Chapter 4

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

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1 Introduction

1.1 Background

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

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

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

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

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

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

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

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

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

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

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

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

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

1.2 Research questions and hypothesis

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

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

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

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

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

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

2 Literature overview

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

2.1.1 The etiology of endometriosis

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

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

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

2.1.2 The pathophysiology of endometriosis and ovarian cancer

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

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

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

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

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

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

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

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

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

Table 4.21: Classical risk factors for endometriosis.

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

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

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

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

2.1.3 The association between endometriosis and malignancy

2.1.3.1 Gynaecological cancer

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

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

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

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

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

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

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

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

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

2.1.3.2 Non-gynaecological cancer

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

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

2.1.4 Evidence that endometriosis can be a pre-malignant disease

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

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

2.1.5 The malignant potential of different forms of endometriosis

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

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

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

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

2.1.6 Endometriosis as a genetic disease

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

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

2.2 Histopathology of endometroid ovarian lesions and ovarian neoplasms

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

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

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

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

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

2.2.1 Endometriosis and related non-malignant lesions

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

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

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

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

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

2.2.2 Endometriosis related malignant lesions

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

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

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

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

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

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

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

2.2.3 Ovarian neoplasms

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

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

2.2.3.1 Ovarian endometroid carcinoma

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

2.2.3.2 Ovarian clear cell carcinoma

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

2.2.3.3 Mucinous adenocarcinoma and mullerian mucinous borderline tumour (MMBT)

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

2.2.3.4 Endometrial stromal sarcoma

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

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

2.2.3.5 Other non-endometroid epithelial carcinomas

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

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

2.3 Genetic changes in endometriosis and ovarian neoplasms

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

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

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

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

2.3.1 Cytogenetic changes in endometriosis and ovarian carcinoma

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

2.3.2 Specific genetic alterations in endometriosis and ovarian carcinoma

2.3.2.1 K-ras

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

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

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

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

2.3.2.2 Beta-catenin

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

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

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

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

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

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

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

2.3.2.4 P 53

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

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

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

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

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

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

2.3.2.5 BRCA 1 and BRCA 2

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

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

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

2.3.2.6 Bcl-2

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

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

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

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

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

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

2.3.2.8 PTEN

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

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

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

2.3.2.9 Phenotype and genotype in ovarian cancer

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

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

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

2.4 The PTEN gene in endometriosis and ovarian neoplasms

2.4.1 PTEN germline mutations in ovarian cancer

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

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

2.4.2 PTEN somatic mutations and pten protein expression

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

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

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

2.4.3 PTEN in normal endometrium and ovary

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

2.4.4 PTEN in ovarian endometriosis

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

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

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

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

2.4.5 PTEN in ovarian epithelial cancer

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

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

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

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

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

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

2.4.6 PTEN and loss of heterozygosity (LOH)

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

2.4.6.1 Frequency of LOH (10q23) in ovarian lesions

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

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

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

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

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

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

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

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

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

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

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

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

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

2.4.6.3 Importance of LOH (10q23) in ovarian lesions

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

2.4.7 PTEN and other genetic changes

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

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

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

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

2.4.8 PTEN involvement in the genetic sequence of carcinogenesis

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

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

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

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

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

2.4.9 PTEN related genetic anomalies

2.4.9.1 The tyrosine kinase-signalling cascade

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

2.4.9.2 Akt or Protein kinase B (PKB)

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

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

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

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

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

3. Materials and methods

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

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

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

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

3.1 Materials

3.1.1 Sampling and clinical material

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

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

3.1.2 Histology reports

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

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

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

3.1.3 Tissue for DNA analysis

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

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

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

3.2 Methods

3.2.1 DNA extraction

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

3.2.2 DNA amplification

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

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

3.2.3 PTEN mutation analysis

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

3.2.3.1 Sequence analysis

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

4. Results

4.1 Clinical data

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

4.1.1 Age distribution and population group

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

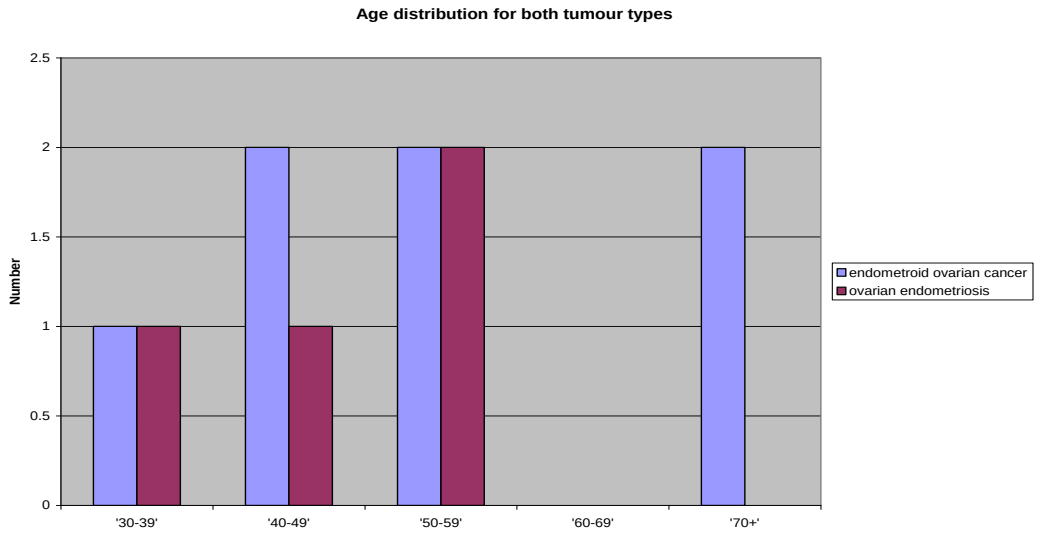

Figure 4.14: Age distribution of ovarian endometroid lesions.

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

4.1.2 Menopausal status and associated disease

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

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

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

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

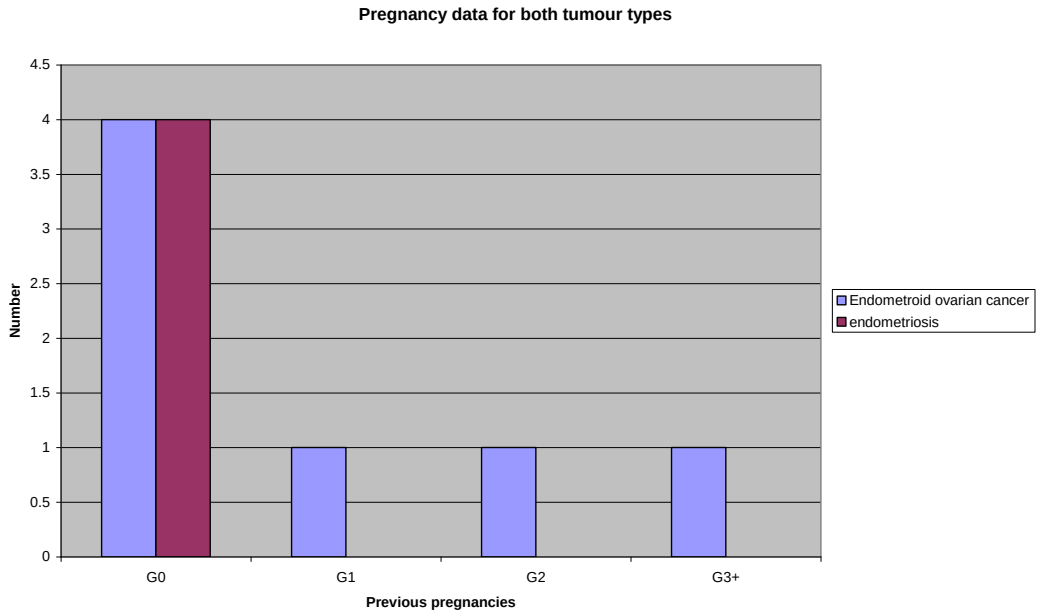

Figure 4.15: Pregnancy data for ovarian endometroid lesions.

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

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

4.2 Histology data

4.2.1 Stage distribution and differentiation grade

The FIGO stage and histological differentiaition grade is shown in table 4.2.

Table 4.22: Histological and clinical parameters.

Study number	Differentiation grade	FIGO stage	Synchronous endometriosis	Co-morbidity
EOC 01	2	2	Ovarian	Infertility
EOC 02	2	2		Colorectal Ca
EOC 03	3	2		DM, HT
EOC 04	1	2	Ovarian	DM
EOC 05	3	4		HT

EOC 06	2	?	Peritoneal	Unopposed estrogen
EOC 07	1	2	Ovarian	Infertility

Other than the typical picture of papillary serous carcinoma, most patients had disease confined to the pelvis at the time of surgery. Five of seven patients were diagnosed in stage 2 and only one patient had disseminated disease.

In spite of the early stage at presentation, four patients died of disease within the four to five years follow up.

The three patients that survived all had pre-existing endometriosis.

4.2.2 Tumour size

Endometroid ovarian cancer tends to form a more solid and larger primary tumour than papillary serous carcinoma. The tumour sizes varied from 1cm to 15cm, average 6 cm.

4.3 Mutation screening

Due to problems with the PCR amplification due to both low DNA quality and insufficient DNA quantity, all samples were sequenced directly without mutation screening. The results will be discussed here.

4.4 Sequence analysis

4.4.1 Non-malignant tissue samples

No PTEN mutations were found in any of the endometriosis samples.

In the one patient who had a PTEN mutation in the ovarian cancer, the associated endometriotic lesion did not display the same mutation, suggesting PTEN mutation was an event associated with malignant behaviour in this tumour.

In the second patient no concurrent endometriotic lesion was available for analysis; this patient was elderly (76 years).

4.4.2 Endometroid ovarian carcinoma

Two patients displayed somatic mutations in the ovarian malignant tumours. Both mutations are considered to be disease causing.

Table 4.23: Mutations in the PTEN gene in endometroid ovarian carcinomas.

Tumour	Mutation type	Exon	Nucleotide change	Effect
EOC 4	nonsense	5	c.388C to T	Arg130Stop
EOC 3	frameshift	6	c.497delT	Stop at 178

4.4.3 Polymorphisms

We did not find any polymorphisms in the PTEN gene in this small group of tumours.

4.5 Correlation between PTEN gene mutations and clinico-pathological findings

The group of tumours where mutation analysis could be completed were too small to correlate these to clinical and pathological findings.

The one mutation positive tumour were poorly differentiated and the other well differentiated, while both patients were diabetic, had stage 2 disease and were post-menopausal (ages 58 and 76).

5. Interpretation and importance

5.1 Ovarian endometriosis

It was expected that PTEN mutations may be present in this benign counterpart of endometroid ovarian cancer if it was indeed found in the malignant tumour. No mutations were however found in this group of tumours. The importance of this finding is severely limited by the small amount of DNA that was available for analysis.

Some other mutation analysis studies in the literature have concordant findings (Obata et al 1998), while others found some mutations (Sato et al 2000) and PTEN involvement on immunohistochemistry.

In one patient the availability of DNA from the benign tumour made it possible to determine that the PTEN mutation was indeed only present in the malignant tissue. This finding is of some importance as it points towards PTEN mutation being a late event in carcinogenesis in this tumour.

We could not correlate PTEN mutation with the finding of cellular atypia. It will be much easier to study the timing of the involvement of the PTEN gene with a method that does not require DNA extraction from these very small lesions.

5.2 Endometroid ovarian carcinoma

This study demonstrated that PTEN mutation plays a role in early stage endometroid ovarian cancer of different etiology. We found evidence of involvement of PTEN in a significant part of this small study population. Two of seven tumours (~29%) had mutations.

The findings concurred with the groups of Obata (1998), Sato et al (2000) and Catasus (2004) who found mutations in between 14 and 24% of endometroid carcinomas. These findings are discussed above (2.4.5) in more detail.

5.3 Epithelial ovarian carcinoma

It is becoming increasingly clear that the developmental pathways for the three major subtypes of ovarian epithelial cancer are fundamentally different. The subtypes are serous, mucinous and endometroid carcinoma, with the molecular characteristics of the latter studied in this project. Evidence is accumulating to demonstrate that serous papillary tumours often arise from the lumen of the fallopian tube, while the etiology of mucinous tumours remains a dilemma.

It is known that endometroid ovarian carcinoma can arise from ectopic endometrial implants that undergo malignant transformation, but it is unclear what proportion arise *de novo* via cellular metaplasia of the ovarian surface epithelium. In the same way that the PTEN gene is involved in malignant transformation of endometrial cells in the endometrium, this gene is probably intimately involved in somatic mutation and transformation of endometrial implants on the ovary.

While PTEN mutation plays a limited role in the majority of ovarian tumours, this study and some others have demonstrated that it is intimately involved in the pathogenesis of endometroid ovarian carcinoma. The precise nature, level and chronology of involvement will remain the subject of study in the near future.

Generally the incidence of PTEN mutations reported in a group of ovarian epithelial cancers is determined by the proportion of endometroid and clear cell tumours in the study.

5.4 Strengths, limitations and recommendations

This study was one of the first in the world to study PTEN involvement in ovarian carcinoma and endometriosis. As far as we could establish, it is the only South African study.

The findings of this study shows important involvement of this tumour suppressor gene in the development of ovarian endometroid carcinoma. We could not, however, demonstrate when in the carcinogenetic pathway (early or late) these mutations occur. It would be hugely interesting to answer this question, but a suitable scientific model of study is outstanding.

The significance of the findings of this study is limited by small numbers but the results are in line with the findings of other research groups. When all the mutation analysis studies are considered together, results are concordant and about 20% of endometroid ovarian cancers show mutations (16 of 82). The results of endometriosis studies are less consistent (demonstrating the difficulty to perform these studies) and results vary between 0% and 20%).

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

It is interesting to compare the findings of this study to that of the endometrial carcinoma and hyperplasia studies done on the same population and described in chapter 2. The results of the different tumour types will be correlated and discussed in the final chapter.