Methods to study the role of PTEN 51
4.2.1 Detecting somatic genetic mutation 51
4.2.2 Detecting the aberrant protein product 52
5 Conclusion 52

List of figures for Chapter 1

Figure 1.1: The function of the protein kinases and phosphatases. 34
Figure 1.2: Integrin function and signalling. 35
Figure 1.3: The function of the lipid kinases and phosphatases. 36

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)..

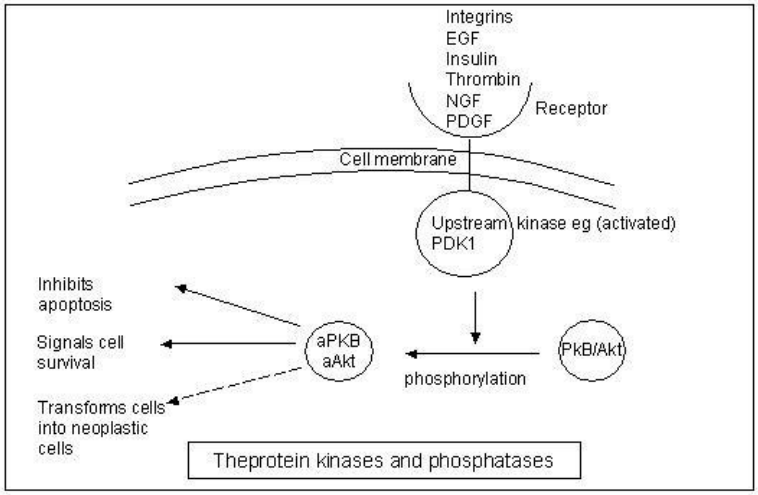
Protein kinase B/Akt (PKB/Akt), which is a serine/ treonine kinase, has been shown to be an extremely important physiological mediator of the effects of insulin, several growth stimuli and growth factors and it protects cells against natural cell death. Activated PKB/Akt will provide a cell survival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

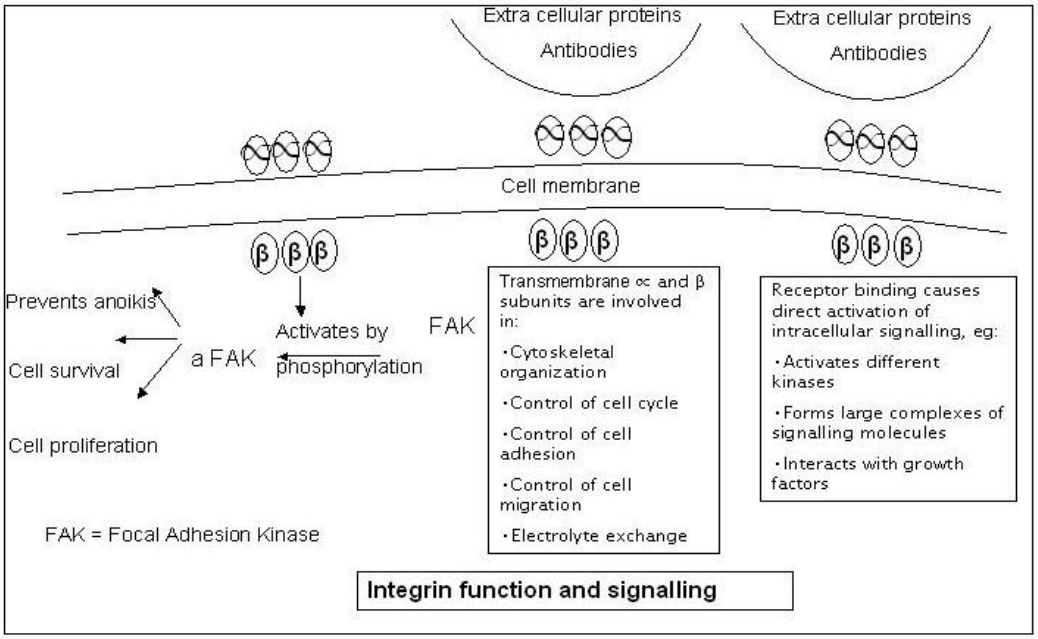


Figure 1.2: Integrin function and signalling.

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

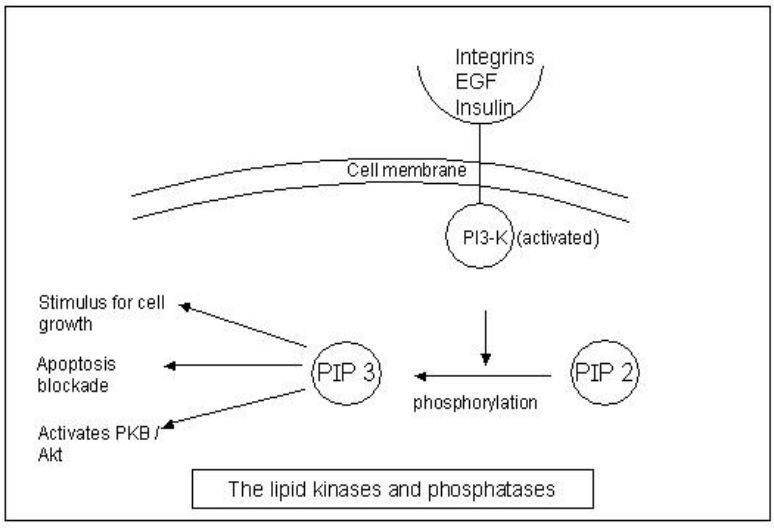
In the phospholipid metabolism pathway, phosphatidylinositol 3,4-biphosphate (PI(3,4)P2 or PIP2) is phosphorylated to phosphatidylinositol 3,4,5-triphosphate (PIP3 or PI-P3) and this reaction is catalysed by the phosphatidylinositol 3-kinase (PI3-K) enzyme. The reaction is stimulated, as in the case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