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

List of figures for Chapter 5

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.

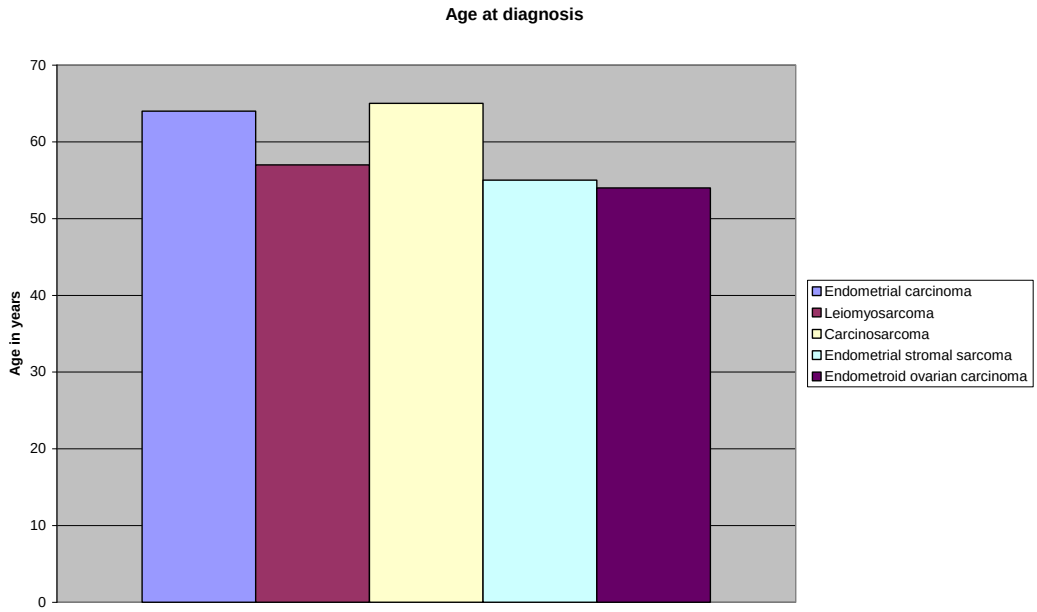

Figure 5.16: Average ages at diagnosis of all malignant tumours included in PTEN mutation analysis.

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.

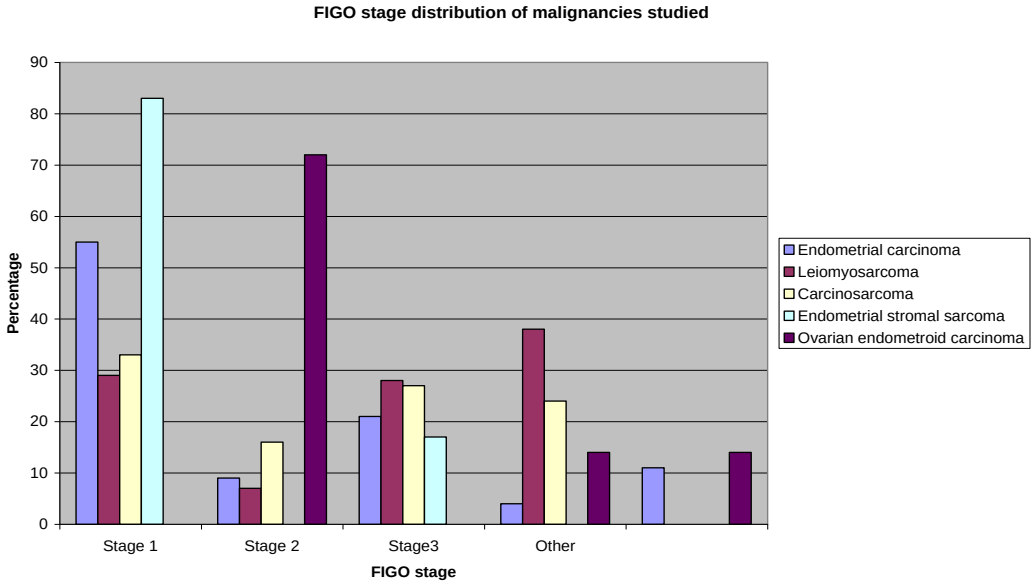

Figure 5.17: FIGO stage distribution of all malignant tumours included in PTEN mutation analysis

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

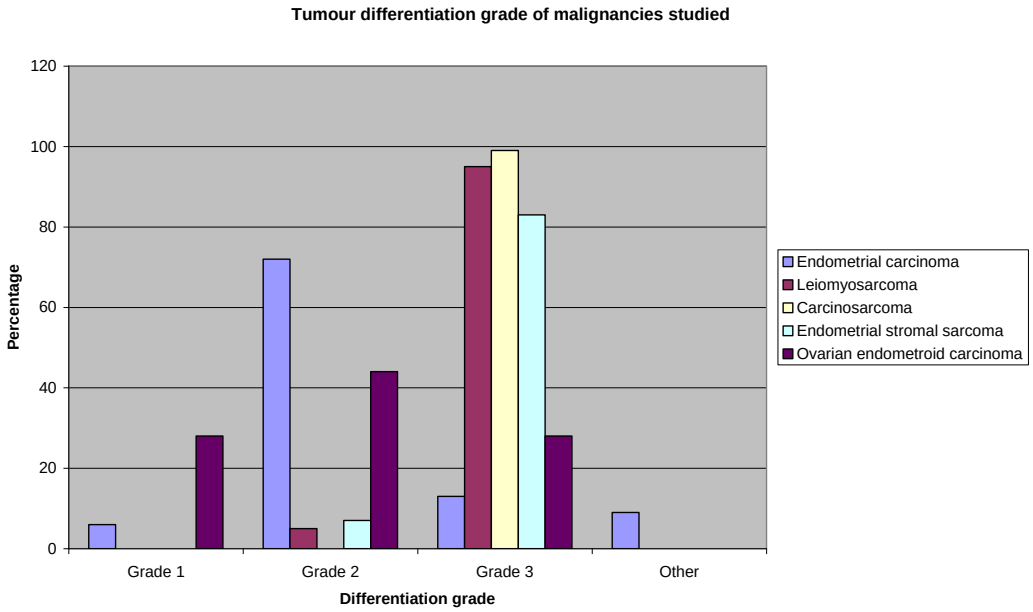

Figure 5.18: Histological differentiation grades of all malignant tumours included in PTEN mutation analysis.

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.

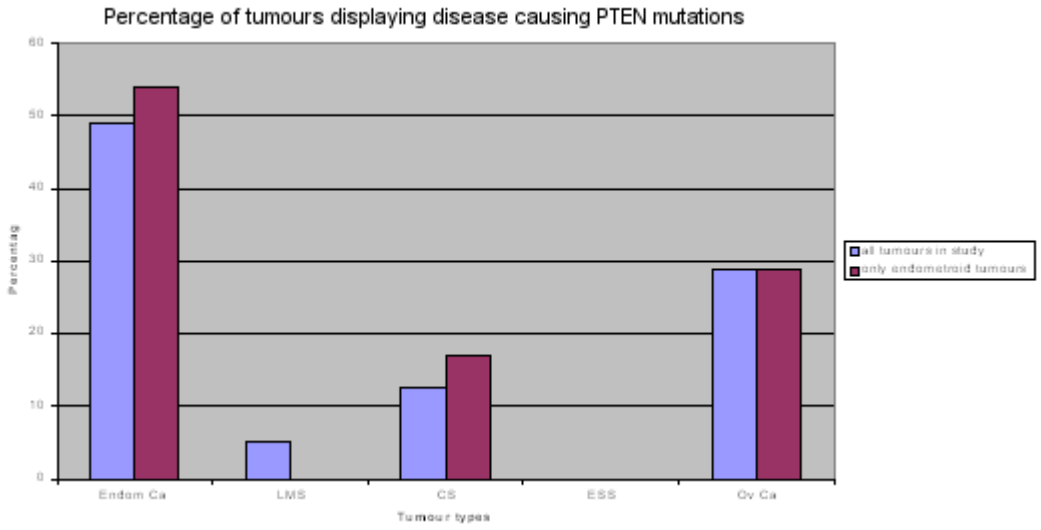

Figure 5.19: Percentage of malignant tumours in this study that displayed mutations considered to be disease causing

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.

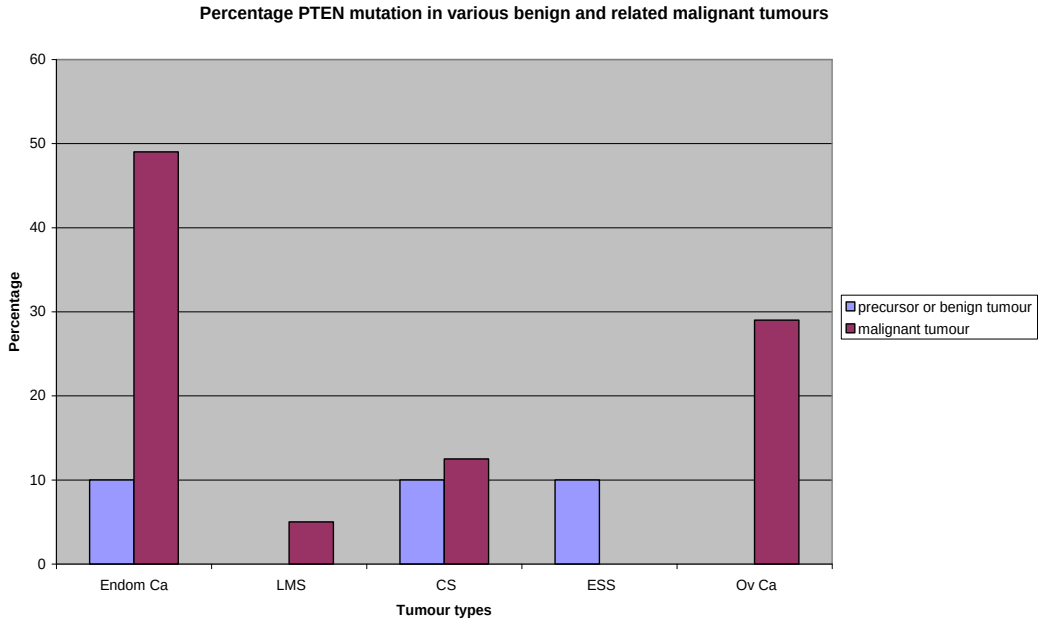

Figure 5.20: Percentage of precursor lesions or benign tumours vs. related malignant tumours that displayed mutations considered to be disease causing.

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.